Two nitridergic peptides are encoded by the gene capability in Drosophila melanogaster

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Kean, Laura, William Cazeneve, Laurence Costes, Kate E. Broderick, Shirley Graham, Valerie P. Pollock, Shireen A. Davies, Jan A. Veenstra, and Julian A. T. Dow. Two nitridergic peptides are encoded by the gene capability in Drosophila melanogaster. Am J Physiol Regulatory Integrative Comp Physiol 282: R1297–R1307, 2002. First published January 17, 2002; 10.1152/ajpregu.00584.2001.—A Drosophila gene (capability, capa) at 99D on chromosome 3R potentially encodes three neuropeptides: GANMGLYAFPRV-amide (capa-1), ASGLVAFPRV-amide (capa-2), and TGPSASSGLWGPL-amide (capa-3). Capa-1 and capa-2 are related to the lepidopteran hormone cardioacceleratory peptide 2b, while capa-3 is a novel member of the pheromone biosynthesis-activating neuropeptide/diapause hormone/pyrokinin family. By immunocytochemistry, we identified four pairs of neuroendocrine cells likely to release the capa peptides into the hemolymph: one pair in the subesophageal ganglion and the other three in the abdominal neuromeres. In the Malpighian (renal) tubule, capa-1 and capa-2 increase fluid secretion rates, stimulate nitric oxide production, and elevate intracellular Ca2+ and cGMP in principal cells. Capa-stimulated fluid secretion, but not intracellular Ca2+ concentration rise, is inhibited by the guanylate cyclase inhibitor methylene blue. The actions of capa-1 and capa-2 are not synergistic, implying that both act on the same pathways in tubules. The capa gene is thus the first to be shown to encode neuropeptides that act on renal fluid production through nitric oxide.

THE NEUROPEPTIDE cardioacceleratory peptide (CAP) 2b (CAP2b) has a unique mode of action, involving the generation and autocrine action of nitric oxide (NO). It is one of a number of cardioactive peptides (CAP1a, CAP1b, CAP2a, CAP2b, and CAP2c) found in the hawk moth Manduca sexta (35, 36). Manduca CAPs have been shown not only to affect heart rate in insects and Crustacea (31) but also to modulate hindgut contractions in insects (34). Sequence is not yet available for CAP1a, CAP1b, or CAP2c, although CAP2a (PFCNAF-TGC) has been shown to be identical to crustacean CAP (1). Purification and sequencing of CAP2b from ventral cords of adult pharate moths showed that this peptide exists as an octapeptide, pyro-ELYAFPRV-amide (11). A peptide with physicochemical properties very similar to CAP2b has been demonstrated in Drosophila and was shown to have potent action on epithelial fluid transport by Malpighian (renal) tubules (3). None of the other M. sexta CAPs modulates fluid transport by Drosophila tubules (3).

In tubules, CAP2b stimulation of fluid transport is triggered by activation of the NO-cGMP signaling pathway. CAP2b treatment results in increased intracellular cGMP concentration (3), and application of NO synthase (NOS) (2). Furthermore, the soluble guanylate cyclase inhibitor methylene blue inhibits CAP2b-stimulated transport and abolishes the rise in cGMP, suggesting that synthesis of cGMP is important in CAP2b action. Soluble guanylate cyclase is the major intracellular target of NO, which is synthesized by NO synthase (NOS) (2).

Drosophila tubules express the single-copy gene for Drosophila NOS (dNOS) (4), encoding a Ca2+/calmodulin-sensitive NOS that has most similarity to vertebrate neuronal NOS (27). We previously showed that NOS activity increases on CAP2b stimulation (4). More recently, NOS has been immunolocalized to only principal cells (2), suggesting compartmentalization of the NO signaling pathway in tubules.

