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LOCALIZATION OF BURSICON IN CCAP-IMMUNOREACTIVE CELLS IN THE THORACIC GANGLIA OF THE CRICKET *GRYLLUS BIMACULATUS*

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Summary

Bursicon is a neuropeptide that induces tanning of the cuticle in freshly moulted insects. In an earlier investigation, we demonstrated that bursicon activity can be detected throughout the ventral nerve cord of the cricket *Gryllus bimaculatus*. This study aims at identifying the neurosecretory cells within the thoracic ganglia that produce bursicon. When homogenates of anterior pieces of thoracic ganglia were separated using SDS gel electrophoresis, proteins with bursicon activity could be eluted only from a slice of the gel spanning the 28–33 kDa region. In the anterior lateral cortex of the thoracic ganglia, there are two bilaterally paired neurosecretory cells with large vacuoles that project contralaterally to neurohaemal release sites associated with segmental nerves N5 and N6. These cells and their processes in N5 and N6 were labelled using antisera against crustacean cardioactive peptide (CCAP). The cell projecting into N6 showed a Tyndall effect (i.e. appeared opaque under oblique illumination) in older adults, and single isolated somata contained bursicon activity. Homogenates of nerves N5 and N6 also showed bursicon activity, but neither bursicon activity nor CCAP-immunoreactive processes were found in segmental nerve N4. The thoracic connectives, which contain three major CCAP-immunoreactive processes, also showed bursicon activity. Homogenates of posterior pieces of the thoracic ganglia did not contain bursicon activity. Western blots demonstrated that the anti-CCAP serum does not recognize the 30 kDa bursicon-active protein fraction. These results suggest that a CCAP-like neuropeptide and a protein with bursicon activity are co-localized in the anterior lateral neurosecretory cells of the thoracic ganglia and in their segmental homologues in the other ganglia. Additionally, we have shown using western blots that a monoclonal antibody raised against a 56 kDa protein from the housefly *Musca domestica*, a protein thought to be bursicon, does not label the 30 kDa bursicon-active protein of crickets. However, this antibody does label an unidentified 56 kDa protein isolated from anterior as well as posterior pieces of thoracic ganglia.

Key words: insect, cricket, *Gryllus bimaculatus*, neuropeptide, bursicon, CCAP, immunohistochemistry, western blot.

Introduction

Bursicon is an insect neurohormone that initiates sclerotization of the newly formed cuticle following each moult (Truman, 1973, 1981; Reynolds *et al.* 1979). Bursicon has been found in all ganglia of the ventral nerve cord of several insects (Mills and Lake, 1966; Vincent, 1972; Taghert and Truman, 1982; Honegger *et al.* 1988; Kostron *et al.* 1995). In the tobacco hornworm *Manduca sexta*, two pairs of neurosecretory cells that contain bursicon have been identified in the abdominal ganglia (Taghert and Truman, 1982; Tublitz and Sylwester, 1990), and the most anterior of these neurones (neuroendocrine cell L1) is immunoreactive to an antiserum raised against crustacean cardioactive peptide (CCAP) (Davis *et al.* 1993) and, at the time of ecdysis, to an anti-cyclic-GMP antiserum (Ewer *et al.* 1994). The lateral white (LW) neurones of the abdominal ganglia in the American cockroach *Periplaneta americana* (Adams and O'Shea, 1981; O'Shea and Adams, 1981) have been reported to contain bursicon (Adams and Phelps, 1983), and the LW cells are also CCAP-immunoreactive (N. T. Davis, personal communication).

In *Locusta migratoria*, anti-CCAP antiserum stains a number of different cells in the ventral ganglia, including a pair of large anterior lateral cells designated as type 1 and type 2 neurones (Dircksen *et al.* 1991). Because the somata of both the type 1 neurones and the LW cells (1) have one or several large vacuoles, (2) are CCAP-immunoreactive, (3) are located

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in comparable positions in the ganglia, and (4) have similar projections, they appear to be homologous cells. The ventral ganglia of *Manduca sexta* also contain paired lateral cells (L1 and In704) that are CCAP-immunoreactive and comparable to the types 1 and 2 neurones of locusts (Davis *et al.* 1993; Ewer *et al.* 1994; Klukas *et al.* 1996).

These various studies suggested that CCAP-immunoreactive lateral neurosecretory cells may also be present in *Gryllus bimaculatus* and that these cells may contain bursicon. Previously, we reported that bursicon activity is found throughout the ventral ganglia of this insect, but the cells responsible for this activity were not identified (Kostron *et al.* 1995). We undertook the present investigation to determine whether CCAP-immunoreactivity could be used as a means of locating the bursicon-containing cells. As part of this study, it was necessary to determine whether the anti-CCAP antiserum recognized bursicon and to perform bursicon bioassays on pieces of ganglia, on peripheral nerves containing CCAP-immunoreactive axons and their projections, and on single isolated somata. Furthermore, the protein fraction responsible for bursicon activity from the ganglion pieces containing the CCAP-immunoreactive cells was isolated. The development of these methods for the isolation of bursicon will serve as the first phase of our long-term goal to isolate and determine the primary structure of bursicon from *G. bimaculatus*.

Materials and methods

For all experiments, last-instar larvae and adult male and female crickets *Gryllus bimaculatus* were taken from our own laboratory culture. Animals were immobilized by chilling them on ice, and the entire nerve cord or single ganglia were removed in ice-cold cricket physiological saline solution (Honegger and Schürmann, 1975).

Preparation of homogenates for bursicon bioassays

Immediately after removal, thoracic ganglia were collected on Parafilm in 5 μl of inhibitor buffer [Tris–HCl buffer, pH 7.4, containing a cocktail of protease inhibitors without phenylmethylsulphonyl fluoride (PMSF); Kaltenhauser *et al.* 1995] and frozen on dry ice. At least 10 frozen ganglia were pooled and stored at −80 °C until used for homogenization.

In order to cut anterior lateral and posterior lateral pieces of thoracic ganglia precisely (see Fig. 1B, inset), the thoracic ganglia were placed onto a thin brass plate which was then transferred to dry ice for 1 min. Pieces were cut from the frozen ganglia with a microscalpel under a dissection microscope, collected in drops of inhibitor buffer, refrozen on dry ice, and processed in the same manner as for the whole ganglia.

Homogenization was performed on ice in a glass homogenizer. Homogenates consisted of one ganglion in 7.5 μl of phosphate buffer (PB; 0.1 mol l^{-1}, pH 7.4) or one pair of anterior or posterior ganglion pieces in 4 μl of PB. For each experiment, between 50 and 300 ganglia or paired structures were homogenized. The homogenates were centrifuged at 15 000 ***g*** at 4 °C for 10 min. The supernatant was either used directly in bursicon bioassays or was partially purified before use (see below).

