Functional and Evolutionary Relationships Among the RPCH-AKH Family of Peptides[1]

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Synopsis. The RPCH-AKH peptide family is a group of structurally similar peptides which are apparently widely distributed in arthropods, and which serve a variety of functions in different settings. The first three recognized members of this family were detected and purified based on endocrine activities including color change in crustaceans (RPCH) and effects on energy metabolism in insects (AKH and Compound II). The most recently identified family members, MI and MII, were found on the basis of neuromuscular activity as well as endocrine effects, and a combination of histological and physiological evidence strongly suggests that at least some of these peptides are localized in neurons including motor neurons which use them as transmitters. Thus this is one of several neuropeptide families with highly conserved structures, but diverse endocrine and neural functions. The significance of structural similarities between family members is unclear. Fortunately the arthropod preparations in which these peptides have been identified lend themselves to detailed developmental, anatomical, and physiological analysis, so there is every reason to suppose that molecular biological and physiological investigations currently in progress will shed significant light on the meaning of the phenomenon of structurally conservative peptide families.

INTRODUCTION

There are now five recognized members of the RPCH-AKH peptide family, four of which have been sequenced. Each was originally purified from a neuroendocrine organ. It has become clear over the past couple of years that at least some of the family members occur in neurons. The family members all show similar activities in endocrinological and neuromuscular assays. Thus the AKH-RPCH peptides are neuropeptides, and are one of several examples of a group of peptides all with similar structures and overlapping bioactivities.

I will briefly describe the characterization of the various family members concentrating on the most recently recognized ones. The story is just now unfolding so that no final conclusions can be drawn, but I hope to make it clear why a number of researchers are eagerly looking forward to the next chapters.

RPCH

Red pigment concentrating hormone (RPCH) was the first of the peptides to be sequenced. The activity for which it is named has been recognized in crustaceans for many years (Perkins, 1928) but it was not until the 1960s that Fernlund and Josefsson (1968) succeeded in using an erythropore pigment assay in shrimp *Palaemon adsepersus* to guide the purification of RPCH from eyestalks of the prawn *Pandalus borealis*. Sequencing the small quantity available of this totally blocked peptide proved quite a challenge, but by 1972 Fernlund and Josefsson had managed this task as well as confirming the sequence with synthetic material. The sequence of this octapeptide is shown in Table 1.

Though the first of the family members to be characterized was derived from a crustacean, the remaining four were all isolated from insects, and it has been in insects that neural as well as endocrine activities of various family members have been observed.

AKH and Compound II

In 1969 both Mayer and Candy as well as Beenakkers reported that a search for a

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Table 1. *The RPCH-AKH family.*

RPCH	pGlu	Leu	Asn	Phe	Ser	Pro	Gly	Trp	NH$_2$		
AKH	pGlu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr	NH$_2$
MI	pGlu	Val	Asn	Phe	Ser	·Pro	Asn	Trp	Gly	Thr	NH$_2$
MII	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	NH$_2$		
Compound II*	pGlu	Leu	Asn	Phe	Ser	Thr	Gly	Trp	NH$_2$		

* Speculative sequence by analogy to RPCH.

lipid mobilizing hormone responsible for the increase in locust hemolymph diglycerides during flight led them to an active extract of a neuroendocrine gland the corpus cardiacum (c.c.) from *Schistocerca gregaria* and *Locusta migratoria*, respectively. The activity which was christened adipokinetic hormone (AKH) by Mayer and Candy was apparently a peptide since it was destroyed by proteases. Sephadex chromatography of c.c. extracts and hemolymph from flown animals revealed bioactivity eluting at the same position suggesting the active peptide was released into the hemolymph during flight. The extract produced diglyceride release from isolated fat body *in vitro*, suggesting this as its locus of action in the whole animal. Seven years later, Stone *et al.* (1976) reported the purification to homogeneity and the sequencing of AKH. As soon as the structure of this decapeptide was determined, the striking similarity between AKH and RPCH was noted (Table 1), explaining the long standing observation that c.c. extracts had pigment concentrating activity (Brown and Meglitsch, 1940; Hanstrom, 1940). Indeed AKH has potent pigment concentrating activity in a variety of crustaceans while RPCH has considerable adipokinetic activity in locust (Mordue and Stone, 1976; Herman *et al.*, 1977; Mordue and Stone, 1977).