Thus CAP2b is a particularly intriguing insect neuropeptide: the first such peptide to be defined as an extracellular modulator of NO signaling in insects and one that also stimulates Ca2+ signaling via Ca2+ entry. The Drosophila genome project is now essentially complete, and sequence for the 130 MB of euchromatic DNA is publicly available. This allows the genetic correlates of physiological properties to be established unambiguously. We show here that the Drosophila genome contains a neuropeptide gene, capability (capa),
which encodes two CAP2b-related peptides (capa-1 and capa-2), together with a third (capa-3), which is most closely related to Bombyx diapause hormone. Similar to lepidopteran CAP2b, the authentic Drosophila peptides capa-1 and capa-2 act on the Malpighian tubule to stimulate fluid production through intracellular Ca\textsuperscript{2+}, NO, and cGMP.

**EXPERIMENTAL PROCEDURES**

**Drosophila stocks.** Drosophila were maintained on a 12:12-h light-dark cycle on standard corn meal-yeast-agar medium at 25°C. The Oregon R strain (8) and the P(GALA) (30) and upstream activating sequence G (UAS\textsubscript{G})-aequorin lines (28) have been described previously. To produce flies in which apoaequorin was expressed in a particular spatial or temporal pattern, the appropriate GAL4 driver line was crossed with a line carrying the apoaequorin transgene under control of the yeast UAS\textsubscript{G} promoter, as previously described (28). In the resultant progeny, apoaequorin is expressed only in cells in which GAL4 is being expressed. For these experiments, the c42 and c710 lines were used to drive expression to the principal and stellate cells of the main segment, respectively (28, 33); such "c42-aeq" and "c710-aeq" flies were maintained as homozygous lines. For tubule dissection, flies were cooled on ice and then decapitated before isolation of whole tubules.

**Materials.** Coelenterazine was purchased from Molecular Probes and dissolved in ethanol before use. Anti-cGMP antibody and fluorescein-conjugated secondary antibody were purchased from Calbiochem, cGMP RIA kits (Amerlex-M) from Amersham Pharmacia, Schneider's medium and Ca\textsuperscript{2+}-free Schneider's medium from GIBCO Life Technologies, and zaprinast, the cGMP-dependent phosphodiesterase inhibitor, from Calbiochem. The neuropeptides pyro-ELYAFPRV-amide (CAP\textsubscript{2b}) (3), GANMGFLYAFPRV-amide (capa-1), and ASGLVAFPRV-amide (capa-2) and the precursor peptide SDSPLASLNDGLEGAVLDG were synthesized by Research Genetics. All other chemicals were obtained from Sigma.

**Identification of capability.** The CAP\textsubscript{2b} peptide is COOH-terminally amidated and is presumably flanked by cleavage sites in the prepropeptide but is too short for straightforward similarity searching. Accordingly, the "baat" peptide ELYAFPRVKRELYAFPRVKR was used to search the available Drosophila genomic and cDNA sequence at the Berkeley Drosophila Genome project site (www.fruitfly.org) using the TBLASTN search at lowest stringency. In early 2000, this produced hits against a single expressed sequence tag (clone GH21009) and to the extreme 3' end of a previously published genomic sequence for the transient receptor potential (trp) gene at 99D. The clone was obtained and sequenced fully on both strands. The deduced peptide was compared with known proteins using the National Center for Biotechnology Information BLASTP similarity search, again at low stringency, and was found to contain two peptides that resembled CAP\textsubscript{2b}, together with a peptide that resembled Bombyx mori diapause hormone. All sequences were flanked with monobasic or dibasic cleavage sites (37).

The searches were continued until submission of the manuscript in June 2001, without detection of any further matches. Inasmuch as the euchromatic genome of *D. melanogaster* is known to high accuracy, the probability that there is any further gene encoding peptides closely similar to CAP\textsubscript{2b} is very low.

**Generation of antisera to capa-encoded peptides.** Antisera were raised to two different peptides: ASGLVAFPRV-amide (capa-2) and SDSPLASLNDGLEGAVLDG, part of the capa gene product encoded by the second exon. Conjugates were prepared by coupling 2 mg of each peptide to 5 mg of thyroglobulin, with difluorodinitrobenzene used as the coupling reagent, as described elsewhere (32). A single female New Zealand White rabbit was injected with each conjugate. The first injections were performed with complete Freund's adjuvant; for subsequent injections, incomplete Freund's adjuvant was used. The rabbit injected with the capa-2 conjugate was injected every 6 wk for a total of four injections. The rabbit injected with the capa-precuror peptide was injected every 2–4 wk for a total of four injections. Rabbits were bled, and serum was collected 10 days after each booster injection.