Partial purification of bursicon

To increase the relative amount of bursicon in the homogenates, a two-step prepurification was performed. This involved a heating step (75 °C for 10 min) followed by a trichloroacetic acid (TCA, 10 %) precipitation. The precipitated pellet was redissolved in PB, and the resulting solution was tested for bursicon activity; the amount of protein was measured according to Bradford (1976; see Kostron *et al.* 1995).

SDS–polyacrylamide gel electrophoresis and gel elution

The methods are described in detail in Kostron *et al.* (1995). Briefly, the bursicon-active protein solutions were heated in sample buffer (see Kostron *et al.* 1995) without 2-mercaptoethanol and loaded onto a 10 % polyacrylamide gel containing 0.1 % SDS (Laemmli, 1970). After electrophoresis, the gels were cut into slices aligned with prestained marker proteins (Sigma SDS 7b and Amersham rainbow high molecular mass). The slices were homogenized and agitated in elution buffer. After centrifugation, the proteins were precipitated in TCA (10 %), washed briefly in acetone, frozen at −80 °C and dissolved in 5–10 μl of saturated urea solution. The urea solution was diluted with PB to a final volume of 80 μl. This solution was tested for bursicon activity.

Bioassay

Bursicon activity was assayed using the ligated fly bioassay (Fraenkel and Hsiao, 1965) as described in Honegger *et al.* (1992) and Kaltenhauser *et al.* (1995). In brief, 5 μl of a test solution was injected into flies neck-ligated at hatching, and the amount of tanning was judged 3 h later. A completely tanned fly received a score of 6 points. The bursicon scores given are the average scores for 10 flies for each test. Control scores are from flies injected with the buffer solutions alone and may reach up to 0.5 points. Therefore, this score is taken to indicate the absence of detectable levels of bursicon in a solution.

Immunocytochemistry

Whole-mount preparations were fixed overnight at 4 °C in 4 % paraformaldehyde in PB (0.1 mol l^{-1}, pH 7.4). Phosphate-buffered saline (0.154 mol l^{-1} NaCl in 0.01 mol l^{-1} PB, pH 7.4; PBS) containing 0.3 % Triton X-100 was used for the preliminary washes, and 0.1 % bovine serum albumin (BSA) was added for diluting the primary and secondary antisera. Before incubation in the primary antiserum, the tissues were treated for 2 h in a blocking solution containing 3 % normal goat serum in the PBS wash solution. Whole mounts were incubated in a 1:1000 dilution of rabbit anti-CCAP antiserum (2TB; Dircksen and Keller, 1988) overnight at 4 °C. They were then washed in PBS containing 0.3 % Triton X-100 and powdered milk. The rabbit IgG was labelled using the avidin–biotin

(ABC) technique as follows. Tissues were incubated in a 1:250 dilution of biotinylated goat anti-rabbit IgG (Dako) overnight at 4 °C; after washing, the tissues were incubated overnight at 4 °C in a 1:100 dilution of an avidin–biotin–peroxidase solution prepared from a Vectastain ABC kit; tissues were then washed in PBS followed by Tris–HCl buffer (pH 7.6). A solution of 0.025 % 3.3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.005 % H_2O_2 was used as a chromogen and, after immunostaining, the whole mounts were dehydrated in isopropanol, cleared in methyl salicylate, and mounted in Canada Balsam. Photographs were taken using a Leitz Dialux 20 photomicroscope.

The following controls were performed: (1) incubation in the primary antiserum was omitted; (2) whole mounts incubated in neither the primary nor the secondary antiserum were reacted with the DAB/H_2O_2 solution; (3) a sample of the primary antiserum at the working dilution was preabsorbed for 18 h at 4 °C with 40 μmol l^{-1} synthetic CCAP (Sigma). No specific staining of neurones occurred after these three control treatments.

During the course of these experiments, a new anti-CCAP antiserum developed by H. Agricola at the University of Jena, Germany, became available. This antiserum immunostained the same neurones and projections as did the anti-CCAP antiserum of Dircksen and Keller (1988). The Agricola antiserum was used at dilutions of 1:5000 and 1:10 000 and gave less background staining, allowing for a better visualization of the projections within the ganglia. To use this antiserum, all the procedures were the same as those described above except that the tissue was fixed overnight at 4 °C in aqueous Bouin's solution. Enzyme-linked immunosorbent assays (ELISAs) showed that the Agricola antiserum did not cross-react with several other peptides, including proctolin (H. Agricola, personal communication). Preabsorption of the Agricola antiserum at a dilution of 1:10 000 with 10 μmol l^{-1} synthetic CCAP abolished all specific staining. The CCAP-positive neurones shown in Fig. 2 were stained with this antiserum.

A mouse monoclonal antibody (IgM) which recognizes a 56 kDa neurosecretory polypeptide from the housefly *Musca domestica* (Song and Ma, 1992) was also used in immunocytochemistry. The ganglia were fixed overnight at 4 °C in a PB solution containing 2 % paraformaldehyde and 0.15 % saturated picric acid solution. They were then either treated as whole mounts or cryosectioned at 20 μm. The tissues were washed and incubated in the primary antibody solution (supernatant, used at a dilution of 1:50) as described above for immunostaining with the anti-CCAP antiserum. The secondary antibody, a FITC-conjugated goat antiserum raised against mouse IgM (Sigma), was diluted 1:50 in PBS containing 0.03 % Triton X-100. The whole mounts and sections were incubated overnight at 4 °C, washed in PBS, mounted in 80 % glycerol in PBS, and examined using the Dialux 20 epifluorescence microscope.

Western blots

Following polyacrylamide gel electrophoresis of homogenates, gels were washed for 1.5 h in regeneration buffer (50 mmol l^{-1} Tris–HCl, pH 7.5, containing 29 % glycerol) and electroblotted (Towbin *et al.* 1979) onto Immobilon P membranes (PVDF, Millipore). The membranes were blocked with three 10 min washes in Tris-buffered saline (pH 7.5) containing 0.05 % Tween-20 (TBST) and 3 % milk powder, and then incubated for 48 h at 4 °C in the anti-CCAP antisera either from Dircksen and Keller (1988), diluted 1:500 or 1:1000, or from H. Agricola, used at a dilution of 1:10 000 in TBST, containing 1 % BSA. In addition, membranes were incubated under the same conditions with the monoclonal antibody of Song and Ma (1992) diluted 1:50 or 1:100. The membranes were then washed three times in TBST. Those treated with the anti-CCAP antisera were incubated for 3 h at room temperature in alkaline-phosphatase-labelled goat anti-rabbit IgG (Promega) diluted 1:7500 in TBST. Membranes treated with the monoclonal antibody were incubated in alkaline-phosphatase-labelled goat anti-rabbit IgM (Sigma) diluted 1:500. After two washes in Tris-buffered saline (pH 7.5) (TBS) and a wash in substrate buffer (10 mmol l^{-1} NaCl, 50 mmol l^{-1} $MgCl_2$, in 100 mmol l^{-1} Tris–HCl, pH 9.5) for 15 min each, staining was developed in the dark for 4–8 min in a mixture of Nitroblue Tetrazolium chloride (NTB) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (BCIP) in substrate buffer. The reaction was stopped by a wash in distilled water.