While investigating the pigment concentrating activity of AKH, Carlsen *et al.* (1979) came upon another peptide in the c.c. of *Schistocerca gregaria* with similar biological activities. The peptide, which they called compound II, was purified to homogeneity and its amino acid content determined. Compound II apparently is even more closely related to RPCH than AKH. It is an octapeptide with only one difference in amino acid content from RPCH, thr instead of pro. Despite the strong homology to RPCH, compound II is less potent in crustaceans and is, in fact, quite comparable to AKH in its pigment concentrating activity. It is the only peptide in the RPCH-AKH family which does not contain a pro residue, but it is comparable to AKH in hyperglycemic and adipokinetic activities (Josefsson, 1983). The sequence of compound II is not known, but it is tempting to speculate on a structure similar to that of RPCH (see Table 1). This peptide has been synthesized and apparently it does have adipokinetic activity (Yamashiro *et al.*, 1984).

MI AND MII

The c.c. of cockroach, *Periplaneta americana* has long been a subject for investigations, and a number of factors and partially purified peptides have been described. These include hyperglycemic activities (Steele, 1961; Brown, 1965), adipokinetic activities in locust (Downer, 1972), gut and heart stimulatory activities (Cameron, 1953; Brown, 1965); and pigment concentrating activity (Brown and Metlitsch, 1940). At the time I became interested in the cockroach c.c., Bauman and Gersch (1982) had made the most progress in characterizing one of these activities. They were pursuing a cardioacceleratory peptide called neurohormone D. They successfully purified the peptide to homogeneity, demonstrated that it was uncharged and blocked at its amino terminal, and performed an amino acid analysis on it. Although Bauman and Gersch noted that neurohormone D was similar to AKH in being a blocked, uncharged peptide, they confined themselves to assays of its cardiac effects in *Periplaneta americana*, and similarities to AKH in bioactivity were not tested.
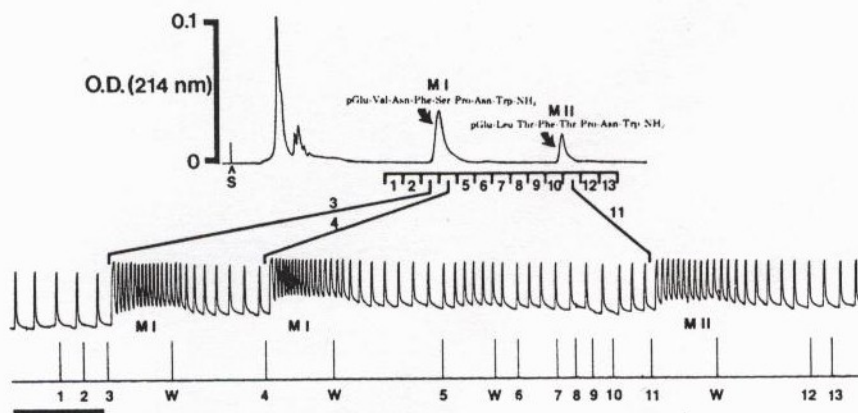
FIG. 1. Optical density profile of reverse phase HPLC (upper trace) and bioactivity (lower trace) of a crude acidic methanol extract of *Periplaneta americana* c.c. MI and MII are the major compounds detected by U.V. absorbance. Fractions corresponding to these peaks cause increase in the baseline of the bioassay reflecting increased skeletal muscle tone, and increase in the frequency of contractions of the leg (a spontaneous activity) reflecting cardioacceleratory activity.

My colleagues, Drs. Michael O'Shea and Jane Witten, and I reasoned that the c.c. might also prove to be a source of peptides active on skeletal muscle. We were interested in myoactive peptides because Dr. O'Shea and his collaborators had recently demonstrated that another peptide, proctolin, was an active muscle stimulator employed by the motor neurons innervating certain skeletal muscles (Bishop *et al.*, 1981; Adams and O'Shea, 1983). The c.c. of cockroach seemed a likely source, since it was a storehouse for so many activities. We made an initial extract using acidic methanol and after drying and resuspending the residue in saline, we could readily demonstrate myoactivity in the locust leg assay. This is a routine assay in the O'Shea laboratory which can detect both skeletal muscle stimulation and cardioexciatory activities (O'Shea *et al.*, 1984). Rather than employing more traditional purification techniques, we immediately subjected a small scale extract to reverse phase high performance liquid chromatography (HPLC), monitoring the result using very sensitive u.v. absorbance and fluorescence detectors. To our surprise the pattern showed no detectable AKH, but two other major peaks (see Fig. 1), and amazingly both peaks produced muscle activity as well as cardioacceleration (Fig. 1). Because of the

