The Journal of Experimental Biology 204, 2011–2020 (2001)
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JEB3340

N-ACETYLNEURAMINIC ACID (NANA) STIMULATES *IN SITU* CYCLIC AMP PRODUCTION IN TENTACLES OF SEA ANEMONE (*AIPTASIA PALLIDA*): POSSIBLE ROLE IN CHEMOSENSITIZATION OF NEMATOCYST DISCHARGE

V. HAKTAN OZACMAK[1], GLYNE U. THORINGTON[1], WILLIAM H. FLETCHER[2] AND DAVID A. HESSINGER[1,*]

[1]*Department of Physiology and Pharmacology, School of Medicine, Loma Linda University, Loma Linda, CA 92354, USA* and [2]*Department of Anatomy, School of Medicine, Loma Linda University, Loma Linda, CA 92354, USA*

*Author for correspondence (e-mail: dhessinger@som.llu.edu)

Accepted 12 March 2001

Summary

Cnidocytes, the stinging cells of cnidarians, optimally discharge nematocysts in response to combined physical contact and stimulation of specific chemoreceptors. In the tentacles of certain sea anemones, the primary chemoreceptors bind *N*-acetylated sugars, such as *N*-acetylneuraminic acid (NANA). Sensitization with NANA predisposes contact-sensitive mechanoreceptors (CSMs) to trigger discharge in response to physical contact. In the ectoderm of sea anemone tentacles, cnidocyte/supporting cell complexes (CSCCs) control and trigger nematocyst discharge. Previous findings have implicated cyclic AMP (cAMP) as a second messenger in NANA-sensitized nematocyst discharge. However, no reports have directly demonstrated that the cAMP content of tentacles changes in response to NANA stimulation. We now show that NANA elevates *in situ* cAMP levels in a dose-dependent manner in the ectoderm of tentacles from the sea anemone *Aiptasia pallida*. However, the endoderm of tentacles shows no detectable cAMP response to NANA. The effect of NANA on the cAMP content of the ectoderm is biphasic. Micromolar NANA increases the *in situ* cAMP level, with a maximal response occurring at 1.8×10^{-5} mol l^{-1} NANA. At higher NANA concentrations, the cAMP content decreases to that of controls. Because the cAMP dose/response curve to NANA coincides precisely with the dose/response curves of NANA-sensitized nematocyst discharge and nematocyst-mediated adhesive force, a second-messenger role for cAMP in NANA-sensitized nematocyst discharge is strongly suggested. The addition of isobutyl-1-methylxanthine (IBMX) to the medium with sea anemones increases tissue cAMP levels both in the absence and in the presence of NANA. However, anesthetizing anemones in sea water containing high levels of Mg^{2+} blocks the NANA-stimulated cAMP response of the ectoderm. In addition, our results suggest that NANA-stimulated cAMP may activate endogenous cAMP-dependent protein kinase (PKA) in broken cell preparations of tentacles. Thus, NANA-stimulated cAMP may function as a second messenger in the NANA chemosensory signaling pathway controlling nematocyst discharge.

Key words: sea anemone, *Aiptasia pallida*, nematocyst discharge, *N*-acetylneuraminic acid, cyclic AMP, cyclic-AMP-dependent protein kinase A.

Introduction

Cnidarians capture swimming prey by discharging nematocysts located on their fishing tentacles. Nematocyst discharge is one of the most dramatic and rapid of single-cell events. Discharge is completed in less than 3 ms (Holstein and Tardent, 1984). Despite a large number of studies on nematocyst discharge, the mechanism and control of discharge are poorly understood. *In situ* nematocyst discharge requires sensory stimulation that involves intracellular and intercellular events, indicating that it is a well-orchestrated process. In general, a combination of appropriate prey-derived chemical and mechanical stimuli initiate maximum discharge (Pantin, 1942).

In sea anemones, the unit of nematocyst discharge is an ectodermal cellular receptor/effector complex called the cnidocyte/supporting cell complex (CSCC) (Thorington and Hessinger, 1988b; Watson and Hessinger, 1989a; Watson and Hessinger, 1989b). The CSCCs consist of individual cnidocytes surrounded by two or more supporting cells (Fig. 1). Two general classes of chemoreceptor detect substances derived from prey and predispose a subpopulation of CSCCs (type B CSCCs) to discharge nematocysts in response to suitable mechanical stimuli. One class of receptor detects free and conjugated *N*-acetylated sugars and another detects amino compounds such as certain amino acids (Thorington and Hessinger, 1988a). Chemoreceptors for *N*-acetylated sugars (e.g. *N*-acetylneuraminic acid, NANA) are

located at the apical plasma membrane of supporting cells (Watson and Hessinger, 1988), as are chemoreceptors for proline (Watson and Roberts, 1994).

Two classes of mechanoreceptor have been implicated in nematocyst discharge: contact-sensitive mechanoreceptors (CSMs; Thorington and Hessinger, 1988a), which presumably trigger discharge and are common to all CSCCs, and vibration-sensitive mechanoreceptors (VSMs; Watson and Hessinger, 1989a; Watson and Hessinger, 1989b), which are frequency-tuned by chemoreceptors and presumably detect the swimming movements of nearby prey (Watson and Hessinger, 1991), thereby preparing a subpopulation of CSCCs (type A CSCCs) for discharge.

Agents that increase intracellular cAMP levels (e.g. dibutyryl-cAMP, forskolin and cholera toxin) sensitize the CSMs of type B CSCCs and tune the VSMs of type A CSCCs to lower frequencies, as does NANA. Furthermore, NANA appears to stimulate adenylyl cyclase activity in supporting cells (Watson and Hessinger, 1992). In the present study, we demonstrate that NANA increases *in situ* cAMP levels in a dose-dependent manner in the ectodermal layer of tentacles from the sea anemone *Aiptasia pallida*, while having no effect on the cAMP content of tentacle endoderm. Furthermore, NANA-induced cAMP appears to activate endogenous cAMP-dependent protein kinase (PKA) in a NANA chemosensory signaling pathway that may sensitize nematocyst discharge.

