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The Roles of Ca^{2+} and cAMP in the Nematocyst Discharge of the Sea Anemone Tentacle

by

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Philosophy in Physiology

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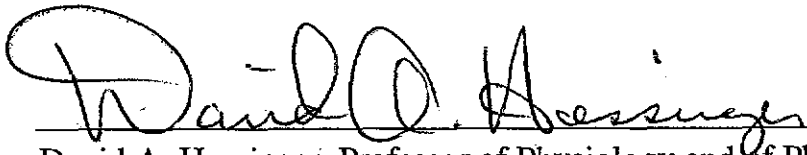

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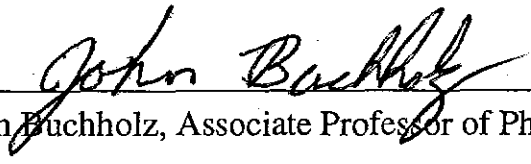

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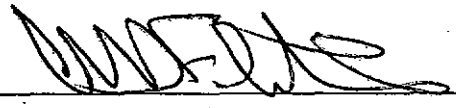

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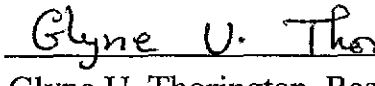

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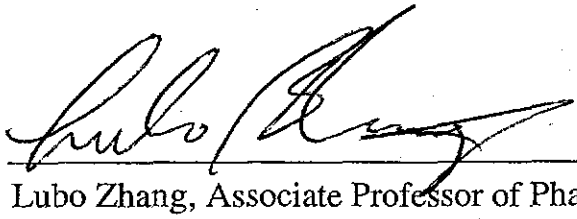

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LIST OF ABBREVIATIONS

ASW	Artificial seawater
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BME	β-mercaptoethanol
Ca^{2+}	Ionic calcium
cAMP	Cyclic adenosine 3', 5'-monophosphate
Cd^{2+}	Ionic cadmium
cpm	Count per minute
CSCC	Cnidocyte/supporting cell coplex
CSM	Contact-sensitive mechanoreceptors
CTX	Cholera toxin
Db-cAMP	Dibutyryl-cAMP
df-ASW	Divalent-free artificial seawater
DHP	Dihydropyridine
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine triphosphate
IBMX	Isobutyl methylxanthine
K^+	Ionic potassium

MES	2-[N-morpholino]ethanesulfonic acid
Mg^{2+}	Ionic magnesium
Mg-ASW	Magnesium-artificial seawater
MgSW	Magnesium-natural saewater
Mn^{2+}	Ionic manganese
NANA	N-acetyl neuraminic acid
PKA	Cyclic-AMP-dependent protein kinase (protein kinase A)
PKI	Protein kinase A inhibitor protein
SB	Stereociliary bundle
VSM	Vibration-sensitive mechanoreceptors

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Physiology
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The phylum Cnidarians are aquatic animals, including jellyfish, hydra, sea anemones, and corals. They are the simplest metazoans having a nervous system and are diploblastic. Cnidarians are obligate predators that capture prey using specialized stinging cells called cnidocytes. The cnidocyte contains a nematocyst, which is a capsule containing an inverted, hollow tubule. Prey contacting the tentacle triggers the nematocyst tubule to rapidly evert; a process called discharge. The everting tubule penetrates and envenomates the prey, which is carried to the mouth by tentacle movements.

Generally both chemical and mechanical stimuli are needed to trigger discharge. The cnidocytes, in sea anemones, are surrounded by two or more supporting cells that have chemoreceptors and possibly contact-sensitive mechanoreceptors (CSMs). Therefore, the cnidocyte/supporting cell complex (CSCC) controls nematocyst discharge.

This project explores the chemoreceptor signaling pathway controlling nematocyst discharge from tentacles of the sea anemone, *Aiptasia pallida*. Chemoreceptors respond to N-acetylated sugars, such as N-acetylneuraminic acid (NANA), which occur on prey surfaces. When stimulated, this chemosensory pathway sensitizes CSMs to trigger nematocyst discharge in response to physical contact.