myoactivity, we named the compounds MI and MII. This first round of HPLC produced virtually pure peptides. A second HPLC step produced material which was entirely suitable for chemical characterization. Acid hydrolysis followed by amino acid analysis showed that MI and MII were indeed both peptides. The analysis disclosed seven amino acids in each, however trp is destroyed in this procedure. Because of the spectral properties of MI and MII and the absence of tyr on amino acid analysis we were confident each peptide must contain trp as well. So MI and MII were apparently octapeptides. Their amino acid compositions suggested that the two peptides were structurally similar, and that both were related to AKH. That idea was supported by the observations that the peptides were uncharged and had blocked amino termini (Witten *et al.*, 1984a). Further, it was striking that MI had the same amino acid composition on HCl hydrolysis as neurohormone D.

One bit of modern technology, HPLC, had greatly facilitated the purification of these compounds, and another solved the difficult problem of determining the sequence of these scarce, blocked peptides. Mass spectrometry (MS) has traditionally been used to assist in the sequencing of a blocked peptide (such as AKH), but until
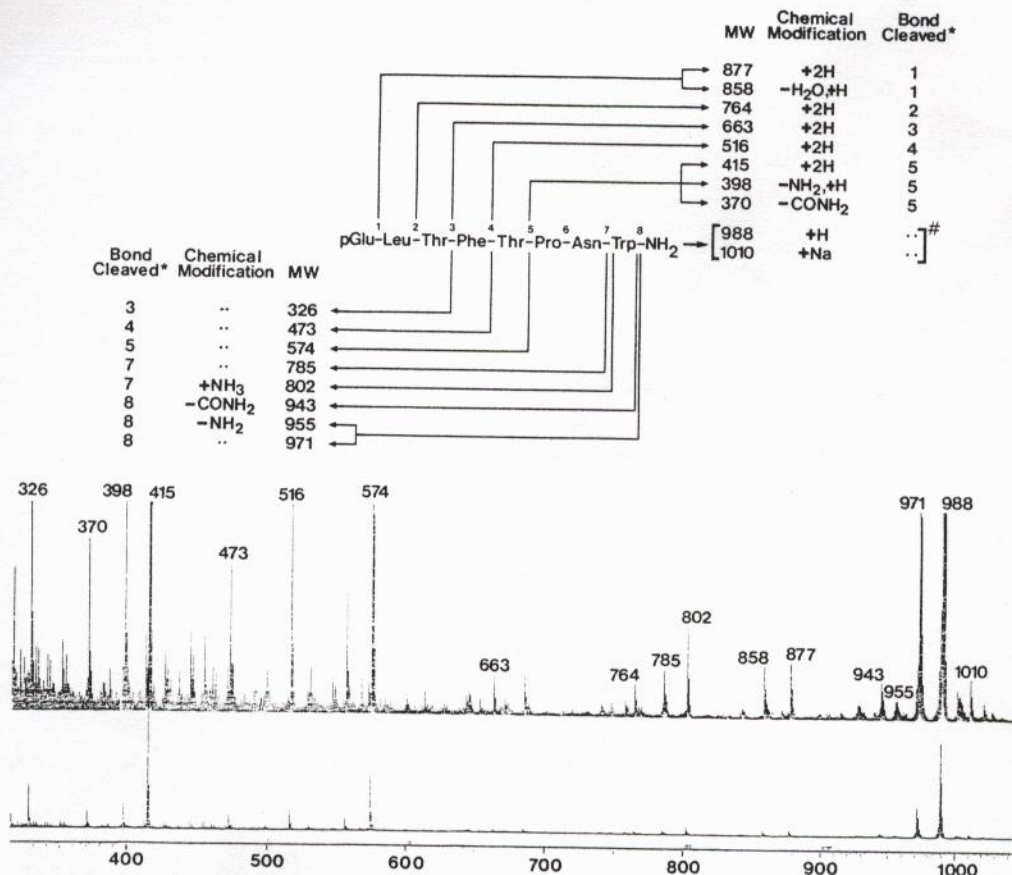
	MW	Chemical Modification	Bond Cleaved*
	877	+2H	1
	858	−H$_2$O,+H	1
	764	+2H	2
	663	+2H	3
	516	+2H	4
	415	+2H	5
	398	−NH$_2$,+H	5
	370	−CONH$_2$	5

pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH$_2$ →

	MW	Chemical Modification	Bond Cleaved*
	988	+H	..#
	1010	+Na	..