The antiserum to capa precursor peptide gave some background immunoreactivity and, therefore, was purified on a capa precursor HiTrap (Amersham Pharmacia Biotech) affinity column according to the manufacturer's instructions. Both acid- and base-sensitive affinity-purified antibodies gave good immunostaining, but the acid-sensitive antibodies were used here. IgG against capa-2 and the precursor peptide were purified from the respective antisera with octanoc acid, diethylaminoethyl HPLC-grade water, and Schliiter-Allen columns. Aliquots were then labeled with 6-((7-amino-4-methylcoumarin-3-acetyl)amino) hexanoic acid succinimidyl ester (AMCA) and/or 5-((and-6)-carboxytetramethylrhodamine succinimidyl ester (tetrarhodamine) (Molecular Probes), as described elsewhere (38a).

**Transport (fluid secretion) assays.** Malpighian tubules were isolated into 10-μl drops of a 1:1 mixture of Schneider's medium and Drosophila saline (in mmol/l: 117.5 NaCl, 20 KCl, 2 CaCl\textsubscript{2}, 8.5 MgCl\textsubscript{2}, 10.2 NaHCO\textsubscript{3}, 4.3 NaH\textsubscript{2}PO\textsubscript{4}, 15 HEPEs, and 20 glucose) under liquid paraffin, and fluid secretion rates were measured as described in detail elsewhere (8) under the conditions described. All peptides were added as solutions in assay medium. Methylene blue, when used, was added as solution in assay medium.

**Measurements of intracellular Ca\textsuperscript{2+} concentration using aequorin transgene.** For each assay, 20 tubules from 4- to 14-day-old c42-aeq adults were dissected in Schneider's medium. Tubules were pooled in 160 μl of the same buffer containing the apoaequorin cofactor coelenterazine (2.5 μM final concentration); reconstitution of aequorin occurred on incubation in the dark for 4–6 h (28, 33). Bioluminescence recordings were made using a luminometer (model LB9507, Berthold Wallac); recordings were made every 0.1 s for each tube. Each tube, containing 20 tubules, was used for a single data point; after intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) was recorded, tissues were disrupted in 350 μl of lysis solution [1% (vol/vol) Triton X-100 and 100 mM CaCl\textsubscript{2}], causing complete discharge of the remaining aequorin and allowing estimation of the total amount of aequorin in the sample. Ca\textsuperscript{2+} concentration was calculated as previously described (28). Mock injections with Schneider's medium were applied to all samples before treatment with neuroneptides.

**Determination of NOS activity by the Griess reaction.** Fifty intact tubules were dissected into 300 μl of Schneider's medium. Before stimulation with peptide, medium was removed and replaced with fresh Schneider's solution. Capa-1, capa-2, or CAP\textsubscript{2b} was applied at 10\textsuperscript{-7} M for 10 min. Samples were chilled on ice and homogenized. The Griess assay was used to detect formation of NO\textsubscript{2} , a stable product of NO, in stimulated and control tubule homogenates. Modified Griess reagent (100 μl; catalog no. G4410, Sigma) and 2.6 ml of water were added to the homogenates, and the reactions were incubated at room temperature in the dark for 20 min. Standard curves were generated using 0–20 μM sodium nitrite (Sigma) standards in Schneider's medium. Absorbance was measured in standards and samples at 540 nm.
Concentrations of NO$_2$ in samples were determined from the standard curve, which was linear over the entire concentration range tested. The Griess assay carried out on tubules did not utilize NADPH and nitrate reductase, inasmuch as initial experiments showed that these reagents did not alter the amount of NO$_2$ detected in control and stimulated samples (data not shown); this suggests that NO is primarily metabolized to NO$_2$ in tubules.