Intracellular recording and staining

Crickets were anaesthetized by chilling, and the thoracic ganglia dissected from the torso. Ganglia were pinned ventral side up in a Sylgard-lined dish, covered with saline, and illuminated from the side to visualize cells showing a Tyndall effect (see Results). Somata were impaled through the sheath with microelectrodes filled with 0.1 mol l^{-1} hexammine cobaltic chloride (resistance 30–40 MΩ). After identification of cells by antidromic stimulation of nerves 5 or 6 contralateral to the soma, they were stained for 20–30 min with a current of 3–5 nA (200 ms depolarizing pulses at 2.5 Hz). Staining was developed in ammonium sulphide and the preparation fixed in alcoholic Bouin's for 2 h. After fixation, cobalt staining was intensified with silver (Bacon and Altman, 1977), and the ganglia dehydrated and cleared in methyl salicylate.

In the double-staining experiments, somata showing a Tyndall effect were impaled and identified as being Al1 neurones by antidromic stimulation from the contralateral nerve 6. They were injected with Lucifer Yellow for 10 min with 5 nA hyperpolarizing current pulses, and ganglia were subsequently fixed overnight in 4 % formaldehyde in PB at 4 °C. Whole-mount immunostaining was performed using the anti-CCAP antiserum from H. Agricola at a dilution of 1:5000 and a Cy3-labelled secondary antiserum at a dilution of 1:200.

Single cell isolation

Thoracic ganglia were dissected and illuminated as described above. After desheathing the ganglia, the cells appearing opaque were exposed by carefully teasing away their

neighbours using sharpened insect pins. A quick flick with a pin through the primary neurite severed the somata from the ganglion. The cells usually adhered loosely to the pin and could be moved to the surface of the saline. Here, cells detached from the pin and could be collected using a small wire loop. Together with a small amount of saline held in the loop by surface tension, they were transferred into 1 μl of distilled water, where they burst open. The drop was then mixed with 5 μl of PB and immediately injected into one head-ligated fly. As controls, somata close to the soma showing the Tyndall effect were isolated and treated in identical fashion.

Results

We have shown recently that cricket bursicon in the thoracic ganglia is a protein of approximately 30 kDa (Kostron *et al.* 1995; Fig. 1A). In order to localize the source of the bursicon activity in the prothoracic ganglion (TG1), the ganglion was divided into pairs of antero-lateral and postero-lateral pieces (as shown in the inset in Fig. 1B), and homogenates of these were then used in SDS gel electrophoresis. As shown in Fig. 1B, the bursicon activity could be found only in homogenates from anterior sections, and only in the eluate of one gel slice that spanned the molecular mass region between 28 and 33 kDa (a result comparable to that obtained using the whole prothoracic ganglion). No activity could be found in homogenates of posterior sections (Fig. 1C). These results support the hypothesis that most of the bursicon-active protein is located in the anterior lateral region of the ganglion.

The bursicon activity in the meso- (TG2) and metathoracic (TG3) ganglia was localized by assaying homogenates of whole ganglia and homogenates of anterior and posterior ganglion pieces. The results are similar to those obtained for the prothoracic ganglion; i.e. bursicon activity was found almost entirely in the anterior lateral part of the TG2 (Table 1). Because TG3 is a composite ganglion, posterior pieces cut as shown in Fig. 1 (inset) include parts of the abdominal neuromeres. When TG3 was divided such that a cut between the origin of nerves 5 and nerve 6 divided the ganglion into an anterior and a posterior part, homogenates of the anterior part scored 4.3 points (Table 1), whereas the homogenate of the posterior part, i.e. a fragment that included the two abdominal neuromeres (homogenized in 8 μl of PB), scored a mean value of 4.5. We therefore deduced that bursicon is contained in neuronal somata in the anterior region of the three thoracic ganglia and also in the abdominal ganglia.

We had previously shown that peripheral nerves N1 and N3 of TG1 contain bursicon activity (Garcia-Scheible and Honegger, 1989). We now extended our search for bursicon activity to the other nerves (N4–N6) of TG1 and its anterior and posterior connectives. Because we wanted to detect very small amounts of bursicon activity, we used a low dilution factor in these assays, i.e. one pair of nerve pieces or connectives was homogenized in 1 μl of PB, rather than in the usual 7.5 μl. The results (Table 2) indicate that nerves N5 and N6 and the connectives do contain bursicon activity, but that nerve N4 does not. The level of bursicon activity is higher in N6 than in N5, and it is higher in the neck connectives than in the first thoracic connectives (see Discussion).