Materials and methods

All chemical reagents, including *N*-acetylneuraminic acid (NANA, type VI), 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide and $MgCl_2{\cdot}6H_2O$ were obtained from Sigma Chemical Co. (St Louis, MO, USA). Kemptide was obtained from Calbiochem Corp. (San Diego, CA, USA) and γ-[^{32}P]-ATP from Amersham Life Sciences (Arlington Heights, IL, USA). The Kerckoff Marine Laboratory of the California Institute of Technology generously provided natural sea water at Corona del Mar, CA, USA. Encysted embryos of *Artemia salina* were purchased from San Francisco Bay Brand (Newark, CA, USA).

Maintenance of sea anemones

Monoclonal specimens of the sea anemone (*Aiptasia pallida*, Miami strain) were reared *en-masse* in flat-bottomed, glass trays and maintained individually in glass finger bowls containing natural sea water at 23±1 °C. The animals were fed daily with freshly hatched brine shrimp nauplii and cleaned 4–6 h after feeding. Anemones were kept on a 12 h:12 h light:dark daily photoperiod using white fluorescent lights at an intensity of 5.5×10^3 lx (Hessinger and Hessinger, 1981).

Experimental anemones and test solutions

Anemones of the same size were selected and starved for 72 h prior to experiments to maximize responsiveness (Thorington and Hessinger, 1988a). During each day of starvation, the natural sea water in which they were kept was replaced. During the last 48 h of starvation, anemones were kept under continuous fluorescent light at 4.5×10^3 lx. This provided optimal uniformity of anemone behavior (Thorington and Hessinger, 1988a; Thorington and Hessinger, 1988b).

Immediately before each experiment, sea anemones were gently rinsed with natural sea water to remove soluble wastes, and the medium was then exchanged with the test solution. Test solutions of NANA at specified concentrations, containing 10^{-4} mol l^{-1} IBMX, were prepared in natural, filtered (type 1, Whatman) sea water adjusted to pH 7.65 with 1 mol l^{-1} HC1 or 1 mol l^{-1} NaOH. Artificial sea water (ASW) consisted of

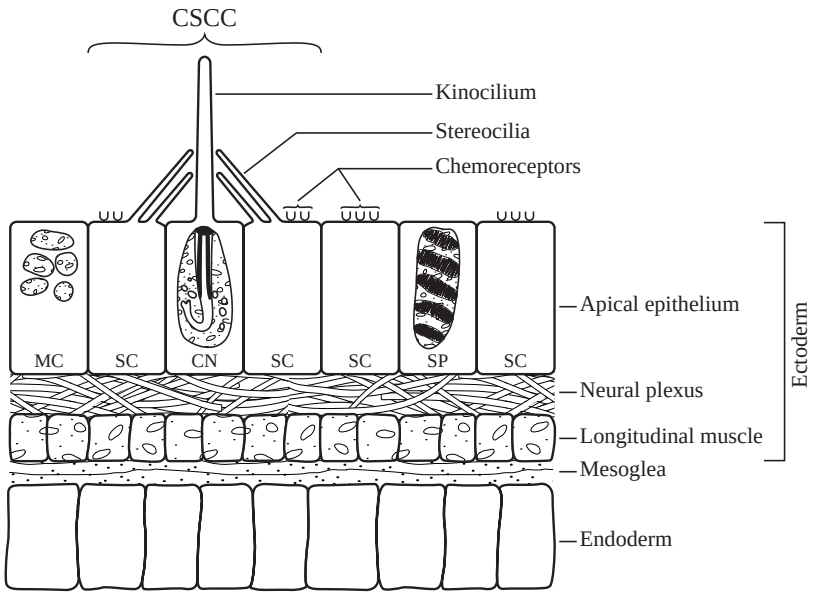

Fig. 1. Diagram of part of an anemone tentacle in cross section (cells not drawn to scale). The tentacle of *Aiptasia pallida* is composed of two epithelial layers; ectoderm (epidermis) and endoderm (gastrodermis) separated by a basement membrane termed the mesoglea. The ectodermal layer consists of three regions: a monolayer of longitudinal muscle cells closest to the mesoglea; an intermediate layer of neuritic processes and neurons termed the neural plexus; and the compositionally diverse apical epithelial layer, which includes mucus-secreting cells (MC), multicellular complexes known as cnidocyte/supporting cell complexes (CSCCs) and relatively rare sensory cells. The CSCCs consist of a cnidocyte about 35 μm in length surrounded by two or more supporting cells (SC). Three different functional types of CSCC have been identified. Two kinds of cnidocyte occur in the tentacle: the nematocyst-containing nematocyte (CN) and the spirocyst-containing spirocyte (SP). The nematocyte sports a single kinocilium from its apical surface which, in turn, associates closely with a bundle of stereocilia contributed from the adjacent supporting cells of the same CSCC. Together, the kinocilium and stereocilia form vibration-sensitive hair bundles. Sensitizing chemoreceptors for *N*-acetylated sugars, such as *N*-acetylneuraminic acid (NANA), occur on the apical surfaces of the supporting cells.

423 mmol l^{-1} NaC1, 10 mmol l^{-1} KC1, 10 mmol l^{-1} $CaC1_2$, 24 mmol l^{-1} $MgC1_2$, 25 mmol l^{-1} $MgSO_4$ and 1.2 mmol l^{-1} $NaHCO_3$, adjusted to pH 7.65. High-Mg^{2+}/seawater (Mg-ASW) was prepared by diluting (1:1) ASW with 0.6 mol l^{-1} $MgCl_2$ in distilled water.

Collection of tentacles

Individual anemones were chemosensitized with NANA solutions containing 10^{-4} mol l^{-1} IBMX for 15 min after preincubation with 10^{-4} mol l^{-1} IBMX alone for 20 min in filtered, natural sea water at room temperature (22±2 °C). Individual primary tentacles were rapidly excised and transferred using fine forceps onto the polished ends of aluminum rods (2.2 cm in diameter) standing in liquid nitrogen. The snap-frozen tentacles were then placed individually in marked Eppendorf tubes (1.5-ml) and stored in racks at –80 °C until extracted and analyzed for cAMP.