**Immunocytochemistry.** The protocol used for immunohistochemistry was the same as that described elsewhere (38). Antiserum to capa-2 was diluted 1:1,000–1:3,000, and the antiserum to the capa precursor peptide was diluted 1:2,000–1:4,000, or, in the case of affinity-purified antibodies, 1:500. Antiserum to pheromone biosynthesis-activating neuropeptide (PBAN) was a kind gift from Dr. G. Fabrias (16) and was used in a dilution of 1:2,000. Incubations in the primary antibodies were performed overnight. A Texas red-conjugated affinity-purified goat anti-rabbit antibody (Jackson Immunologics) was used for visualization of the primary antiserum. As double and triple labelings, the tissues were incubated subsequently with unlabeled peptide antibody, a fluorescein-labeled F(ab) fragment of goat anti-rabbit IgG (Jackson Immunologics) to visualize the first peptide antibody, and AMCA- and/or tetramethylrhodamine-labeled purified IgG to visualize one or two of the other peptides.

For immunocytochemical detection of cGMP induced by exposure to capa neuropeptides, intact tubules were preincubated with 10$^{-4}$ M zaprinast for 10 min and then stimulated with the appropriate peptide at 10$^{-7}$ M for 10 min. Tubules were fixed in 4% (vol/vol) parafomaldehyde for 30 min, washed twice for 1 h in PBS containing 1% (wt/vol) cold fraction V bovine serum albumin (Sigma)-1% (vol/vol) Triton X-100 (PAT), and incubated overnight in 5% (vol/vol) normal goat serum containing rabbit polyclonal anti-cGMP antibody diluted 1:3,000 in PAT. After three washes in PAT (1 h), tubules were subsequently incubated with fluorescein-labeled secondary antibody (1:250 dilution; Vector Labs) and washed twice for 1 h in PAT and once for 5 min in PBS. All these procedures were carried out at room temperature. Stained tubules were mounted in Vectashield (Vector Labs). Whole mount tubules were examined with a Molecular Dynamics Multiprobe laser scanning confocal upright microscope. The excitation (488 nm) and emission (515 nm) barrier filters were appropriate to the fluorescein-based label of the secondary antibody. Images were viewed with NIH Image.

**Measurement of tubule cGMP concentration.** cGMP concentrations were measured in isolated tubules by radioimmunoassay using anti-cGMP antibody (Amerlex-M kit, Amersham Pharmacia), as described previously (3). Briefly, 20 tubules per sample were incubated with 10$^{-4}$ M zaprinast for 10 min in Schneider’s medium before stimulation with appropriate neuropeptides for a further 10 min. Samples were quenched with ice-cold ethanol and homogenized. Supernatants were dried and resuspended in 0.05 M sodium acetate buffer (Amersham Pharmacia) and assayed for GMP content.

**Statistics.** Values are means ± SE. Where appropriate, the significance of difference between data points was analyzed using Student’s t-test, with $P < 0.05$ taken as the critical level.

**RESULTS AND DISCUSSION**

The capa gene. The only hits within the Drosophila genome that gave near-perfect matches for the core CAP$_{2b}$ sequence within the “bait” peptide (using TBLASTN) were expressed sequence tags for clones GH21009, and later GH28004 (GenBank accession no. A1517299). The GH28004 cDNA was obtained from Research Genetics and was sequenced fully on both strands; the resulting cDNA and deduced peptide sequences are shown in Fig. 1A. The gene was named “capability” (capa), because it clearly has the ability to encode two neuropeptides of the CAP$_{2b}$ family. The compact gene contains a 592- and a 71-bp intron (Fig. 1B).