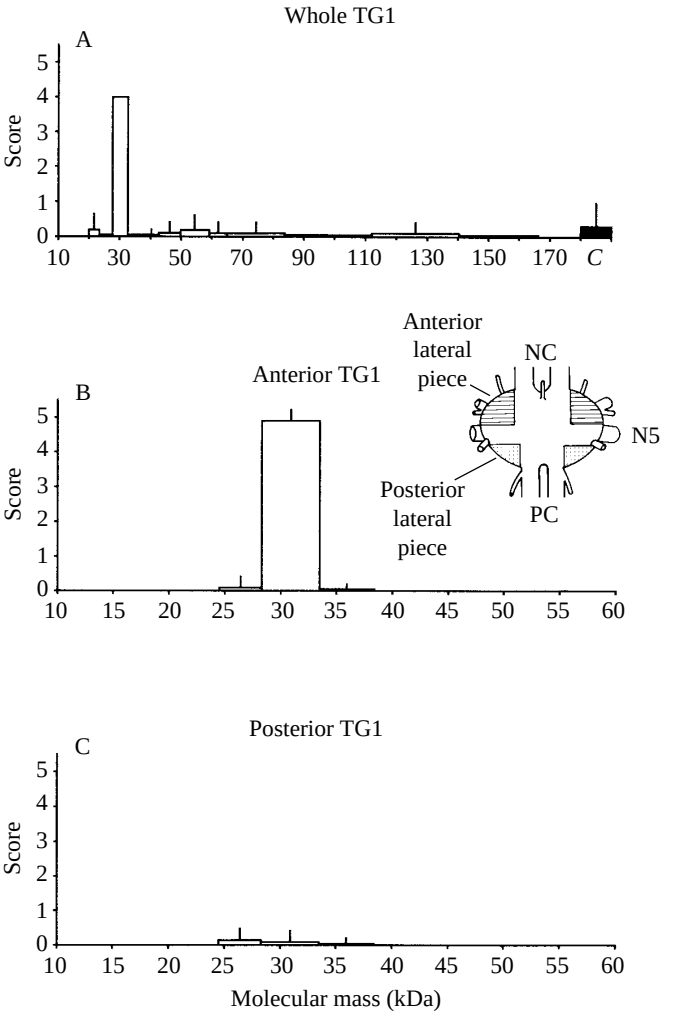

Fig. 1. (A–C) Elution of bursicon activity from SDS–polyacrylamide gels. Prepurified homogenates of 67 whole TG1 (A) or anterior (B) or posterior (C) TG1 pieces (see inset in B), cut from 88 TG1, were run on 10% SDS–PAGE under non-reducing conditions. (A) After electrophoresis, the gel was cut into slices between the dye front and 165 kDa (*x*-axis), and the proteins were eluted from the slices (see Materials and methods). Bursicon activity (score $\geqslant$1.0 points) can only be eluted from a gel slice spanning the molecular mass range between 28 and 32 kDa. Therefore (B,C), slices were cut only in the range between 24 and 38.4 kDa (*x*-axis). Bursicon activity can only be eluted from a gel loaded with homogenates of anterior ganglion pieces and only from the slice cut between 28 and 33 kDa, as for the intact TG1 (A). In A 115 μg, in B 22.8 μg and in C 4.6 μg of total protein was loaded. In C, less protein was loaded onto the gel because the posterior pieces cut from the 88 TG1 are smaller than the anterior pieces cut from the same ganglia (see inset in B). In A, the control score is shown as the shaded column on the right (*C*) (obtained when only phosphate buffer was injected into ligated flies). In B and C, the control scores were 0. Inset: NC, neck connective; PC, posterior connective; N5, peripheral nerve 5; anterior is to the top.

Table 1. *Bursicon activity of homogenates of anterior and posterior pieces of the prothoracic (TG1), mesothoracic (TG2) and metathoracic (TG3) ganglia*

	TG1		TG2		TG3	
	Ant.	Post.	Ant.	Post.	Ant.	Post.
I	3.8	0.5	4.6	0.9	–	–
II	4.8	–	4.9	–	4.3	–
III	4.8	0.7	–	–	–	–
IV	4.6	0.7	–	–	–	–
V	4.7	0.2	–	–	–	–
VI	4.6*	0.1	–	–	4.3†	–

Homogenates of anterior (Ant.) and posterior (Post.) ganglion pieces were tested for bursicon activity in six independent experiments (I–VI). The bursicon scores are means from 10 injected flies (see Materials and methods). One pair of ganglion pieces was homogenized in 4 μl of phosphate buffer. For TG3, only anterior pieces were tested (but see text).

*Bioassay after partial purification of homogenates (see Materials and methods).

†The whole thoracic neuromere was homogenized in 4 μl of PB per ganglion.

Since controls can score up to 0.5 points in the bioassay, only scores above this value are considered to represent bursicon activity (Honegger *et al.* 1992).

Table 2. *Bursicon activity of the connectives and peripheral nerves N4–N6 of TG1*

Tissue	Bursicon score	
NC	3.9	3.9
PC	1.3	1.6
N4	0.1	0.0
N5	3.6	3.6
N6	4.0	4.8
C	0.1	0.2

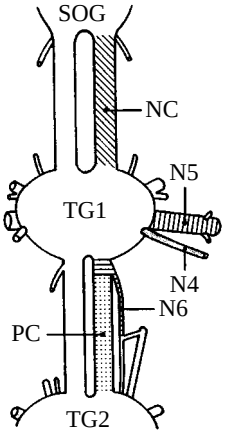

Diagram (right panel) of the prothoracic ganglion (TG1), its nerves and connectives showing the tissues (nerves or connectives) that were tested for bursicon bioactivity.

In each bioassay, one pair of nerves or connectives was homogenized in 1 μl of buffer. As in Table 1, scores are means for 10 injected flies. C, control scores (phosphate buffer). Note that, of the tissues tested, only N4 is bursicon-negative. In two further experiments, similar scores to those shown here were obtained.

SOG, suboesophageal ganglion; NC, neck connectives; PC, posterior connectives to TG1; N4–N6, peripheral nerves 4–6; TG2, mesothoracic ganglion.

Immunocytochemistry

Use of the anti-CCAP antisera results in intense immunostaining of two pairs of large somata in the anterior lateral region, close to the origin of nerve N3 of TG1 (Fig. 2A,B). Owing to their position, they are designated Al1 and Al2 (anterior lateral cells 1 and 2). Al1, in particular, contained at least one large vacuole (Fig. 2D). The Al1 soma shows a Tyndall effect in older adults (see below). The neurite of Al1 projects through the ventral neuropile towards the midline of the ganglion, where it turns dorsally. Within the dorsal part of the ganglion, it extends diagonally into the contralateral nerve N6 (Fig. 2A). Within the sheath of N6, axon collaterals of Al1 form a network of neurohaemal varicosities (Fig. 2E,F). Branch points of the Al1 axon are frequently detected. The neurite of Al2 follows that of Al1 but extends into nerve N5. In one intracellular cobalt fill of Al2, a fine axon branch was seen to project into the contralateral posterior connective (see below). Axon collaterals form only a few neurohaemal varicosities where N5 extends into the thoracic cavity. We have not followed these CCAP-immunoreactive projections further into the periphery.

In TG2 and the thoracic neuromere of TG3, there are pairs of CCAP-immunoreactive cells that are serial homologues of the Al1 and Al2 cells of TG1. Only one bilateral CCAP-immunoreactive cell is found in each of the two abdominal neuromeres of TG3 and in each of the four unfused abdominal ganglia. They appear to be segmental homologues to the thoracic Al1. The terminal ganglion contains one pair of CCAP-immunoreactive neurones in the most anterior neuromere and three intensely stained cell pairs in its most posterior part between the origins of the cercal nerves. In addition to these sets of neuroendocrine cells, there are a number of smaller somata in TG1–TG3 and in the other ventral ganglia that are CCAP-immunoreactive. These cells appear to be interneurones, but their arborizations and projections could not be traced in whole mounts. There is also extensive CCAP-immunoreactivity in the neuropile in all ganglia, but its origin could not be determined as the intracellular fills of Al1 and Al2 show that these two cells contribute only little to it (see below). In the suboesophageal ganglion (SOG), there are two pairs of CCAP-immunoreactive somata in each of the maxillary and labial neuromeres and one pair in the mandibular neuromere. One pair in each of the maxillary and labial neuromeres appears to be the segmental homologue of the thoracic Al1. The second neurone in each neuromere sends a major axon posteriorly that extends to the terminal ganglion, as does a third axon probably deriving from a soma in the protocerebrum. Thus, three prominent CCAP-immunoreactive axons descend through the entire nerve cord. In addition, fine CCAP-immunoreactive fibres project into all connectives, but the neuronal origin of only one of them (Al2 in TG1) could be determined.