Indirect evidence suggested that the NANA chemosensory pathway acts through the intracellular second-messenger, cyclic-AMP (cAMP). We now show that NANA dose-dependently increases *in situ* cAMP levels in the ectodermal layer of tentacles from *A. pallida*, but has no effect on the endodermal cAMP content. In addition, NANA activates cAMP-dependent protein kinase (PKA) in whole tentacle homogenates.

High levels of extracellular Mg^{2+} are commonly used to anesthetize excised tentacles and to block discharge. We find that high levels of Mg^{2+} block the NANA-stimulated cAMP increase. This supports the fact that high Mg^{2+} levels inhibit nematocyst discharge, but calls into question published findings in which NANA-induced changes in stereociliary bundle length of excised, Mg^{2+}-anesthetized tentacles are attributed to cAMP.

We also find that NANA stimulates calcium influx into isolated tentacle ectodermal cells and that the influx is sensitive to various L-type calcium channel

blockers, including dihydropyridines. The coincidence of the desensitization region of the nematocyst discharge curve with those NANA concentrations that most stimulate calcium influx, suggests a role of NANA-stimulated calcium influx in desensitization.

CHAPTER ONE

INTRODUCTION

General Characteristics of Cnidarians

The phylum Cnidaria (or Coelenterate), having more than 10,000 species and including sea anemones, jellyfish, corals, and hydra, are the simplest metazoans with a nervous system. They are aquatic invertebrates exhibiting primary radial, biradial, or radio-bilateral symmetry. Cnidarians possess two epithelial layers, an ectoderm and endoderm, separated by an acellular mesoglea. This diploblastic epithelium surrounds one internal cavity (gastrovascular cavity or coelenteron) that opens to the external medium by way of a single protostomal mouth. Common to all species of the phylum are eversible secretory products called cnidae contained within specialized cells, cnidocytes. The most common type of cnidae is the nematocysts. Species can exist in either of two adult forms: sessile polyp or free-swimming medusoid type.

The phylum is commonly divided into four classes: 1) Hydrozoa. Hydroids and small craspedote medusae belong to this class. There are approximately 2,700 species. Venomous *Physalia physalis* (Portuguese Man-of-War), a planktonic colony of specialized polyps, is a well–known member of the class in the order Siphonophora. 2) Scyphozoa. There are approximately 200 species, which comprise the jellyfish and acraspedote or large medusae. 3) Anthozoa. Sea anemones, corals, and alcyonarians constitute the class that totals approximately 6,100 species. The medusae stage is absent

in this class and the majority of polyps make colonies. 4) Cubozoa. Sea wasps or deadly cubomedusae belong to this class.

Most cnidarians are harmless to humans. However, some species inflict considerable harm. For instance, the stings of some cubomedusae can kill an adult human within minutes. Likewise, stings of a large colony of Portuguese Man-of-War can be fatal. In these cases, extremely potent nematocyst venoms afflict various vital systems of human beings. Although a number of cnidarian stings occur each year, most of which are not life–threatening, there is no adequate treatment other than symptomatic (Halstead, 1978).

Morphology of the venom apparatus

The means for delivering cnidarian venom are the nematocysts, which are primarily located on the tentacles. The nematocyst was first described by Trembley (1774). Moebius (1866) was the first to study its structure. The term nematocyst, meaning "stinging cell", is actually not correct because it is not a cell, rather a cell organoid or secretory product of the cell. Much of its structural parts are made up of a mini-collagens (Lentz, 1966; Anderluh *et al.*, 2000). Nematocyst size varies from 5 micron to 1.12 mm in length. The largest known nematocyst is found in a siphonophore and has a tubule length of several mm.

Among the cnidarians, diversity in nematocyst morphologically provides a taxonomic characteristic. The nematocyst is a highly structured capsule containing a coiled-eversible tubule. The rapidly eversible, hollow tubule is used to inject toxins into prey. Prey capture, defense, and aggression are the major functions of nematocysts.