Extraction and measurement of cAMP

Each frozen tentacle was extracted individually by adding 1 ml of ice-cold 1 mol l^{-1} formic acid, pH 2.0 (Payne and Ames, 1982). After incubation for 15 min with formic acid on ice followed by brief vortexing, samples were centrifuged at 4000 ***g*** for 5 min to separate the still intact endoderm from the soluble extract. The extraction method inactivates tissue enzymes, such as phosphodiesterases, and solubilizes the ectoderm of freeze-thawed tentacles, as judged by enzymatic assay, light microscopy and the protein content of the supernatant medium. The endoderm remains intact and is surrounded by the denuded, intact, sac-like mesoglea. To measure endodermal cAMP content, the endoderm with the mesoglea was homogenized in 1 ml of ice-cold 1 mol l^{-1} formic acid using a pestle connected to a motor-driven mixer (Kontes Glass Co., Vineland, NJ, USA). Formic acid was removed from the supernatant and homogenate samples by drying overnight on a Speed-Vac (Savant Instruments Inc., Farmingdale, NY, USA). Each dried extract was then dissolved in 300 µl of 50 mmol l^{-1} acetate buffer (pH 5.8). Individual samples of 100 µl were taken for separate measurements of cAMP and protein content.

The cAMP content of samples was measured using a commercial competitive radioimmunoassay kit (RPA 509, Amersham Life Sciences, Arlington Heights, IL, USA) using 5.9×10^4 Bq of adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester and a gamma counter. The protein content of tissue extracts was measured spectrophotometrically by the enhanced BCA protein assay with bovine serum albumin as the standard (Pierce Chemical Co., Rockford, IL, USA).

[^{3}H]cAMP (adenosine-8-[^{3}H]3′,5′-cyclic monophosphate; Sigma Chemical Co.; 3.7×10^7 Bq ml^{-1}) was used to measure the efficiency of recovery of cAMP from tentacle extraction. [^{3}H]cAMP (3.7×10^4 Bq≈2000 cts min^{-1}) was added to replicate single tentacles, extracted normally and then counted by liquid scintillation. As a control for percentage recovery, the same amount of [^{3}H]cAMP, without extraction and without tentacle, was counted by liquid scintillation, and the percentage recovery was calculated [(cts min^{-1} in extract/total cts min^{-1})×100]. The percentage recovery of [^{3}H]cAMP was consistently between 90 and 95 %. Assayed tentacle samples were corrected for recovery from extraction in each experiment.

Measurements of adhesive force and counting discharged nematocysts

Measuring adhesive force and counting discharged nematocysts quantifies the *in situ* cnidocyte response to combined chemical and mechanical stimulation. These measurements in response to NANA sensitization were determined using previously described methods (Giebel et al., 1988; Thorington and Hessinger, 1988a; Thorington and Hessinger, 1988b). Briefly, test probes consisted of insect pins, the nylon head (0.8±0.01 mm in diameter) of which was coated with 0.06 mm of 30 % (w/v) gelatine. They were stored at 4 °C under 100 % humidity and used within 24 h. Single bowls containing individual sea anemones in various concentrations of NANA were raised by hand until the distal third of a tentacle contacted the probe attached to a force transducer (FT-03, Grass Instruments, Quincy, MA, USA) and a strip-chart recorder. The resistance springs were removed from the transducer to maximize sensitivity, and the transducer was calibrated gravimetrically. The bowl was lowered gently and smoothly after 5 s of contact until the tentacle separated from the probe. The force necessary to separate the probe from the tentacle was recorded and expressed in units of newtons (N) since there is negligible acceleration (Miller, 1959).

After the probes had been used once to measure adhesive force, they were processed for counting nematocysts. Individual gelatine-coated probes were placed in separate flat-bottomed microtiter wells (Microtest 11, Falcon Plastics) each containing 40 µl of 1 % enzyme/detergent mixture (Trizyme; Amway Products, Ada, MI, USA). After incubation for 4 h at room temperature, the probes were removed and the nematocysts released from the hydrolyzed-gelatine were visually counted using an inverted light microscope at a final magnification of 512×.

Preparation of tentacles for assaying cAMP-dependent protein kinase activity

The cAMP-dependent protein kinase (PKA) activity was assayed both in cell-free tentacle supernatants and in broken cell preparations of tentacles. For the preparation of cell-free supernatant solutions, 10 freshly harvested tentacles were pooled and homogenized by mortar and pestle under liquid nitrogen. Ground tentacles were quickly transferred into an Eppendorf tube with 1 ml of ice-cold extraction buffer (10 mmol l^{-1} sodium phosphate buffer, pH 6.8, containing 5 mmol l^{-1} EDTA, 0.5 mmol l^{-1} IBMX, 75 mmol l^{-1} NaCl, 10 mmol l^{-1} $NaVO_4$ and 10 µmol l^{-1} leupeptin). The sample was sonicated for 2–4 s on ice (Sonic Dismembrator, Fisher model 60) followed by centrifugation at 20 000 ***g*** for 20 min at 4 °C. The supernatant was removed and immediately used as the source of PKA activity.

For tentacle broken cell preparations, 20 tentacles were harvested and transferred by Pasteur pipette into a Dounce glass homogenizer kept on ice. After removing most of the sea water, 100 µl of extraction buffer was added. Following gentle cell rupture by hand with 10 strokes on ice, the total volume was increased to 2 ml. GTP and ATP at final concentrations of 0.1 mmol l^{-1} were added to the preparation, which was then divided into two 1 ml fractions. An 18 µl volume of 10^{-3} mol l^{-1} NANA was added to one fraction to a final concentration of 1.8×10^{-5} mol l^{-1}, while the other fraction received 18 µl of the extraction buffer. Broken cell preparations with and without NANA were incubated on ice for 15 min, and samples were then taken for the assay of PKA.

Assay for PKA activity

PKA activity was measured according to modifications of the assay described by Cherrington et al. (Cherrington et al., 1976; Byus and Fletcher, 1982). PKA activity was evaluated with kemptide as the substrate, and assays were performed with and without cAMP, with and without PKA inhibitor (PKI), with and without kemptide and in the presence of various doses of kemptide. Positive controls consisted of purified catalytic subunit from bovine heart and negative controls of PKI purified from rabbit skeletal muscle (Byus and Fletcher, 1982).