The monoclonal antibody raised against a 56 kDa haemolymph protein reported to have bursicon activity in *Musca domestica* (Song and Ma, 1992) was also used in immunostaining of whole mounts or frozen sections of TG1 of

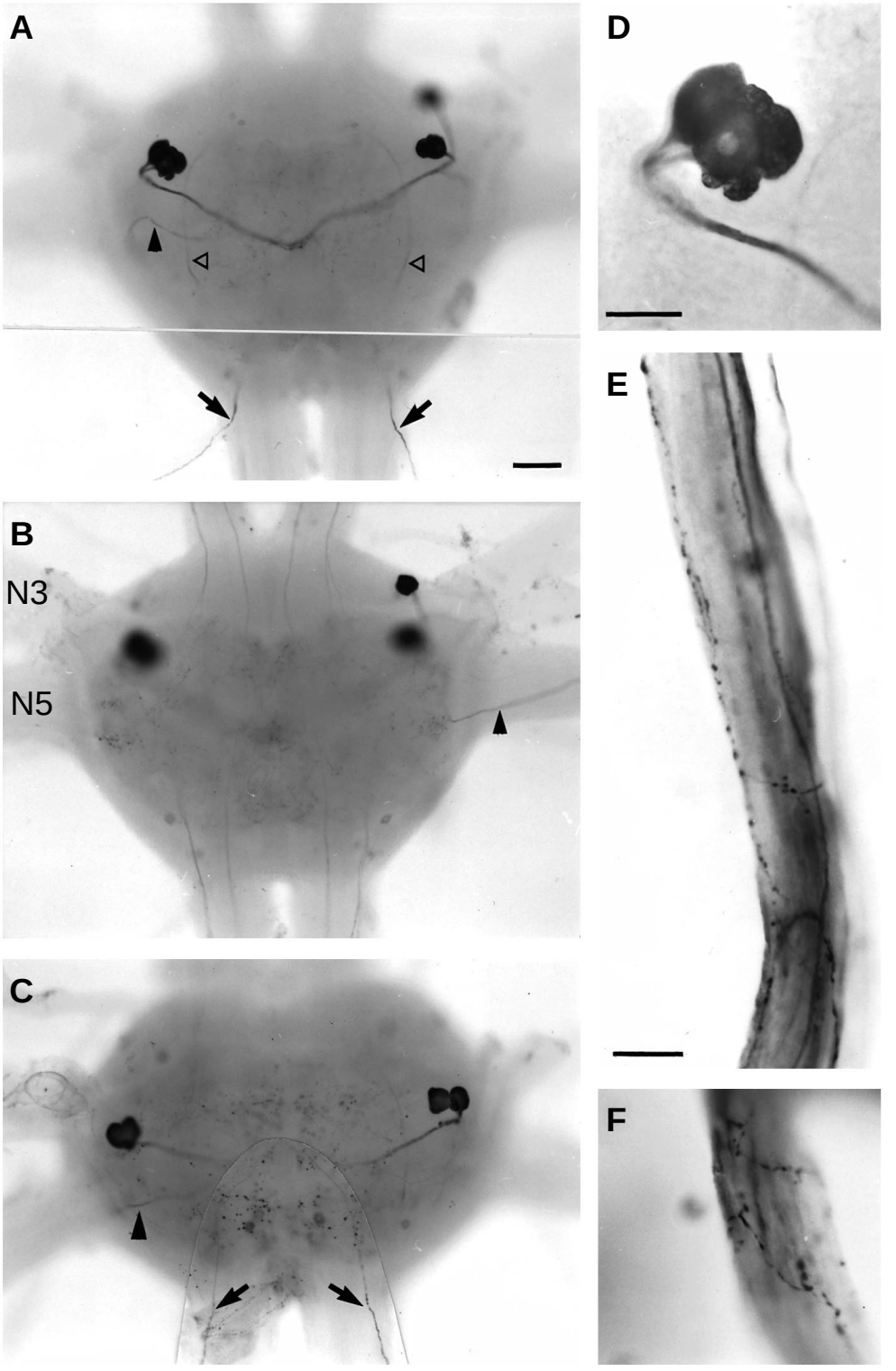

Fig. 2. (A–F) CCAP-immunoreactive cells in whole mounts of the prothoracic (TG1) and mesothoracic (TG2) ganglia from the last larval instar of *Gryllus bimaculatus* (about 1 day before moulting) (anti-CCAP antiserum from H. Agricola, 1:5000 dilution). (A) Ventral view of TG1 showing two immunoreactive somata on either side of the ganglion; on the right side, the smaller one is out of focus. The axon of one cell in each hemiganglion projects into the contralateral nerve N6 (arrows), and the axon of the other cell projects into the contralateral nerve N5 (arrowhead, see also B). Open triangles point to axons of CCAP-immunoreactive interneurones running through TG1 and TG2. (B) The same preparation as in A but with a more dorsal focus showing two of the three prominent CCAP-immunoreactive axons in the anterior and posterior connectives. In this focal plane, the smaller of the two CCAP-immunoreactive somata on the right is in focus, as is the CCAP-immunoreactive axon in nerve N5 (arrowhead). (C) CCAP-immunoreactive cells and projections in TG2 showing an organization homologous to that in TG1. The origin of the axon projections in N6 can be followed to the midline of the ganglion. Note also the fine CCAP-immunoreactive varicosities in the neuropile. Dark dots are four of approximately 10 small weakly stained cells. (D) Higher magnification of the two CCAP-immunoreactive somata on the left side of the ganglion shown in A. Note that one of the two cells has large intensely stained vacuoles. The multiple vacuoles shown here may be a fixation artefact as only one large vacuole can usually be seen. (E,F) Two focal planes of nerve N6 showing the labelled axon and its collaterals with varicosities in the neural sheath. Scale bars, 100 μm in A–C, 50 μm in D, 30 μm in E, F.

G. bimaculatus. However, no somata or processes were stained.

In adult animals older than 5 weeks after the imaginal moult, but not in young adults and larvae, one large soma in the antero-lateral region of TG1–TG3 was found to display a distinct Tyndall effect, i.e. it appeared whitish and opaque under oblique illumination. When eight such somata were impaled with microelectrodes and stained, all the cells resembled Al1 morphologically and they all projected into the contralateral N6. Likewise, cobalt backfills of nerve 6 (N=15) revealed only one large soma in the anterior TG1 contralateral to the stained nerve. Double labelling with intracellularly injected Lucifer Yellow and subsequent CCAP-immunostaining showed that the opaque soma was CCAP-