Although the primary location is on the tentacles, nematocysts are also found in the ectoderm of the oral disc and such internal structures as gastric filaments, acontia, and septal filaments. The nematocysts develop inside immature cnidocytes (cnidoblasts). Cnidoblasts differentiate from multipotent interstitial cells located away from the region in which the mature nematocyst is utilized, i.e., the basal enlargement of the tentacles. Differentiating cnidoblasts migrate to the ectodermal epithelium by amoeboid movement (Weill, 1934, Yanagita & Wada, 1959).

Public Health Aspects

Only a small fraction of cnidarian stings are recorded because cnidarian stings are non–reportable public health cases and most stings are not life–threathening. Therefore, information about global public health aspects of cnidarian stings and its precise geographic distribution is very limited. On the other hand, various species of the phylum Cnidaria can be found in all open seas and oceans of the world. The most dangerous species usually live in tropical and warm waters. Knowledge of the accurate incidence of cnidarian stings is extremely limited. Based on published reports, between 1836 and 1960, more than 50 fatal cases occurred among 500 reported stings, providing a raw 9 percent of fatality rate (Halstead, B. W., 1978)

General Characteristics of Sea Anemones

The class Anthozoa has two subclasses according to Hyman (1940): (1) Alcyonaria, which covers soft corals, sea pens, sea pansies, and sea fans; and (2) Zoantharia, which includes sea anemones and true corals. The sea anemones are

members of the order Actiniaria. They are one of the most abundant seashore animals. There are approximately 1,000 species, inhabiting a range extending from the tidal zone to depths of more than 17,000 feet. While most live in warm tropical waters, some species live in polar seas. Their size varies between a few millimeters to a half-meter in diameter. They have a cylindrical body and a flat oral disc margined with a number of tentacles around a slitlike mouth. The base of the pedal disc serves for attachment to objects. They are sessile and attach to various objects, yet they are able to crawl about to some extent. The interior surface of the body has radially-arranged compartments formed by septa that are in multiples of six. Inner margins of the septa comprise the gastric filaments which, in the suborder Aconitidae, are continued as threadlike acontia. In the acontiate anemones, the acontial nematocysts can sometimes aid in catching prey when the acontia extend through the mouth or through pores located in the body wall. The anemones are passive predators feeding upon mollusks, crustaceans, other invertebrates, and fish. When they are covered by seawater and allowed to relax, their tentacles and body are expanded, exhibiting a flowerlike shape, often displaying a variety of colors. If disturbed, the anemone contracts its body and tentacles. Sea anemones reproduce either sexually or asexually or both. They have no medusal stage in their life cycle and do not exhibit alteration of generations, as do many other types of cnidarians.

Sea anemone stings, in particular, and anthozoan stings, in general, can exhibit from moderate to no symptoms. Itching, burning, swelling, and erythema may be initially experienced at the sting site, with local necrosis and ulcerations developing later.

In some cases, generalized symptoms, such as fever, abdominal pain, nausea, headaches, vomiting, and total weakness, may occur.

In some parts of the world, sea anemones are eaten by native people, causing intoxication due to the ingestion of anemone poisons. In Samoa and other parts of the tropical Pacific, certain species of sea anemones have been reported to be eaten (Farber & Lerke, 1963). Acute gastritis characterized with nausea, vomiting, and abdominal pain are early symptoms of intoxication, accompanied with cyanosis and total exhaustion. In a few cases, victims experience prolonged shock and die with pulmonary edema.

A variety of approaches has been used for treating nematocyst stings. They include topical or oral cortisone preparations, oral antihistaminics and topical antihistaminic creams, morphine sulfate, intravenous hypertonic glucose solutions, calcium gluconate, and subcutaneous epinephrine in severe cases. None of the numerous remedies, however, are not radical treatments directed toward either prevention or diminishing the neurotoxic and hemolytic effects of the venom. Another approach is to develop active or passive immunization against fatal envenomations, i.e. the sea wasp *Chironex fleckeri*, and *Physalia physalis*. There have been some studies on the development of an antivenin for humans.