Interestingly, the first splice site coincides exactly with the putative signal peptide cleavage site (Fig. 1). There was no evidence from the genomic sequence or the sequenced cDNAs for multiple transcripts from the gene. Capa sits very close to the trp gene (Fig. 1B); only 268 bases separate the end of the published trp cDNA from the start of the capa cDNA. This means that most upstream regulatory sequences for capa could be concentrated in a relatively small area. Within this short upstream region, there is a high density of putative binding sites for Drosophila transcription factors, as assessed by MatInspector (http://transfac.gbf.de/) (26); there are one or more matches for each of deformed, fushi-tarazu, crocodile, broad complex, hunchback, snail, and delta transcription factors. Promoter analysis (http://www.fruitfly.org/seq_tools/promoter.html) reveals a good match, including a TATA box 47 bp upstream of the start of the cDNA sequence. The predicted transcriptional start site is 17 bp upstream of the start of our cDNA sequence. Within the capa cDNA (Fig. 1), there are three potential initiator codons (ATG) in frame with the putative peptides. The first initiator codon (ATG) that is in-frame with the major open reading frame (nt 72–74 of the cDNA; Fig. 1A) is the most likely translational start site, because it produces a prepropeptide with a plausible signal peptide, as is required for secreted proteins (14, 15). The automated annotation of the cDNA sequence by the Berkeley Drosophila Genome Project, however, starts the translation with the initiator codon (ATG) at nucleotides 171–173 of the cDNA (Fig. 1A), but there would be no plausible signal peptide for this initiator site.

The three putative neuropeptides encoded by capa were named capa-1, capa-2, and capa-3 in order of their position within the prepropeptide. Both CAP$_{2b}$-like peptides, capa-1 and capa-2, are appropriately flanked with upstream dibasic and downstream monobasic convertase cleavage sites (37), and both have COOH-terminal amidation signals (Fig. 1A). Surprisingly, the same gene also encoded capa-3, a peptide that is closely related to the PBAN and diapause hormone, which at least in Lepidoptera are encoded by the same gene. The deduced peptide capa-3 (Fig. 1, A and C) is flanked by dibasic cleavage sites, has a COOH-terminal amidation signal, and is clearly a member of the FxPRL-amide group, also known as pyrokinins. It is most closely related to the B. mori diapause hormone, sharing the COOH-terminal LWFGPRL-amide with this peptide (Fig. 1C), and pyrokinins 5 and 6 from the American cockroach Periplaneta americana (24, 25). Diapause hormone is responsible for inducing embryonic diapause, which does not occur in Drosophila, and the function of cockroach pyrokinins 5 and 6 is un-
known. There is no other significant match to the Helicoverpa PBAN peptide sequence within the Drosophila genome, at the deduced peptide level (searching with BLASTP) or at the nucleotide level (searching with TBLASTN). Nevertheless, capa-3 is probably not the Drosophila PBAN homolog, inasmuch as peptides more closely related to PBAN are potentially encoded by the hugin gene (Fig. 1C). The short neuropeptide of hugin and capa-3 share the pyrokinin signature (Fx-PRL-amide) with the PBAN family but have no other similarities to PBAN (Fig. 1C). Consequently, the function of capa-3 is far from clear. Although we did not synthesize the predicted capa-3 peptide, we assayed Helicoverpa zea PBAN and leukopyrokinin (a kind gift

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of Dr. R. Jurenka) and found that neither had an effect on tubules (data not shown). Thus it seems that the role of capa-3 differs from that of the other two peptides encoded by the capa gene.

**Localization of the capa peptides by immunocytochemistry.** The location of the capa precursor was mapped with three different antisera: one against capa-1, which was expected to recognize capa-1 and capa-2 and, to a lesser degree, peptides with similar COOH-terminals, e.g., capa-3, eclosion-triggering hormone (ETH), and PBAN, a second antiserum against PBAN (expected to recognize PBAN and capa-3 and probably to cross react with ETH’s capa-1 and capa-2), and a third against a linking region of the prepropeptide that does not encode any biologically active neuropeptide. The antiserum to capa-1 and PBAN recognized several cells in the larval nervous system (Fig. 2) that appear identical to myomodulin peritracheal cells (19). The latter cells are likely to contain ETH and, not necessarily, the capa peptides. Cross-reactivity of the capa-1 antiserum with *Drosophila* ETH would not be surprising, inasmuch as the COOH termini of these two peptides have similar structures (Fig. 1C).