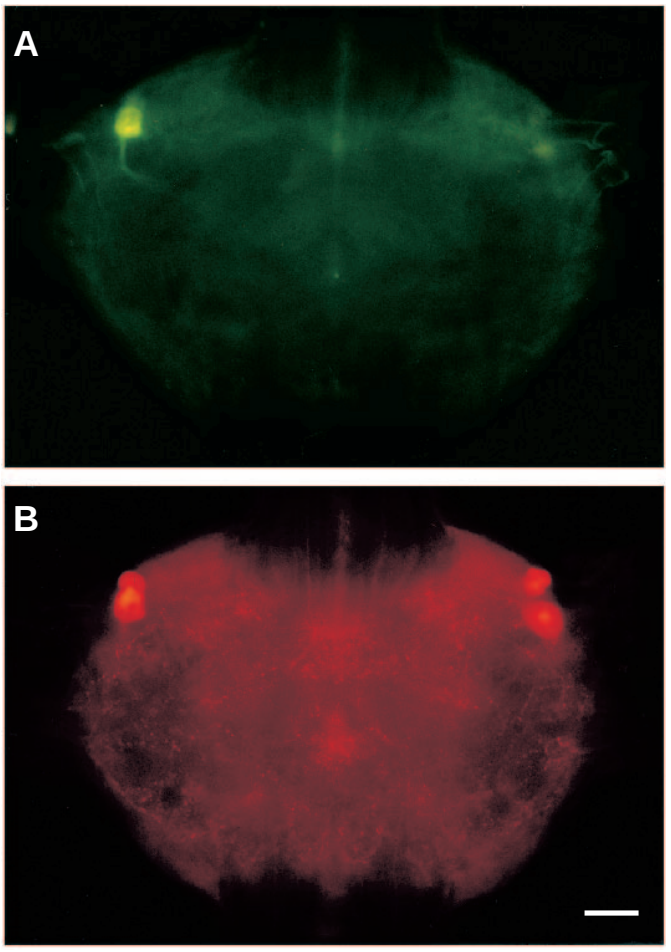

Fig. 3. (A,B) Double labelling of the Al1 neurone. Ventral views of the same prothoracic ganglion. (A) The left Al1 was injected with Lucifer Yellow and photographed. Subsequently (B), the same ganglion was processed for immunohistochemistry using the anti-CCAP antiserum from H. Agricola and a Cy3-labelled secondary antibody. Al1 and Al2 on both sides of the ganglion are immunolabelled. Note the large vacuole of Al1 in A. Scale bar, 100 μm for A and B.

immunoreactive (Fig. 3A,B). Al2 was also stained intracellularly by probing with the microelectrode in the vicinity of the Al1 soma for a neurone that could be stimulated *via* the contralateral nerve 5. The morphology of Al1 and Al2 is shown in Fig. 4A,B. Their soma position and size, as well as the course of their primary neurites and axons, exactly match those observed in CCAP-immunoreactive cells. We conclude, therefore, that Al1 and Al2 are unique neurones and not members of populations of cells with similar morphology. The amount of cobalt hexammine chloride injected was calculated to reveal the central projections of Al1 and Al2. It was not sufficient to identify the Al1 axon collaterals and the network of neurohaemal varicosities shown in Fig. 2E,F after immunostaining.

Bioassays for bursicon activity in a single Al1

The 30 kDa bursicon-active protein of TG1 is found primarily in the anterior lateral portions of the ganglion and in nerves N5 and N6 (Table 1). Correspondingly, CCAP-immunoreactivity is found in antero-laterally located cells that project into these nerves. Therefore, Al1 and Al2 in the thoracic ganglia (and their homologues in the other ganglia) seemed to be the likely source of bursicon activity. Since Al1 in the antero-lateral TG1–TG3 can be identified by its Tyndall effect in older adults, this hypothesis was tested for Al1 in the thoracic ganglia. Seven single opaque Al1 somata were isolated from TG1 and TG2 of three crickets and each one was diluted in 6 μl of PB and injected each into one head-ligated fly. The bursicon scores of the seven injected flies were 3, 1, 4, 2, 4, 5 and 4. Control somata from the vicinity of Al1, also diluted in 6 μl of PB, showed scores of 0, 0, 1, 0, 0, 0.5 and 0. This results supports the hypothesis that the CCAP-

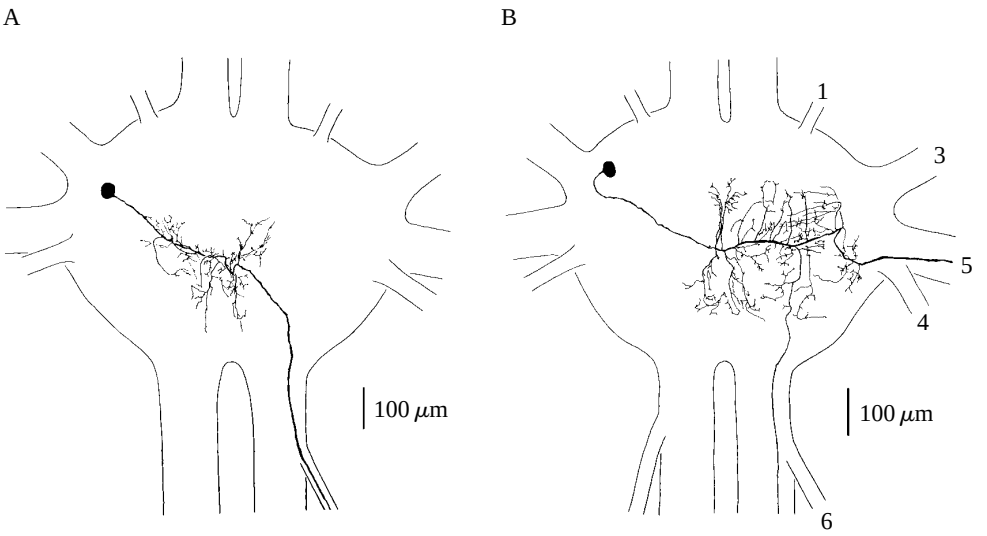

Fig. 4. (A,B) Morphology of prothoracic antero-lateral neurones Al1 (A) and Al2 (B) as revealed by intracellular staining with cobalt hexammine chloride (anterior is to the top). Note that the dendritic ramifications of both neurones are not very dense and major branches emerge near the sagittal plane of the ganglion. In the case of Al2, most ramifications are on the side contralateral to the soma and are located close to the ventral edge of the neuropile. The vacuole(s) are not shown because, after cobalt ionophoresis and silver intensification, it is difficult to distinguish the vacuoles from adjacent cell bodies that are weakly stained during probing for the somata of Al1 and Al2. 1–6, nerves 1–6.

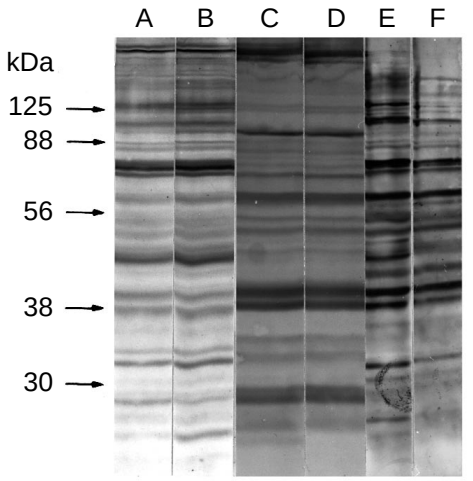

Fig. 5. (A–F) Western blots of homogenates of whole TG1 (lanes A–D) and of the anterior (lane E) and posterior (lane F) ganglion pieces. Electrophoretic separation of proteins was on a 10 % PAGE under non-reducing conditions. PVDF membranes were incubated with anti-CCAP antisera either from Dircksen and Keller at a dilution of 1:500 (A,B,E,F) or from Agricola at a dilution of 1:10 000 (C,D). (B) The anti-CCAP antiserum was preabsorbed with 40 μmol l^{-1} synthetic CCAP. (D) The antiserum was preabsorbed with 10 μmol l^{-1} synthetic CCAP. Total protein loaded in A–E was 50.7 μg; E,F 10 μg. Bursicon scores of the homogenates were A and B, 4.1 points; C and D, 4.0 points; E, 4.6 points; F, 0.7 points.