Nematocyst discharge is triggered by an appropriate stimulation of chemo- and mechanoreceptors. The Hessinger laboratory has pioneered the study of the sensory control of discharge. In the present study, the sea anemone, *Aiptasia pallida* (Miami strain), is used because they: (1) can be asexually cloned and reared under standardized conditions to minimize variability among animals (Hessinger and Hessinger, 1981); (2)

are big enough to be individually manipulated for measurement of nematocyst discharge (Thorington and Hessinger, 1990); and (3) have the most characterized nematocyst toxins (Hessinger, 1988).

The Unit of Nematocyst Discharge

In anemone tentacles, each cnidocyte is surrounded by two or more accessory cells called supporting cells (Thorington and Hessinger, 1988; Watson and Hessinger, 1989). Contrary to the independent effector hypothesis, which states that the cnidocyte possesses the chemo- and mechanoreceptors involved in nematocyst discharge, it has been shown that the adjacent supporting cells possess the chemo- and mechanoreceptors which detect prey and trigger nematocyst discharge when suitably stimulated (Watson and Hessinger, 1988, 1991). Thus, the multicellular cnidocyte/supporting cell complex (CSCC) is the unit of nematocyst discharge in sea anemones.

Three types of CSCC have been identified : Types A, B, and C (Thorington and Hessinger, 1990). Type C CSCCs are induced to discharge by mechanical contact only. Type B CSCCs are induced to discharge by a combination of both mechanical and chemical stimulation. Stimulation of chemoreceptors for free and conjugated *N*-acetylated sugars predispose contact-sensitive mechanoreceptors (CSM) to trigger discharge in response to tactile stimulation (Thorington and Hessinger, 1988). Type A CSCCs involve vibration-sensitive mechanoreceptors (VSM), as well as CSMs and chemoreceptors. In the absence of *N*-acetylated sugars, the VSMs sensitize maximal discharge at 30, 50, 55, and 75 Hz frequencies, while in the presence of *N*-acetylated sugars, the VSMs sensitize maximal discharge in response to 5, 15, 30, and 40 Hz

stimulation, which match the movements produced by swimming prey (Watson and Hessinger, 1989). The frequency shifting process is referred to as "tuning" and involves concomitant elongation of the stereocilium hair bundles comprising the VSMs (Watson and Hessinger, 1991). Thus, chemoreceptors for *N*-acetylated sugars tune associated VSMs, and discharge is initiated by simultaneous stimulation of the CSMs and VSMs.

The three distinct types of CSCC provide versatility and specificity of cnidocyte responsiveness in fishing tentacles of sea anemone. Considering the cellular and molecular mechanisms underlying the chemosensory control of discharge, this study focuses on the control of discharge.

Type B CSCCs are associated with two general classes of chemoreceptors. In other words, two groups of low molecular weight substances sensitize cnidocytes in the tentacles of sea anemone, *Aiptasia pallida* : (1) A variety of amino compounds, such as glycine, proline, histamine, glutamine, and alanine; and (2) *N*-acetylated sugars, such as *N*-acetyl neuraminic acid (NANA), *N*-acetylglucosamine, and *N*-acetylgalactosamine.

Control of Sensitization

Sensitization appears to be controlled by two signaling pathways.

1. cAMP and G proteins. Agents known to elevate intracellular cAMP, such as cholera toxin, caged GTP-γS, forskolin, and dibutrylyl-cAMP sensitize Type B CSCCs. Reponses to such agents mimic the effects of chemoreceptor stimulation by *N*-acetylated sugars, such as NANA (Watson and Hessinger, 1992). In the presence of NANA, stimulation of endogenous adenylyl cyclase activity at the apical plasma membrane of

supporting cells has been detected by cytochemical methods (Watson and Hessinger, 1992).

These results suggest that stimulating chemoreceptors with NANA activates adenylyl cyclase in supporting cells, thereby elevating intracellular cAMP levels. According to my model, the increased levels of cAMP either directly or indirectly, via cAMP-dependent protein kinase, convert a contact-sensitive mechanoreceptor (CSM) from an inactive to an excitable state. Thus, in response to mechanical stimulation, the CSM initiates discharge.