The assay mixture contained 35 µmol l^{-1} kemptide, 0.2 mmol l^{-1} ATP, γ-[^{32}P]ATP (total specific activity 7.4×10^{6} Bq pmol^{-1}) and 200 mmol l^{-1} Mes buffer, pH 6.8, containing 40 mmol l^{-1} $MgCl_2$ and 28 mmol l^{-1} β-mercaptoethanol. Volumes of 40 µl of either cell-free supernatant or tentacle broken cell preparation were added to reaction tubes containing 40 µl of assay mixture to start the enzyme reaction. The reaction was incubated for 10 min (unless indicated otherwise) at 30 °C. The reaction was terminated by pipetting 40 µl of the reaction volume onto Whatman P81 filters (2 cm in diameter) and then immediately placing the filter papers in ice-cold 30 % acetic acid. The assay filters were washed in cold 30 % acetic acid at 4 °C followed by a cold wash in 15 % acetic acid and then 15 % acetic acid at room temperature for 10 min. Filter papers were then rinsed in 100 % acetone for 5 min, dried and counted in a liquid scintillation counter (3800 Beckman scintillation counter).

Protein concentrations were determined spectrophotometrically using the enhanced BCA protein assay (Wiechelman et al., 1988) with bovine serum albumin as the standard (Pierce Chemical Co., Rockford, IL, USA).

Data analysis

Results of cAMP experiments were determined by generating a standard curve with each experiment. Data were corrected for recovery from extraction of [^{3}H]cAMP and then normalized as cAMP content per microgram of protein (fmol cAMP µg^{-1} protein). PKA activity was expressed as pmoles of phosphate transferred per minute. All results are given as means ± S.E.M. Statistical analyses of paired data were performed using Student's *t*-test. Two-way analysis of variance (ANOVA) was used to compare multiple groups of means with repeated measures and *post-hoc* Fisher PLSD tests (Stat-View Software, Abacus Concepts Inc., Berkeley, CA, USA). The differences were considered to be significant at $P<0.05$. The total number of replicate samples (n) and the number of replicate experiments (N) are indicated in the figure legends. The number of sea anemones used in each experimental condition ranged between four and 20.

Results

Basal cAMP contents of tentacle ectoderm and endoderm

The basal cAMP content of individual *A. pallida* tentacles was measured following excision and rapid freezing. Tentacles were thawed by adding either 1 ml of ice-cold 0.05 mol l^{-1} acetate buffer (pH 5.8) or 1 mol l^{-1} formic acid (pH 2.0). The soluble extracts were of ectodermal origin, while the insoluble tentacle residues consisted of intact endodermal layers encased within intact mesoglea. The ectodermal cAMP content of the formic-acid-extracted tentacles yielded approximately five times the cAMP content of the acetate-extracted tentacles (Fig. 2; $P<0.0001$). The endodermal cAMP contents prepared by the two extraction methods, however, were indistinguishable ($P=0.98$). In view of the higher cAMP recovery from the

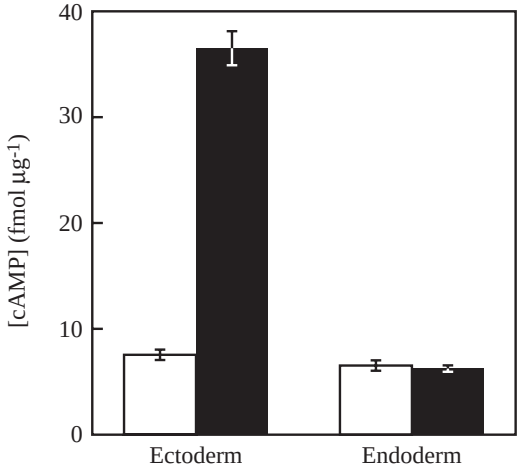

Fig. 2. Effects of extraction buffer on the cAMP content of untreated ecto- and endodermal layers from *Aiptasia pallida* tentacles. Tentacles of sea anemones starved for 72 h were individually excised, snap-frozen, stored at –80 °C and later extracted in two different media. For measurement of ectodermal cAMP levels, either 1 ml of ice-cold 1 mol l^{-1} formic acid, pH 2.0 (filled columns), or 1 ml of ice-cold 0.05 mol l^{-1} acetate buffer, pH 5.8 (open columns), was added to each frozen tentacle. The cAMP and protein contents of the soluble, freeze-thawed ectodermal layers were assayed. For measurement of endodermal cAMP levels, the insoluble residue, consisting of intact endoderm plus mesoglea, was homogenized in 1 ml of 1 mol l^{-1} formic acid or 1 ml of 0.05 mol l^{-1} acetate buffer, pH 5.8, on ice, and then assayed for cAMP and protein. Data are expressed as fmol cAMP µg^{-1} protein. Twenty-eight sea anemones were used. Values are means ± S.E.M. ($N=2$ experiments; $n=17$ samples).

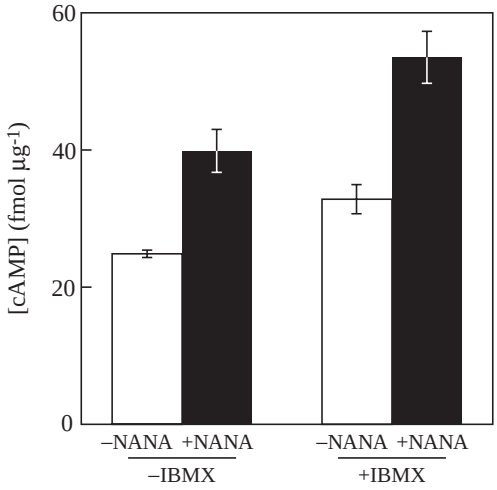

Fig. 3. Effects of IBMX on tentacle ectodermal cAMP content following treatment with and without *N*-acetylneuraminic acid (NANA). A group of sea anemones starved for 72 h was incubated in filtered, natural sea water, pH 7.65, with or without 10^{-4} mol l^{-1} IBMX for 20 min (open columns). A second group of sea anemones starved for 72 h was incubated in filtered, natural sea water, pH 7.65, containing 1.8×10^{-5} mol l^{-1} NANA with or without 10^{-4} mol l^{-1} IBMX for 20 min (filled columns). Individual tentacles from each group were snap-frozen, extracted and assayed for ectodermal cAMP using 1 mol l^{-1} formic acid. Data are expressed as fmol cAMP μg^{-1} protein. Four anemones were used. Values are means ± S.E.M. (n=8 samples).