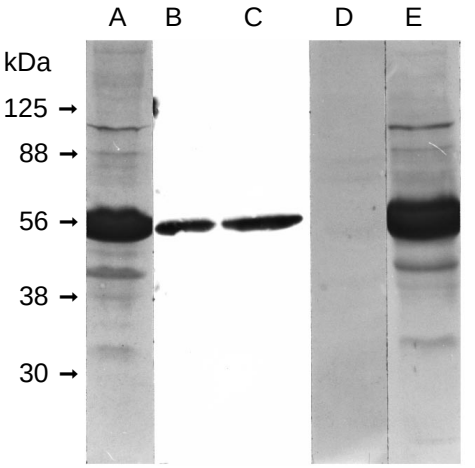

Fig. 6 (A–E) Western blots labelled with a monoclonal antibody against the *Musca domestica* 56 kDa haemolymph protein (Song and Ma, 1992) diluted 1:100 in A, D and E, and 1:50 in B and C. Electrophoresis of proteins was on a 10 % PAGE under reducing conditions in A–D, and under non-reducing conditions in E. (A) Bursicon-active homogenate of whole ganglia (96 μg of protein loaded, score 4.2 points). (B) Homogenate of anterior TG1 pieces (6.4 μg of protein, bursicon score 4.8 points). (C) Homogenate of posterior TG1 pieces (6.4 μg of protein, bursicon score 0.6 points). (D) Homogenate of whole ganglia (96 μg of protein loaded) with the primary antibody omitted. (E) Homogenate of whole ganglia (96 μg of protein, bursicon score 4.2) electrophoresed under non-reducing conditions. Note that the same major protein is labelled under both non-reducing (E) and reducing (A–C) conditions.

immunoreactive Al1 indeed contains bursicon. The differences in the scores may be explained by the fact that not all the cells completely emptied their contents with our methodology.

Western blots

Bursicon activity occurs in CCAP-immunoreactive cells. Yet bursicon is unlikely to be CCAP since CCAP does not mimic bursicon activity at concentrations between 10^{-8} and 10^{-3} mol l^{-1} (Kaltenhauser, 1994). To obtain further evidence that bursicon is different from CCAP, it was of interest to determine whether the anti-CCAP antisera cross-react with bursicon. This question was studied using western blots. If the antisera to CCAP cross-react with bursicon, they should react with the 30 kDa bursicon-active protein band in SDS gels (CCAP itself is not retained on the gel).

Western blots of homogenates of whole TG1 and TG2, and of anterior and posterior sections of TG1, were incubated with the anti-CCAP antisera (see Materials and methods). As controls, blots of homogenates of whole TG1 were incubated with antisera that had been preabsorbed with CCAP (40 μmol l^{-1}) or incubated without the primary antisera. No bands were stained in the blot in which the antisera were omitted (data not shown). The blots treated with the antisera (Fig. 5A,C,E,F) and with preabsorbed antisera (Fig. 5B,D) demonstrated that the antisera bind non-specifically to many proteins. A faint band can be seen at 30 kDa in all four blots incubated in the antiserum from Dircksen and Keller, and a double band can be seen in the blots incubated with the antiserum from Agricola, including the two preabsorption control blots for both antisera. Therefore, the staining at 30 kDa must be interpreted as non-specific staining (see Discussion).

The gels of electrophoretically separated proteins of homogenates of anterior and posterior sections of TG1 were also used in western blots to test the monoclonal IgM from Song and Ma (Song and Ma, 1992). As a control experiment, the monoclonal antibody was omitted. The results from the western blots showed that this antibody also recognizes a 56 kDa protein from anterior and posterior pieces of TG1 in *G. bimaculatus* but does not recognize the 30 kDa bursicon-active protein of this insect (Fig. 6A–C,E). There was no labelling in the control experiment (Fig. 6D). The failure of this antibody to recognize the 30 kDa bursicon of crickets conforms with its failure to immunostain neurones in TG1–TG3 (see above).

Discussion

We have shown recently that highly purified bursicon from the meal beetle *Tenebrio molitor* has a molecular mass of 30 kDa (Kaltenhauser *et al.* 1995). Furthermore, we have shown that the ventral nerve cords of *Periplaneta americana*, *Gryllus bimaculatus*, *Locusta migratoria* and *Calliphora erythrocephala* all contain bursicon, and that this bursicon has a similar molecular mass to that of *Tenebrio molitor*, i.e. approximately 30 kDa (Kostron *et al.* 1995).

The monoclonal antibody produced by Song and Ma (1992)

reacts with a 56 kDa haemolymph protein from *Musca domestica* that has bursicon activity. Therefore, it seemed possible that it might detect bursicon in *Gryllus bimaculatus* and in other species as well. Our experiments now indicate that this monoclonal antibody does not recognize cricket bursicon. In our western blots (Fig. 6), it labels intensely a protein of about 56 kDa, as it does in *Musca domestica*. However, this staining is seen even in lanes loaded with homogenates from posterior pieces of TG1 that do not contain bursicon activity. Moreover, the monoclonal antibody does not detect proteins in the 30 kDa range from which bursicon is exclusively eluted. These results are comparable with those of Kaltenhauser (1994), who found that the monoclonal antibody did not detect highly purified bursicon from *Tenebrio molitor* in western blots. Since bursicon seems to be a protein of similar molecular mass (30 kDa) in various insects, including the fly *Calliphora erythrocephala* (Kostron *et al.* 1995), it is surprising that bursicon from the housefly *Musca domestica* is a 56 kDa protein. Song and Ma (1992) suggested that bursicon might exist in different molecular forms. Progress in the investigation of the molecular structure of bursicon is needed to reveal whether closely related species may have different forms.

Our present study shows that, in *Gryllus bimaculatus*, bursicon activity is found mostly in the antero-lateral parts of TG1 and TG2, not in the postero-lateral parts. Immunostaining of these two thoracic ganglia demonstrates that pairs of CCAP-immunoreactive neuroendocrine cells (Al1 and Al2) are located in the anterior lateral region of each ganglion, and this is the same region in which bursicon activity is found. Up to now, Al1 and Al2 are the only peptidergic and neuroendocrine cells detected in this part of these two ganglia (as also shown using a conventional staining method; Gaude, 1975), and we show here that the isolated soma of Al1 contains bursicon. The conclusion that Al2 also contains bursicon is supported by our finding that bursicon activity is found in nerves N5 and N6, into which the CCAP-immunoreactive processes of Al2 and Al1 project. To our knowledge, no anastomoses exist between nerves N6 and N5 that would allow axon collaterals from Al1 to project onto nerve N5. In addition, we do not know of any projections onto nerve N5 of peripheral neurosecretory cells that contain bursicon activity (Garcia-Scheible and Honegger, 1989). We are confident, therefore, that the bursicon activity in homogenates of nerve 5 is represented by bursicon in Al2. N6 contains a much higher bursicon activity than N5. This may be explained by the fact that nerve 6 carries many axon collaterals with numerous varicosities (see Fig. 2E,F) whereas nerve 5 has only a few varicosities. We cannot explain the difference in the bursicon score between the neck connectives and first thoracic connectives.