Bond Cleaved*	Chemical Modification	MW
3	..	326
4	..	473
5	..	574
7	..	785
7	+NH$_3$	802
8	−CONH$_2$	943
8	−NH$_2$	955
8	..	971

Fig. 2. FAB-Mass spectrometry of synthetic MII (Peninsula Laboratories) with the sequence of MII and assignment of the peaks illustrated above. #988 peak and 1010 peak are derived from the molecular ion plus H$^+$ or Na$^+$, respectively.

recently this technique required prior derivation of the peptide and yielded limited information. The prospects for MS sequencing of peptides were notably improved with the introduction of a new ionization technique, fast atom bombardment (FAB) (Rinehart, 1982). Dr. Kenneth Rinehart and his colleagues at the University of Illinois at Urbana-Champaign became interested in helping us determine the sequences of MI and MII, and with their skilled help, the structures for both peptides were determined (Witten et al., 1984a) (see Table 1). Figure 2 shows the analysis of synthetic MII and illustrates the power of FAB-MS.

The sequences do indeed confirm the marked similarity between MI, MII, AKH, and RPCH. This can be demonstrated biologically as well. On the one hand AKH shows similar myoactivity on the locust leg assay (M. O'Shea and J. L. Witten, 1983, unpublished observation), while MI and MII both have adipokinetic activity, MII apparently being the more potent (O'Shea et al., 1984). It seems quite likely that these peptides explain the activity of Periplaneta c.c. extracts on crustacean chromatophores as well.

Using HPLC analysis, we were able to demonstrate that the cockroach c.c. not only contains large amounts of MI and MII, but also releases that material on depolarization with high levels of potassium by a calcium dependent mechanism. The experiment is illustrated in Figure 3. This
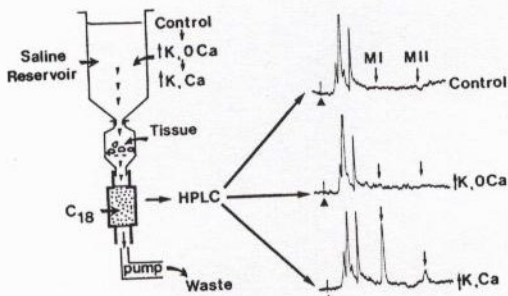
FIG. 3. Release of MI and MII from *P. americana* c.c. Twenty-two c.c.'s were placed in a perfusable chamber and bathed with insect saline followed by high K⁺ saline lacking and then containing Ca⁺⁺. Each perfusate was pumped through a separate disposable reverse phase C-18 cartridge and at the end of the experiment the cartridges were eluted with methanol. The eluates were dried *in vacuo* and analyzed by HPLC. The right side of the figure shows fluorescence profiles of the HPLC analysis. The arrows indicate the elution positions of MI and MII which appear only with High K⁺ in the presence of Ca⁺⁺.

work compliments results on locust c.c. showing that AKH is contained in secretory vesicles within glandular lobe cells (Stone and Mordue, 1979), and that there is an increase in exocytotic pits following a physiological stimulus for AKH release, flight (Rademakers and Beenakkers, 1977). Taken together a rather complete picture of neurosecretion by the c.c. of RPCH-AKH family members is established.

At the same time we were searching for myoactive peptides in *Periplaneta americana* c.c. another group, at Zoecon, was pursuing the cardioacceleratory factors previously reported. Not surprisingly they simultaneously came upon the same peptides which they called periplanetin c.c.-1 and periplanetin c.c.-2 (corresponding to MI and MII respectively) (Scarborough *et al.*, 1984). Using somewhat different techniques they purified and sequenced the peptides with the same results. Scarborough *et al.* demonstrated that MI and MII