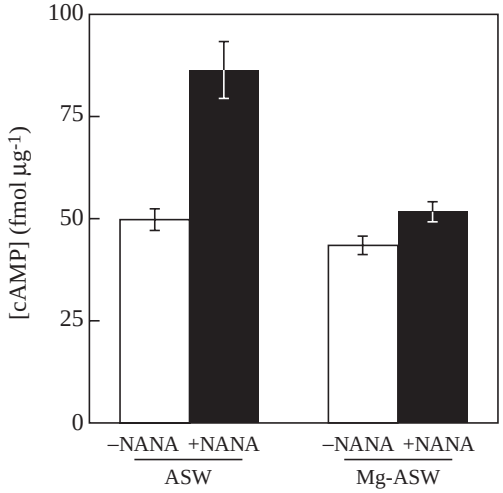

Fig. 4. Effects of *N*-acetylneuraminic acid (NANA) and Mg-ASW on tentacle ectodermal cAMP content. Following 72 h of starvation, one group of four anemones was pre-incubated in artificial sea water (ASW) containing 10^{-4} mol l^{-1} IBMX for 10 min followed by incubation in ASW containing either 10^{-4} mol l^{-1} IBMX and 1.8×10^{-5} mol l^{-1} NANA (filled columns) or 10^{-4} mol l^{-1} IBMX alone (open columns) for 15 min. Individual tentacles from each group were collected, snap-frozen, extracted and assayed for ectodermal cAMP using 1 mol l^{-1} formic acid. Data are expressed as fmol cAMP μg^{-1} protein. A second, similar group of four anemones was treated as above except that Mg-ASW replaced ASW. Values are means ± S.E.M. (n=20 samples).

ectoderm extract and because the formic-acid-extracted tentacles exhibited complete removal of the ectodermal layer, as determined by light microscopic examination, we elected to use formic acid extraction in all subsequent experiments.

Effects of IBMX on cAMP contents of ectoderm

The basal cAMP content of tentacles from animals treated with filtered, natural sea water containing 10^{-4} mol l^{-1} IBMX, a non-specific inhibitor of cAMP phosphodiesterases, was approximately 30 % higher than that of animals incubated in sea water alone (Fig. 3; P=0.04). The cAMP content of tentacles from animals treated with 1.8×10^{-5} mol l^{-1} NANA plus 10^{-4} mol l^{-1} IBMX was approximately 35 % higher than that of animals incubated in sea water containing NANA alone (Fig. 3; P<0.002). Because IBMX enhanced cAMP levels, we pre-incubated anemones in 10^{-4} mol l^{-1} IBMX in all subsequent experiments.

Effects of Mg-ASW on NANA-induced cAMP content

In normal ASW, 1.8×10^{-5} mol l^{-1} NANA stimulates an almost twofold increase in *in situ* cAMP content of tentacle ectodermal layers (Fig. 4; P<0.0001). We tested the effects of anesthetizing levels of Mg^{2+} on NANA-stimulated cAMP content because high levels of Mg^{2+}, usually achieved by mixing equal volumes of ASW and 0.6 mol l^{-1} $MgCl_2$, are commonly used to anesthetize and immobilize spontaneously contracting excised tentacles. In Mg-ASW containing 312 mmol l^{-1} $MgCl_2$ and 12.5 mmol l^{-1} $MgSO_4$, the NANA-stimulated increase in cAMP content was inhibited, with cAMP content not being significantly different from that of controls (Fig. 4; P=0.17).

Effects of NANA on nematocyst discharge, adhesive force and cAMP content

The effect of NANA concentration on *in situ* nematocyst discharge was biphasic and consisted of two regions (Fig. 5): sensitization and desensitization. The sensitization region occurred at the lower tested concentrations of NANA. Half-maximal discharge (EC_{50}) occurred at approximately 10^{-7} mol l^{-1} NANA. Maximum discharge was 3.4 times that of the seawater controls and occurred at 1.8×10^{-5} mol l^{-1} NANA (EC_{100}). The effect of NANA on adhesive force (Fig. 5) was also biphasic and coincided with the nematocyst discharge response curve. Maximum adhesive force was approximately 1.2 times that of the seawater controls and occurred at 1.8×10^{-5} mol l^{-1} NANA (EC_{100}).

NANA also increased the cAMP content of tentacle ectodermal layers in a dose-dependent manner (Fig. 5). The dose/response curve of NANA-stimulated cAMP content was biphasic and coincided with the effects of NANA on nematocyst discharge and adhesive force. Maximum cAMP levels in tentacle ectodermal layers of NANA-stimulated anemones occurred at 1.8×10^{-5} mol l^{-1} NANA, approximately twice that of seawater-treated anemones (P<0.0001). In

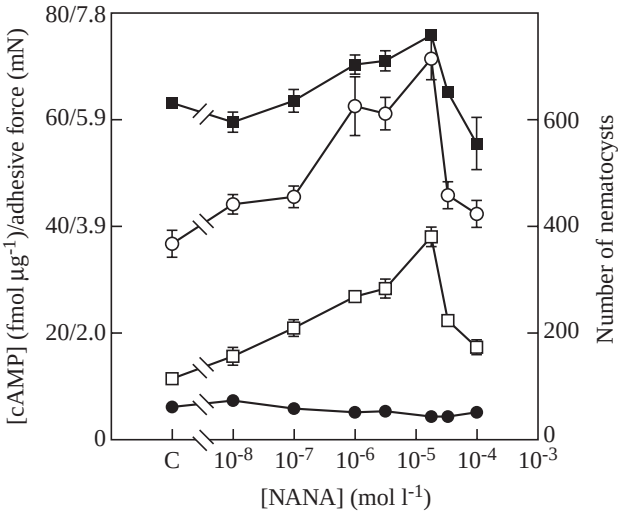

Fig. 5. Effects of *N*-acetylneuraminic acid (NANA) on nematocyst discharge, adhesive force and tentacle cAMP content. Pre-starved anemones were incubated in filtered, natural sea water containing the specified NANA concentrations for 10 min, and *in situ* adhesive force and nematocyst discharge were measured. Nematocysts on six probes were counted for each dose tested, and the results are the mean of three experiments (□, *N*=3 experiments; *n*=18 samples). Values of adhesive force measurements were expressed in newtons as the mean of three experiments (■, *N*=3; *n*=27). For measurement of cAMP, pre-starved anemones were pre-incubated in filtered, natural sea water containing 10^{-4} mol l^{-1} IBMX for 20 min followed by incubation in sea water containing 10^{-4} mol l^{-1} IBMX and different concentrations of NANA for 15 min. Individual tentacles were excised, snap-frozen and subsequently assayed for cAMP content of both ectoderm (○, *N*=2; *n*=20) and endoderm (●, *N*=1; *n*=10) with 1 mol l^{-1} formic acid, as in Fig. 1. Results are expressed as fmol cAMP μg^{-1} protein. Untreated control values (C) are indicated at the left-hand end of the *x*-axis. The number of anemones used in each experiment ranged between 16 and 20. Values are means ± S.E.M.

contrast, endodermal layers showed no significant change in cAMP content over the tested range of NANA concentrations. The mean cAMP content of endodermal layers from all NANA doses was 5.2±0.5 fmol μg^{-1} protein (Fig. 5), a value approximately one-seventh that of ectodermal layers from control anemones and one-fourteenth that of ectodermal layers from anemones exposed to 1.8×10^{-5} mol l^{-1} NANA.