2. Calcium. Extracellular calcium is required for discharge from all CSCC types (Watson and Hessinger, 1994), indicating a common dependence on calcium. Optimal discharge is observed at 10mM calcium, the normal level in sea water, while inhibition of discharge occurs at both higher and lower calcium levels.

Specific Goals and Objectives

Nematocyst discharge in anemones is regulated by chemoreceptors for *N*-acetylated sugars (e.g. *N*-acetylneuraminic acid, NANA). Since *N*-acetylated sugars are present on the surfaces of all aquatic prey, chemoreceptors for NANA are considered the primary receptors in prey capture. Possible involvement of the cAMP second-messenger system in the NANA signaling pathway of Type B CSCCs was suggested in a previous study (Watson and Hessinger, 1992). Furthermore, studies implicate dihydropyridine (DHP)-sensitive calcium channels in the NANA signaling pathway (Watson and Hessinger, 1994). My aims are to show that NANA dose-dependently increases the

cAMP content of anemone tentacles and that calcium has an antagonistic role on both cAMP content and sensitization and discharge.

My specific hypotheses are:

A. Micromolar levels of NANA elevate intracellular cAMP levels in sea anemone tentacles;

B. Millimolar levels of NANA elevate intracellular calcium levels in sea anemone tentacles.

I have tested these hypotheses using monoclonal sea anemones, *Aiptasia pallida* (Miami clone) reared under controlled conditions.

Significance of Intracellular Control of Nematocyst Discharge

Despite the fact that the cnidarians are at a diploblastic level of multicellular organization, cnidocytes and their supporting cells, which constitute the CSCCs, have a structural and functional complexity which is unique in the animal kingdom. When triggered, the nematocysts rapidly evert an eversible and hollow tubule containing a toxin. The chemosensory control of this spectacular event has been neglected so far.

Among several kinds of sensitizing chemoreceptors, the NANA chemoreceptor is considered to be the primary receptor since *N*-acetylated sugars occur on the surfaces of aquatic prey. The proposed investigations include the partial characterization of the NANA chemosensory signaling pathway and the modulatory role of calcium channels in this pathway. Therefore, this proposal on the intracellular control of nematocyst

discharge will develop our understanding of basic biologic events at the cellular level, which are applicable to all metazoans.

Prey capture and defense are the main functions of nematocysts. Besides being extremely harmful, the nematocyst stings of some cnidarians, such as *Physalia* (Portuguese Man-of-war) and *Chironex* (sea wasp) are fatal to human. However, no effective therapy exists due to insufficient knowledge on the nematocyst toxins and their functions. The study of mechanisms by which the discharge of nematocyst is controlled may suggest means to protect humans at risk from stinging cnidarians.

Relation to Present State of Knowledge

Prey capture is mediated by nematocyst discharge. Cnidarians are exclusively aquatic and predatory animals. They capture prey by discharging nematocysts. Nematocyst discharge is regulated by two sensory processes: chemoreceptor control and mechanoreceptor triggering. (1) In chemoreceptor control, activation of chemoreceptors "sensitize" (or predispose) contact-sensitive mechanoreceptors to respond to appropriate mechanical stimuli. (2) In mechanoreceptor triggering, the stimulation of contact mechanoreceptors "triggers" nematocyst discharge.

The chemosensory control of nematocyst discharge consists of three processes: (1) sensitization, which requires activation of the NANA chemosensory pathway that predisposes contact mechanoreceptors; (2) desensitization, which involves dose-dependent inhibition of sensitization by higher levels of NANA; and (3) modulation, which involves the interaction of the NANA signaling pathway with negative signaling

pathways from other remote receptor systems. The net interaction of sensitizing, desensitizing, and modulating pathways sets the threshold for mechanoreceptor triggering. The present proposal deals with aspects of all three processes controlling discharge.

Research on the nematocyst discharge has obstacles. Knowledge of the cellular and molecular mechanisms has been hampered by two difficulties. These are (1) lack of standardized experimental animals and lack of controlled experimental conditions and (2) failure to quantitate nematocyst discharge. The Hessinger laboratory has developed methods and techniques to manage these difficulties. Laboratory-cultured and asexually-cloned species of anemones have been used to ensure reproducibility of experimental results (Hessinger and Hessinger, 1981). The nematocysts discharged into artificial targets of defined size and composition are counted by a technique developed in the Hessinger laboratory (Thorington and Hessinger, 1990).