To localize the capa peptides more definitively, we raised an antiserum to part of the capa gene product with no structural similarity to the ETH or hugin precursors. The latter antibody recognizes a subset of the capa-1 immunoreactive cells and did not recognize the peritracheal cells. The most strongly immunoreactive cells in the nervous system recognized by the capa precursor antibody have the morphology of neuroendocrine cells and are divided into two different groups. The first group consists of usually three pairs of ventral neuroendocrine cells in the abdominal neuromeres. In the larva, these cells use the surface of three pairs of abdominal median nerves as their neurohemal release site, while in the adult, the dorsal surface of the thoracoabdominal ganglion is used. The second group consists of a single pair of very large neuroendocrine cells in the labial neuromere. In larvae and adults, the axons of these cells project to the cerebral ganglion and leave the brain by means of the nervi corporis cardiaci to the retrocerebral complex (Fig. 2). We believe that only these cells produce the capa precursor and are, hence, the only cells capable of producing capa-1 and capa-2. Significantly, we did not see staining with any of the antisera in the midline mesodermal cells previously implicated in CAP function (35). Thus it seems

![Fig. 2. Immunocytochemical localization of the capa peptides.](image-url)
that the capa gene and its products are genuinely new members of the family.

All members of the CAP2b family stimulate epithelial fluid transport. Fluid secretion by Drosophila tubules is stimulated by all members of the CAP2b family, albeit to different extents (Fig. 3). By inspection, capa-1 is the most potent stimulator of fluid secretion (Fig. 3B). By contrast, fluid secretion rates are stimulated to the same extent by capa-2 at 10^{-10}–10^{-7} M, with a dramatically diminished response at 10^{-8} M; furthermore, compared with capa-1 and CAP2b, there is no discernible rise in fluid secretion rates at 10^{-9} M. CAP2b stimulates fluid secretion maximally at 10^{-4}–10^{-5} M, with dose-dependent stimulated secretion rates at lower concentrations. Inasmuch as capa-1 and capa-2 are encoded by the same gene, it is possible that they are coreleased, and so there is the potential for synergistic interaction between them; however, this appears not to be the case (Fig. 3C). Mixed peptides produce a response intermediate between each peptide individually.

There is a distant structural similarity between the CAP2b-like peptides and ETH (Fig. 1C). ETH is known to act through NO, and so it was conceivable that the NO-stimulating properties of CAP2b merely reflected a cross-reaction with an ETH receptor. Accordingly, we synthesized ETH and found that it stimulated fluid secretion, but far less effectively than CAP2b (Fig. 3D); additionally, these peptides do not increase [Ca^{2+}]_i in principal cells. Therefore, the COOH-terminal PRxamide motif common to the ETH and CAP2b families is not solely responsible for the physiological effects of CAP2b. It thus seems clear that the diuretic effects of capa-1 and capa-2 cannot be ascribed to cross-reaction with an ETH receptor.

All members of the CAP2b family increase [Ca^{2+}]_i in principal cells. Previous work described the CAP2b-induced rise in [Ca^{2+}]_i in principal, but not stellate, cells of the main segment (28). Here we show that these new members of the CAP2b family also induce a [Ca^{2+}]_i rise in principal cells, with similar kinetics (Fig. 4).

All concentrations (10^{-4}–10^{-11} M) of capa-1, capa-2, and CAP2b cause a rise in [Ca^{2+}]_i in principal cells, with no significant differences in the magnitude of the responses induced by all three members of the CAP2b family at 10^{-6}–10^{-11} M. Capa-2, however, is significantly less effective than capa-1 or CAP2b at 10^{-4} and 10^{-5} M. At 10^{-11} M, capa-2 does not cause an elevation of [Ca^{2+}]_i. Surprisingly, given the efficacy of capa-1 in fluid transport assays, there is no discernible difference between the rise in [Ca^{2+}]_i induced by capa-1 and that induced by CAP2b.
Stellate cell \([\text{Ca}^{2+}]_i\) increases significantly after stimulation with all CAP2b-like peptides at \(10^{-7}\) M, although only capa-1 elicits a large enough change to be likely to be physiologically significant. However, there is no significant effect at \(10^{-8}\) M. Interestingly, a capa-1-induced rise in \([\text{Ca}^{2+}]_i\) in stellate cells may account, in part, for the dramatic increase in fluid secretion rates induced by this peptide. This effect of a CAP2b-like peptide on stellate cells has not been documented previously.