Time course of NANA-stimulated ectodermal cAMP levels

The ectodermal cAMP content of individual tentacles was measured from sea anemones incubated in 1.8×10^{-5} mol l^{-1} NANA for different times (Fig. 6). No significant increase in cAMP levels occurred during the first 3 min of incubation with NANA. At 4 min, the *in situ* cAMP content increased significantly ($P<0.01$), and between 4 and 10 min the cAMP content did not change. The maximum increase in cAMP content occurred at 15 min; approximately 1.8 times that averaged for 0–3 min. For incubation times longer than 15 min, the cAMP content declined steadily, reaching basal levels at 30 min and declining to below basal levels by 45 min ($P<0.05$).

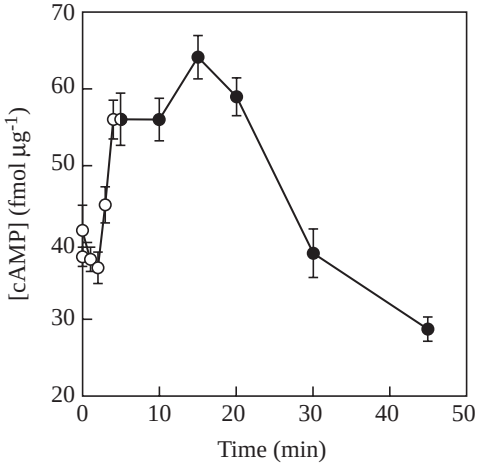

Fig. 6. Time course of *N*-acetylneuraminic acid (NANA)-stimulated cAMP content of tentacle ectoderm. Pre-starved anemones were pre-incubated in filtered, natural sea water containing 10^{-4} mol l^{-1} IBMX for 20 min followed by incubation in filtered, natural sea water containing 10^{-4} mol l^{-1} IBMX and 1.8×10^{-5} mol l^{-1} NANA for specified times. Tentacles were excised, snap-freezen and assayed for ectodermal cAMP content as in Fig. 4. Two sets of experiments were combined: one measured the cAMP contents between 0 and 5 min of incubation with NANA (○, *N*=3 experiments; *n*=36 samples) and the other between 5 and 45 min of incubation with NANA (●, *N*=3; *n*=22). The value for 5 min of incubation was the average of means from the two sets of experiments. Data are expressed as fmol cAMP μg^{-1} protein. The number of sea anemones used in each experiment ranged between 18 and 20. Values are means ± S.E.M.

cAMP-dependent protein kinase activity in cell-free supernatants of tentacles

Basal cAMP-dependent protein kinase (PKA) activity was measured in cell-free supernatants prepared from pooled, excised tentacles. The activity was measured both in the presence and in the absence of 0.1 mmol l^{-1} cAMP (Fig. 7). Approximately 40 % more protein kinase activity was measured in the presence of added cAMP than in its absence. In addition, 1:1 dilutions of supernatants were also assayed, yielding PKA activities approximately 40 % of those of the undiluted supernatants.

To evaluate the effect of kemptide concentration on PKA activity, different concentrations of kemptide were tested with and without 0.1 mmol l^{-1} cAMP. The enzyme activity increased with increasing substrate concentration both with and without added cAMP. Activities with added cAMP were consistently higher than activities without added cAMP at the same substrate concentrations (Fig. 8). The inhibitor PKI at 20 nmol l^{-1} totally inhibited PKA activity both in the presence and in the absence of exogenously added cAMP (data not shown).

cAMP-dependent protein kinase activity in tentacle broken cells

Pooled tentacles had their cells gently ruptured as described in the Materials and methods section. Broken cell preparations

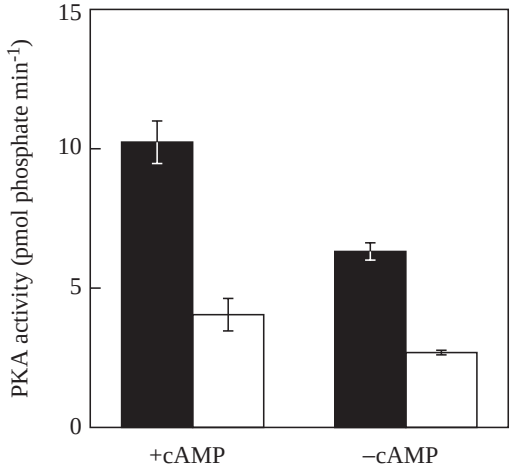

Fig. 7. cAMP-dependent protein kinase (PKA) activity in cell-free extracts of tentacles. Tentacles were collected, pooled and homogenized by mortar and pestle under liquid nitrogen followed by sonication for 2–4 s in 1 ml of ice-cold extraction buffer, pH 6.8. The homogenate was then centrifuged at 20 000 ***g*** for 20 min at 4 °C. The supernatant was immediately used as the source of enzyme activity. PKA activity was assayed with 35 μmol l^{-1} kemptide in the presence and absence of 0.1 mmol l^{-1} exogenous cAMP using undiluted (filled columns) and 1:1 diluted (open columns) supernatants. The protein content of undiluted supernatants was 176 μg ml^{-1}. Data are expressed as pmoles of phosphate transferred per minute. Values are means ± S.E.M. (*N*=2 experiments; *n*=4 samples).