do indeed have the appropriate cardioacceleratory effect on the Gersch assay, making it virtually a certainty that neurohormone D and MI are identical. Further, they showed that both MI and MII cause significant increases in carbohydrate levels when injected into cockroach. This could explain previous reports of a hyperglycemic factor in cockroach and locust c.c. (Steele, 1961; Goldsworthy *et al.*, 1972). This activity is particularly interesting because of the observed similarity between MII and glucagon (Scarborough *et al.*, 1984; Table 2). Although the structural similarity between MII and a segment of glucagon near its amino terminal end is not as striking as that between RPCH-AKH family members, it is considerable, and both peptides' possessing carbohydrate mobilizing activity seems an interesting coincidence. Since it is likely that the MII precursor contains a basic amino acid processing site beyond the MII sequence, an additional homology with lys (12) of glucagon may be present. Further if one rearranges the order of ser and thr in the speculative sequence for compound II shown in Table 1, this too would show a marked similarity to glucagon.

Tager *et al.* (1976) have investigated glucagon-like immunoactivity in the c.c. of *Manduca sexta*, *Plodia interpunctella*, and *Periplaneta americana*. In all cases the immunoactivity was associated with compounds that are apparently larger than MII judging from their behavior on gel permeation chromatography. Glucagon-like immunoactivity from *Manduca*, which is apparently of similar molecular weight to mammalian glucagon, was shown to elevate carbohydrate levels in *Manduca*. Thus it is far from clear that RPCH-AKH family members are the only carbohydrate elevating factors in c.c. One possible explanation of the large molecular weights observed by Tager *et al.* is that the gluca-

TABLE 2. *Homology between MII and glucagon.*

Glucagon	H-His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	...
			*		*	*	*							
MII			pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp-NH₂				

gon antibodies they employed recognized precursor proteins rather than the fully processed peptides. Final judgments on this issue, as well as on the possible relationships between AKH-RPCH family members and glucagon must await characterization of the peptides' precursor molecules and ideally their genes. Such studies are currently underway in my laboratory.

Immunohistochemical evidence strongly suggested a neuropeptide role for an AKH-like peptide just as we began to suspect this based on the myoactivity of MI, MII, and AKH. Schooneveld et al. (1983) reported that the peroxidase antiperoxidase technique used with an antibody developed against the [Tyr¹] analogue of AKH effectively stained the glandular cells of the locust c.c. as well as a variety of neurons in the locust brain and the subesophogeal ganglion. The Schooneveld antibody is also effective in staining P. americana neurons (Witten et al., 1984b; Witten and O'Shea, manuscript in preparation). A variety of neurons stain throughout the cockroach nervous system including a number which are good candidates for motoneurons. Figure 4 illustrates several of the neuron types seen in the abdominal ganglia as well as axons running along the midgut (Fig. 4C). These axons give new meaning to previous reports of c.c. extract effects on gut motility (Cameron, 1953; Davey, 1962; Brown, 1965). Thus one or more of the RPCH-AKH family members may, like proctolin, be active on skeletal, gut, and cardiac muscle. Of course, much work remains to be done to clarify these provocative histochemical findings, including the chemical characterization of the immunoreactive compounds. In addition to immunological techniques, the potential of invertebrate nervous systems for unambiguous neuronal identification and direct chemical analysis (see for example O'Shea and Bishop, 1982) can be exploited to directly identify the peptide(s) in neurons of interest.

Target Cell Studies

The staining of axonal processes and the clear evidence of hormonal release suggest that RPCH-AKH family peptides act in a variety of settings. Unfortunately little is known about the receptors which recognize the peptides. Stone et al. (1978) have studied the adipokinetic activity of various synthetic analogues in Locusta, while Christensen et al. (1979) performed similar studies of pigment concentration in Palaemon. The structure-activity relationships in these two systems are rather different. For pigment concentrating activity, a peptide must have a trp-NH₂ sequence with other parts of the structure influencing potency but being less critical. So gly-trp-NH₂ has ¹⁄₁₀,₀₀₀ the potency of RPCH and pglu-pro-gly-trp-NH₂ has ¹⁄₁₀₀ RPCHs activity. On the other hand adipokinetic activity seems to be more sensitive to the whole molecule. The analogues phe-thr-pro-asn-trp-gly-thr-NH₂ and pglu-leu-asn-phe-thr-pro-trp-NH₂ have less than 0.003 the agonist activity of AKH. Stone et al. (1978) propose that a minimum of 8 residues are required for reasonable activity and noted with interest this is just the size of RPCH (and now MI, MII, and compound II). Amino acid changes in the AKH sequence effect potency but do not point to a critical site. Since these are in vivo assays it is not possible to know to what extent they reflect receptor requirements, however at least some of the analogues produce effects over the same time course as the natural hormones, and so presumably their potencies do not differ on the basis of their rate of metabolic breakdown. The differences in structure-activity relationships between the color change receptor and the adipokinetic receptor may simply reflect the evolutionary distance between two very different organisms. However it might be that these are receptor types which occur in the same organism and which are an important aspect of activity modulation.