All members of the \(\text{CAP}_{2b}\) family elevate NO. \(\text{CAP}_{2b}\) was previously shown to have a unique, nitridergic mode of action; that is, it acts through intracellular \(\text{Ca}^{2+}\) to stimulate the calmodulin-modulated \(d\text{NOS}\) to generate NO in the same cell type that responds to the signal (4). Given that capa-1 and capa-2 peptides appear to act similarly to \(\text{CAP}_{2b}\), it is clearly important to establish whether these peptides are also nitridergic in their action. This is indeed the case (Fig. 4); with the use of the Griess reaction, all three peptides increase...
nitrite generation by Malpighian tubules. Previously, the nitridergic of effect CAP2b had been demonstrated using the arginine-citrulline conversion assay (4); these independent assays give very similar values for CAP2b stimulation of endogenous NOS activity: 1.5-fold by Griess reaction vs. 1.44-fold by arginine-citrulline conversion assay.

If this rise in NO is physiologically relevant, then blockade of the NO signal with the guanylate cyclase inhibitor methylene blue should reduce the effects of capa-1 and capa-2 on fluid secretion, as has been reported for CAP2b (4). Figure 5B shows that methylene blue inhibits diuresis caused by all members of the CAP2b family. Methylene blue fails to suppress the Ca2+/H11001 signal induced by the CAP2b-like peptides (Fig. 5C), so its effect on the capa-signaling pathway is through inhibition of soluble guanylate cyclase, rather than the Ca2+ signal. The fact that the Ca2+ signal is unaffected
may also explain why methylene blue only partially inhibits the effect of CAP2b-like peptides on fluid secretion.

Application of CAP2b, capa-1, or capa-2 at 10^{-7} M results in an elevation of intracellular cGMP levels (Fig. 5D), as has been previously demonstrated for CAP2b (3). All three peptides elevate intracellular cGMP levels significantly above basal; however, capa-1-stimulated cGMP levels (15 ± 2.67 fmol/20 tubules) are significantly lower than capa-2- or CAP2b-stimulated cGMP levels (20 ± 1.2 and 26 ± 3.8 fmol/20 tubules, respectively). This suggests that, as discussed above, the cGMP signal may contribute only in part to the high rates of fluid secretion induced by capa-1. As shown in Fig. 5, E–G, the rise in intracellular cGMP is confined to the principal cells: stellate cells do not show detectable cGMP by immunocytochemistry. This is consistent with the results that the main [Ca^{2+}]; increase is in principal cells and that only principal cells contain NOS.

**Conclusion.** The CAP2b peptide family is unusual in its function. The cardinal CAP2b acts through intracellular Ca^{2+} to stimulate an endogenous NOS, which acts in autocrine fashion through cGMP to stimulate a plasma membrane V-ATPase and, thus, accelerate fluid secretion. This mode of action is unique. Here, we report the first sequence for a gene encoding members of the CAP2b family and show that *Drosophila* contains two peptides, rather than one. We have shown here that the two novel peptides act on *Drosophila* tubules almost indistinguishably from *M. sexta* CAP2b. All three peptides act on the principal cell, and the responses to capa-1 and capa-2 are not additive, implying that multiple peptides, derived from different cells, can converge on a single signaling pathway in a single cell type and thus, quite possibly, but not necessarily, on a single receptor in that cell type.