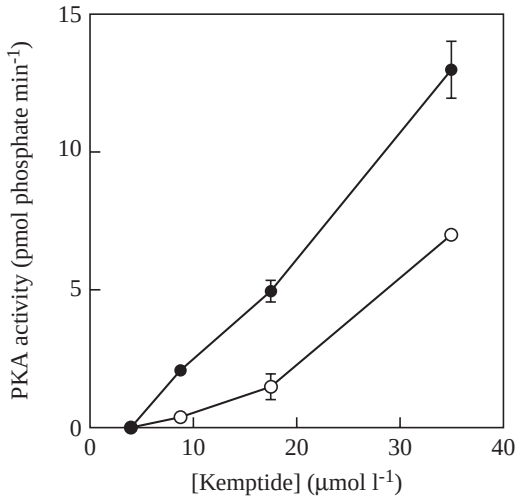

Fig. 8. Effects of substrate concentration on cAMP-dependent protein kinase (PKA) activity in cell-free extracts of tentacles. Tentacles were pooled and processed for the assay of PKA activity as in Fig. 6. The supernatant extract was immediately mixed with different concentrations of kemptide in the reaction mixture in the presence (●) or absence (○) of 0.1 mmol l^{-1} cAMP. The protein content of supernatants was 140 μg ml^{-1}. Data are expressed as pmoles of phosphate transferred per minute. Each data point represents the mean ± S.E.M. (*N*=2 experiments; *n*=4 samples).

were assayed for PKA activity. The enzyme activity in the absence of NANA was 14.08±0.46 pmol phosphate min^{-1}.

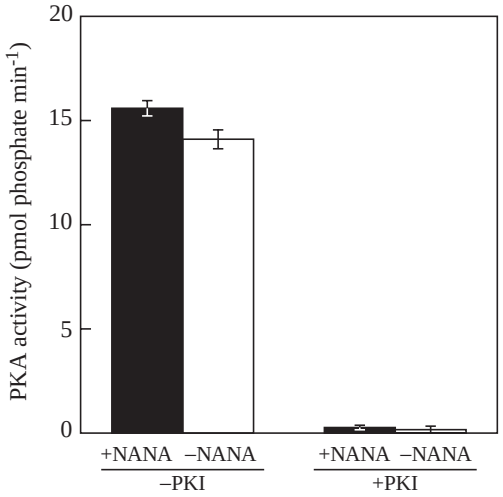

Fig. 9. Effects of *N*-acetylneuraminic acid (NANA) on cAMP-dependent protein kinase (PKA) activity in whole-tentacle homogenate. Tentacles were harvested, pooled and gently homogenized on ice in extraction buffer, pH 6.8, with 10 strokes of a Dounce all-glass homogenizer. Homogenates including 0.1 mmol l^{-1} GTP and 0.1 mmol l^{-1} ATP were stored on ice for 15 min in the presence (filled columns) and absence (open columns) of 1.8×10^{-5} mol l^{-1} NANA, after which 40 μl samples were taken for PKA assay at 30 °C for 10 min. The addition of 20 nmol l^{-1} PKA inhibitor (PKI) completely blocked the PKA activity both in the presence and in the absence of NANA. The protein concentration of the homogenate was 528 μg ml^{-1}. Data are expressed as pmoles of phosphate transferred per minute. Each data point represents the mean ± S.E.M. (*n*=4 samples).

In the presence of NANA, the activity averaged 15.57±0.33 pmol phosphate min^{-1}. The difference between the two groups was statistically significant (*P*=0.02) (Fig. 9). PKA inhibitor (PKI) completely inhibited the enzyme activities, both with and without added NANA.

Discussion

Cnidarians, such as sea anemones, jellyfish, hydra and corals, employ complex secretory products, termed nematocysts, for such functions as prey capture, defense and aggression. The ectodermal layer of sea anemone tentacles is armed with these stinging organelles. Housed within a specialized cell called the cnidocyte, the nematocyst consists of an intracellular capsule containing a highly folded, contiguous and eversible tubule. Eversion of the tubule, through which the venomous contents of the capsule are conveyed, is called nematocyst discharge. In this manner, the discharging nematocyst injects potent toxins into the prey (Hessinger, 1988).

In situ nematocyst discharge is initiated by appropriate stimulation of chemo- and mechanoreceptors (Pantin, 1942; Thorington and Hessinger, 1988a; Thorington and Hessinger, 1988b). Activated chemoreceptors are alleged to predispose contact-sensitive mechanoreceptors to trigger discharge in

response to prey contact with the tentacle. Chemical and mechanical stimuli originating from prey also regulate subsequent feeding behavior and ingestion of prey by sea anemones. In the sea anemone *Anthopleura elegantissima*, concerted tentacle movements towards the mouth are controlled by asparagine, which is presumed to leak from nematocyst-inflicted wounds, while prey-derived reduced glutathione controls the ingestion of food into the mouth (Lindstedt, 1971).

In *A. elegantissima*, reduced glutathione activates adenylyl cyclase in oral disc membrane preparations (Gentleman and Mansour, 1974). In another cnidarian, the sea pansy *Renilla koellikeri*, adenylyl cyclase activity in membrane preparations is stimulated by GTP, GTP-γS, NaF and cholera toxin (Awad and Anctil, 1993). Adenylyl cyclase activity is also found in membrane preparations of hydra (Venturini et al., 1984). Furthermore, cAMP plays an important role in mediating the effect of head activator and in head regeneration in hydra (Fenger et al., 1994; Galliot et al., 1995).

In the sea anemone *H. luciae*, the membrane-permeant cAMP analogue dibutyryl-cAMP biphasically sensitizes *in situ* nematocyst discharge, as do forskolin, cholera toxin and caged GTP-γS. Furthermore, endogenous adenylyl cyclase activity in *H. luciae* is detectable cytochemically at the apical plasma membranes of the tentacle-supporting cells, but only in the presence of *N*-acetylated sugars, such as NANA (Watson and Hessinger, 1992). These findings indirectly implicate cAMP as the second messenger for activated supporting cell chemoreceptors involved in the NANA sensitizing pathway.

In our present experiments, we directly demonstrate that the chemosensitizer NANA increases the *in situ* cAMP content of *A. pallida* tentacle ectoderm. The effects of NANA concentration on cAMP content were biphasic and yielded a dose/response curve that coincided with those of NANA-sensitized nematocyst discharge and nematocyst-mediated adhesive force (Fig. 5). These dose/response curves have similar regions of sensitization, EC_{100} values and regions of desensitization at higher concentrations. The EC_{100} values of NANA-sensitized nematocyst discharge and nematocyst-mediated adhesive force in the present study (Fig. 5) confirm those published previously (Thorington and Hessinger, 1988a; Thorington and Hessinger, 1990; Thorington and Hessinger, 1998).