Several groups have studied the cellular basis of the hyperglycemic and adipokinetic effects. As might be expected from mammalian examples, both appear to be mediated by cAMP which activates protein kinases (Steele, 1963; Goldsworthy, 1970; Hanaoka and Takahashi, 1977; Gade, 1979; Pines et al., 1981). These peptides may not always employ cAMP as a second messenger however, as the case of proc-

tolin illustrates (O'Shea and Schaffer, 1985).

FUTURE DIRECTIONS

I have briefly described the biochemical identification and the best studied bioactivities of the currently recognized members of the RPCH-AKH family. It should not be assumed that this is a complete catalogue of family members. Indeed when one considers that two closely related insects, locust and cockroach, contain at least four distinct family members, and that there are many reports of related bioactivities in different animals, as well as chromatographic evidence of a wide distribution of MI and MII in arthropods (O'Shea et al., 1984), it seems more reasonable to suppose that a good number of family members await discovery. How is it that locust and cockroach both require at least two members each in the same neuroendocrine gland despite the fact that the peptides are structurally so similar and apparently have, at least qualitatively, similar bioactivities? Why is it that cockroach c.c. contains MI and MII but no AKH while locust c.c. contains neither MI or MII (M. Schaffer, unpublished observations, 1983). Even if no more peptides are discovered, the question of why arthropods need so many similar peptides deserves an answer. This situation is not unique to the RPCH-AKH family. For example the opiate peptides in mammals show considerable structural homology and have overlapping bioactivities. Although there are some interesting ideas about what the function of the opiate family might be (Weber et al., 1983) the precise physiological functions of these peptides are not known (Woolf and Wall, 1983), and so it is impossible to judge to what extent these ideas have physiological significance. In order to characterize RPCH-AKH peptide family neural systems to the point of their becoming instructive models of peptide function considerable work must be done on the biochemical, anatomical and neurophysiological levels, but the great value of the relatively simple invertebrate systems is that the currently available powerful biotechnologies permit detailed analysis at each of
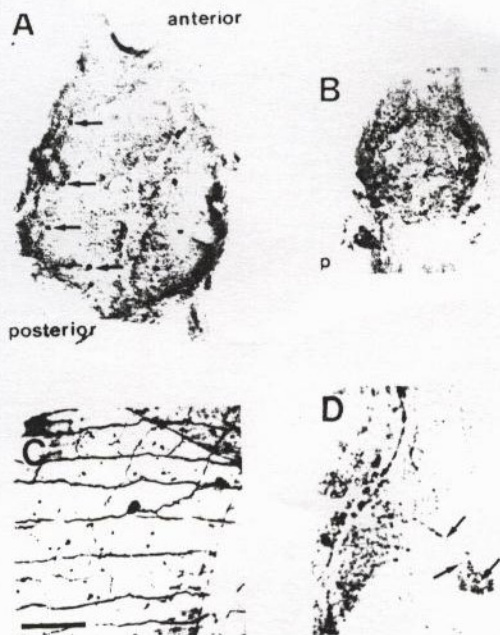
FIG. 4. Immunostaining of AKH-like material in *P. americana* neurons prepared by Dr. Jane L. Witten. The peroxidase antiperoxidase procedure was employed using the Schooneveld antibody. A. Terminal ganglion showing 4 bilaterally symmetrical cell bodies (arrows). B. Another abdominal ganglion showing cell bodies and processes. C. Axons containing AKH-like immunoactivity coursing over the midgut. D. Cell bodies located at the nerve root of an abdominal ganglion with projections into the ganglion.

these levels. As this analysis progresses, I think we will begin to understand why arthropods use so many peptides with such carefully conserved structures. I do not presume to know what the answer to that question will be, but I certainly suspect the answer will be an interesting one that will have implications beyond the arthropods and the RPCH-AKH family.

ACKNOWLEDGMENTS

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