The anti-CCAP antiserum of Dircksen and Keller (1988) has been shown to recognize authentic CCAP in certain crustaceans and in two insect species (Stangier *et al.* 1987, 1989; Lehman *et al.* 1993). We have tested this antiserum using western blots to determine whether it also recognizes bursicon. Because we used a 10 % polyacrylamide gel, CCAP (which consists of nine amino acid residues) is absent from the gel and, as expected, no specific staining for CCAP was detected in these western blots. The anti-CCAP antiserum does bind to several proteins, including weak binding to proteins in the 30 kDa range where bursicon activity is found (Fig. 5). However, the antiserum binds to all of these proteins even after it has been preabsorbed with synthetic CCAP, indicating that the staining that is observed in the western blots is non-specific and that the CCAP-specific antibodies within the serum do not recognize bursicon. Non-specific staining can also be observed when using the anti-CCAP antiserum of H. Agricola (Fig. 5). Data from binding assays (Kaltenhauser, 1994) support the conclusion that the two antisera do not recognize bursicon: anti-CCAP antiserum bound to Concanavalin A–sepharose was unable to remove bursicon activity from solutions. However, the western blots and binding studies do not completely exclude the possibility of cross-reactivity between the anti-CCAP antiserum and bursicon in immunocytochemistry. The lack of cross-reactivity can only be resolved unambiguously when the amino acid sequences of bursicon and the CCAP-related peptides in the cricket are known.

In spite of these uncertainties, our experiments suggest that the neuroendocrine cells Al1 and Al2 co-produce bursicon and a CCAP-like peptide. CCAP-immunoreactivity and bursicon activity may frequently be associated for the following reasons. (1) The lateral region of the labial neuromere of the SOG contains bursicon (Garcia-Scheible and Honegger, 1989), and this is the region where the pair of CCAP-immunoreactive somata are located. (2) The connectives that contain the three major CCAP-immunoreactive axons of intersegmentally projecting interneurones in the brain and SOG also show bursicon activity. (3) Lateral neuroendocrine cells of the ventral nerve cord of *M. sexta* and *P. americana* have been shown to contain bursicon (Taghert and Truman, 1982; Adams and Phelps, 1983) and to be CCAP-immunoreactive (Davis *et al.* 1993; P. Bräunig, unpublished observations). However, in *Gryllus bimaculatus* and *Periplaneta americana*, there are dense CCAP-immunoreactive projections in the entire neuropile of all ganglia, which probably represent the branchings of local interneurones with small somata. Pieces cut from the posterior regions of TG1 and TG2 show low bursicon activity. This activity can be attributed to the few branches of Al2 (compare Fig. 1, inset, and Fig. 4B). Therefore, it is unlikely that these local CCAP-immunoreactive interneurones co-produce bursicon. Thus, the probable co-localization of CCAP-like peptide and bursicon seems to be restricted to the large anterior neurosecretory cells Al1 and Al2 in the thoracic ganglia, to their segmental homologues and to intersegmental CCAP-immunoreactive interneurones in the SOG and the brain.

Our study has focused particularly on the location of bursicon-containing cells in TG1 and TG2 because these two ganglia are well suited for this task in that they are large and not fused with other ganglia. Since we used anti-CCAP antisera as a tool to locate bursicon-containing cells, it was not the intention of this study to work out the detailed neuroanatomy of the CCAP-immunoreactive neurones of *Gryllus bimaculatus.*

However, it has been noted that the two anti-CCAP antisera used in this study stain most prominently two pairs of large lateral neurosecretory cells in each of the thoracic ganglia, one pair of large cells in the abdominal ganglia, and serial homologues in the SOG of *Gryllus bimaculatus*. Comparable CCAP-immunoreactive cells are found in *Locusta migratoria*, *T. molitor* and *M. sexta*, where the anatomy has been worked out in detail (Dircksen *et al.* 1991; Breidbach and Dircksen, 1991; Davis *et al.* 1993; Klukas *et al.* 1996).

We previously demonstrated that the median nerve, and nerves N1 and N3 of TG1, also contain bursicon activity (Garcia-Scheible and Honegger, 1989). We now know that the surfaces of these nerves are covered with many varicose neurohaemal CCAP-immunoreactive processes which derive from CCAP-immunoreactive neuroendocrine Al1 homologues in the SOG (P. Bräunig and H. W. Honegger, unpublished results). Therefore, it appears that bursicon may be released from much of the surface of the peripheral nervous system and not just from well-defined neurohaemal organs. In addition, peripheral neurosecretory cells in the neck region of *Gryllus bimaculatus* appear to contain bursicon activity (Garcia-Scheible and Honegger, 1989), but are not labelled by the anti-CCAP sera. Similarly, in the abdominal ganglia of pharate adult *Manduca sexta*, four lateral neurones contain bursicon (Taghert and Truman, 1982; Tublitz and Sylwester, 1990) but only the most anterior of these neurones (L1) is CCAP-immunoreactive (Davis *et al.* 1993). These observations support the hypothesis that bursicon and CCAP-immunoreactivity may be co-localized in certain neurone populations but not in others.

The co-localization of bursicon and CCAP in neuroendocrine cells may facilitate their simultaneous release in response to the same stimuli. Bursicon triggers the sclerotization of the new cuticle after moulting, and CCAP increases the contractile properties of the visceral muscles of the heart, hindgut and/or oviduct (Dircksen, 1994). Since the new cuticle is expanded immediately after ecdysis by a rise in haemolymph pressure, the increase in the activity of the circulatory organs may play a crucial role in both the expansion process and the distribution of bursicon.

Since bursicon triggers the sclerotization of the newly formed cuticle after eclosion, it is somewhat surprising that the nervous systems of adult *Gryllus bimaculatus* and *Periplaneta americana* contain high levels of bursicon. In *Locusta migratoria*, bursicon activity decreases appreciably with adult age (Kostron *et al.* 1995). In this insect, internal cuticular structures continue to develop for up to 3 weeks after adult emergence (Andersen, 1973) and, during this period, bursicon would still be necessary. Furthermore, in adult insects, bursicon is needed in the process of wound repair when epidermal cells secrete new cuticle (Neville, 1975). The continuing ability to repair wounds in conjunction with the continuous production of bursicon throughout adult life may correlate with different life strategies for survival as well as with differences in longevity among insects.

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