The relevance of NANA-stimulated increases in cAMP concentration is confirmed at the tissue level by the fact that the effect is confined to the ectoderm, where the CSCCs are located. NANA does not affect cAMP levels in the tentacle endoderm, where cAMP contents averaged 14 % of ectodermal control levels and 7 % of ectodermal NANA-stimulated content. That NANA optimally increases cAMP levels in the ectoderm only twofold is probably because the extracted tentacle ectoderm includes contributions from many cells that may not respond to externally applied NANA, including those of the neural plexus and the longitudinal muscle layer (Fig. 1). The fact that the ectodermal cAMP content decreases at desensitizing levels of NANA suggests that desensitization is regulated between the chemoreceptor and cAMP in the NANA chemosensory signaling pathway. Thus, our data strongly suggest that NANA-stimulated cAMP functions as a second messenger in NANA-sensitized nematocyst discharge.

The present direct findings in *A. pallida* confirm and extend the indirect evidence previously provided by us that, in *H. luciae*, the NANA-chemosensitizing system of the anemone employs cAMP as a second messenger. Among higher animals, several examples of chemosensory pathways using cAMP as an intracellular mediator are known, including mammalian olfaction (Nakamura and Gold, 1987), sweet gustation (Avenet et al., 1988) and lobster olfaction (Boekhoff et al., 1994).

High levels of Mg^{2+} prepared by mixing equal volumes of ASW and 0.6 mol l^{-1} $MgCl_2$ to make Mg-ASW have been used to anesthetize *H. luciae* and to immobilize excised tentacles for *in situ* studies of NANA-stimulated effects (Watson and Hessinger, 1991; Watson and Hessinger, 1992; Watson and Hessinger, 1994; Thibodeaux-Mire and Watson, 1993). We find that Mg-ASW blocks the *in vivo* NANA-stimulated increase in cAMP concentration in the tentacle ectoderm (Fig. 4), and it also blocks NANA-sensitized nematocyst discharge in *A. pallida* and *H. luciae* tentacles (G. U. Thorington and D. A. Hessinger, unpublished data). However, in excised *H. luciae* tentacles immobilized with Mg-ASW, stereociliary bundles, which comprise the vibration-sensitive mechanoreceptors (VSMs) of type A CSCCs, are reported to elongate in response to NANA and to agents that commonly increase intracellular cAMP levels (Watson and Hessinger, 1992). Thus, either NANA-induced elongation of stereociliary bundles occurs by a cAMP-independent pathway or *A. pallida* and *H. luciae* differ with respect to the effects of high-Mg^{2+} ASW on NANA-stimulated increases in cAMP concentration. The practice of using Mg-ASW is also questionable because it is a hyperosmotic solution.

The time course of NANA-stimulated cAMP shows that NANA increases cAMP levels above control levels only after 3 min. In contrast, unpublished experiments with gelatine-coated probes pre-equilibrated with solutions of NANA at various concentrations indicate that the sensitizing effect of NANA on nematocyst discharge can occur within a few seconds. In tentacles of *H. luciae*, chemosensitization of nematocyst discharge increases within 1 min, reaches its highest level at 5 min, and then gradually declines by 25 min. The 25 min minimum is then followed by an increase with a smaller maximum at 35 min, which is followed by a lower minimum at 45 min (Watson and Hessinger, 1989a). In experiments in which whole anemones are bathed in medium containing NANA, specimens of *A. pallida*, which are found subtidally in nature, require several minutes to recover from the disturbance of exchanging the medium, whereas *H. luciae*, which are found intertidally in nature, appear to be undisturbed by medium exchanges. Thus, it is possible that the physical disturbance caused by changing the medium in our time course experiments with *A. pallida* causes the NANA-stimulated cAMP pathway to be temporarily inhibited, favoring defensive

withdrawal behavior over prey capture, whereas *H. luciae* are relatively unaffected.

After 45 min, NANA-stimulated cAMP levels fall to a level below that of control ectodermal layers. This suggests that chemosensitization is subject to adaptation in response to prolonged NANA stimulation. Furthermore, the decline in cAMP levels to below control levels by prolonged exposure to NANA indicates that a significant proportion of the basal cAMP content of approximately 40 pmol μg^{-1} protein is maintained by contributions from the NANA-responsive CSCCs.

Our data also indicate the presence of endogenous PKA activity in *A. pallida* tentacles. The monospecific PKA inhibitor protein PKI, or Walsh protein, blocks 98 % of the measured kinase activity, indicating that the activity is due to PKA (Fig. 9). This is further confirmed by the observation that added cAMP increases activity (Fig. 7, Fig. 8). In broken cell preparations of tentacles to which ATP, but not cAMP, has been added, NANA stimulates PKA activity by approximately 10 % (P<0.02). This suggests that stimulated NANA chemoreceptors activate adenylyl cyclase to synthesize enough additional cAMP to stimulate PKA significantly above untreated control levels. That higher levels of NANA-stimulated PKA activity were not measured may be because of problems associated with the PKA assay and the preparation of the broken cells. For instance, diluting the broken cell preparations 40-fold for the PKA assay probably lowered levels of endogenously produced cAMP to values that less than optimally activate PKA. Furthermore, partial physical uncoupling of the membrane-associated chemosensory components of the NANA signaling pathway may have occurred during the preparation of the broken cells.

Our results document for the first time that physiologically relevant levels of NANA stimulate cAMP production in anemone tentacles. This effect of NANA is restricted to cells of the tentacle ectoderm and does not affect cAMP levels in the tentacle endoderm. Our finding that high levels of Mg^{2+} in the bathing sea water block the NANA-stimulated increase in cAMP concentration is consistent with the fact that high levels of Mg^{2+} block nematocyst discharge, but raises questions about the purported role of cAMP in NANA-induced elongation of the stereociliary bundles of vibration-sensitive hair bundles in type A CSCCs. In conclusion, our findings are consistent with cAMP acting as a second messenger in NANA-sensitized discharge of nematocysts from type B CSCCs.

We wish to thank J. A. Hessinger for help in culturing anenomes and Drs J. Buckholz and R. Alburqueque for helpful discussions. Funded in part by a BSRC grant.

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