Do the CAP2b-like peptides act on more than one cell type in Malpighian tubules? Inasmuch as CAP2b-like peptides act through NOS, it is quite conceivable that NO could diffuse to the stellate cells and exert an action there or that the stellate cells have receptors for capa peptides. We were unable to detect increases in stellate cell intracellular cGMP for any of the CAP2b-like peptides (Fig. 5), so any signal in stellate cells is unlikely to be mediated by NO. The targeted aquorin technology allows the resolution of [Ca^{2+}]; signals in the principal and stellate cells. The cardinal CAP2b peptide produces no Ca^{2+} signals in stellate cells (Fig. 4) (28). However, capa-1 and capa-2 significantly increase stellate cell Ca^{2+} when applied at high concentration (Fig. 4B). A rise in stellate [Ca^{2+}]; is necessary for activation of the chloride shunt conductance, although only the capa-1-induced signal is large enough to be likely to modulate cell function. Any activation of the chloride shunt conductance controlled by the stellate cells would act to collapse transepithelial potential difference, as is seen for leukokinin (20, 21). Interestingly, although physiological concentrations of CAP2b increase transepithelial potential difference, very high concentrations (10^{-5} M) have been shown to collapse it (3). It seems, therefore, that capa-1 and capa-2 may act on both cell types, and this parallel activation of cation transport and the chloride shunt pathway may explain its potency in stimulating fluid production, particularly at high concentrations (Fig. 3B). These results are consistent with a model in which CAP2b-like peptides act on G protein-coupled receptors to raise [Ca^{2+}]; in both cell types, although the stellate cell receptor has lower affinity and is capa-1 selective. In principal cells, the [Ca^{2+}]; signal is transduced through the Ca^{2+}/calmodulin-sensitive dNOS (4, 28), whereas in stellate cells, which lack NOS (2), it acts on chloride channels (20, 21). The effect is to produce a broad response to CAP2b-like peptides over a wide concentration range (Fig. 5B). It is not clear, however, whether the concentrations of capa-1 and capa-2 required to stimulate the stellate cell would ever occur physiologically.

When producing antisera to the capa peptides, we anticipated cross-reactivity with *Drosophila* peptides having similar COOH termini; hence, we were not surprised that the capa-1 and PBAN antibodies recognized the same cell. However, unambiguous localization of the capa precursor was obtained using the third antiserum specific for this protein. The capa precursor...
is expressed in two different neuroendocrine cell types: a single pair in the labial neuromere and three pairs in the abdominal neuromeres of the ventral ganglion. It is possible that not all these cells will make capa-1, capa-2, and capa-3, inasmuch as the proteolytic cleavage sites in the precursor are not the same for the three peptides. Significantly, although capa-3 is flanked by dibasic cleavage sites, capa-1 and capa-2 have upstream dibasic cleavage sites but monobasic downstream signals. Expression of different convertases in the two cell types might be responsible for capa-1 and capa-2 being produced in only one of the two cell types expressing the capa precursor. Nevertheless, the neurohemal release sites of the neuroendocrine cells in the abdominal neuromeres are very close to the tissue previously found to contain CAP2b-like biological activity (35); hence, it seems almost certain that these abdominal neuroendocrine cells do indeed produce capa-1 and capa-2.

Why do CAP2b and its related peptides have such a unique mode of action? CAP2b was originally described in the context of a group of cardioactive peptides, and we speculate that CAP2b may contribute to a response orchestrated by the CAPs. NOS has been reported to be induced by parasitic infestation of mosquito Malpighian tubules (5), and taking these lines of argument together, we suggest (7) that CAP2b may act on tubules to modulate tubule NO response and, possibly, to flush the tubules of potentially harmful solutes or microorganisms. Inasmuch as osmoregulation is a critical function in an insect, it is plausible that inputs from multiple hormones will be integrated by the tubules, allowing them to respond appropriately to the needs of the insect. A second modulatory role for capa has recently emerged from a comprehensive survey of clock-regulated genes in Drosophila. Genome-wide microarray analysis has shown that the capa gene is regulated by clock (17), one of the key genes in circadian rhythmicity in Drosophila (10). Tubules are known to contain their own autonomous clock machinery (9) that can be reset by light (12). Capa peptides might thus modulate the tubule clock or tubule function. As we have outlined, the Drosophila Malpighian tube is an ideal tissue for the detailed analysis of clock function, because physiological understanding is more detailed than for almost any Drosophila phenotype (7). This characterization of a gene encoding circadian-regulated neuropeptide may thus prove important in furthering our understanding of peripheral clocks and their function. Inasmuch as peptides closely similar to CAP2b have now been reported in Lepidoptera, Diptera, and molluscs, this peptide family may play an important role across a wide phylogenetic range of animals.

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