It has been suggested that nematocyst discharge takes place as a result of an exocytotic event in which the membrane enclosing the nematocyst fuses with the apical plasma membrane (Lubbock *et al.*, 1981). As for all known exocytotic processes, *in situ* discharge of nematocysts from *Hydra* requires extracellular calcium (Lenhoff and Bovaird, 1959) as does discharge from acontial tissues. Discharge from tentacles of the jellyfish, *Pelagia noctiluca*, is inhibited by gadolinium, a non-specific calcium channel blocker (Salleo *et al.*, 1994). Extracellular calcium is also required for discharge from Type A, B, and C CSCCs of sea anemone tentacles and dihydropyridine-sensitive

calcium channels are involved in discharge from Type B CSCCs (Watson and Hessinger, 1994).

NANA chemoreceptor sensitivity is dramatically affected by the level of extracellular calcium. In normal seawater containing 10 mM calcium, the dose-response is biphasic with an EC_{100} of about 10^{-5} M NANA. In the presence of 1 mM calcium, the biphasic dose-response to NANA shifts to the left by 7-orders of magnitude. In 11 mM, or higher calcium, however, the biphasic response to NANA disappears and discharge from Type B CSCCs is inhibited. These results indicate that NANA-sensitized discharge is both positively and negatively modulated by extracellular calcium.

Studies with drugs that affect calcium channels suggest that calcium modulates NANA sensitivity via dihydropyridine-sensitive calcium channels. In the absence of NANA, organic and inorganic calcium channel blockers sensitize nematocyst discharge, producing biphasic dose-responses. While the blocking (+) enantiomer of Bay K-8644 and the racemic (±) Bay K 8644 sensitize the nematocyst discharge, the activating (-) enantiomer does not. Furthermore, calcium channel blockers shift the NANA dose-response to the left, as does 1 mM calcium, while the effect of (-) Bay K 8644 is similar to that at high extracellular calcium. Thus, the pharmacological studies suggest that dihydropyridine-sensitive calcium channels modulate the sensitivity of Type B CSCCs to NANA.

Biophysical evidence of voltage-gated calcium channels comes from preliminary whole-cell patch-clamp studies of isolated supporting cells. Such studies indicate

positive inward currents with delayed inactivation, which are augmented by replacing extracellular calcium with $BaCl_2$. The barium-carrying inward currents are inhibited by Cd^{2+}.

CHAPTER TWO

N-ACETYLNEURAMINIC ACID (NANA) STIMULATES *IN SITU* cAMP PRODUCTION IN ECTODERM OF SEA ANEMONE (*Aiptasia pallida*) TENTACLES: POSSIBLE ROLE IN CHEMOSENSITIZATION OF NEMATOCYST DISCHARGE

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N-ACETYLNEURAMINIC ACID (NANA) STIMULATES *IN SITU* cAMP PRODUCTION IN ECTODERM OF SEA ANEMONE (*Aiptasia pallida*) TENTACLES: POSSIBLE ROLE IN CHEMOSENSITIZATION OF NEMATOCYST DISCHARGE

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Key words: Sea anemone, *Aiptasia pallida*, nematocyst discharge, NANA, cAMP, PKA.

Summary

Cnidocytes, the stinging cells of cnidarians, discharge nematocysts in response to physical contact in combination with the 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 ectodermal layer of sea anemone tentacles, cnidocyte/supporting cell complexes (CSCCs) control and trigger nematocyst discharge. Previous findings have implicated cAMP as a second messenger in NANA-sensitized nematocyst discharge. However, there are no reports of studies directly demonstrating this possibility. We now show that NANA dose-dependently increases *in situ* cAMP levels in the ectodermal layer of tentacles from the sea anemone, *Aiptasia pallida*. The endodermal layers of tentacles, however, have no detectable cAMP response to NANA. The effect of NANA on the cAMP content of ectoderm is biphasic. Micromolar NANA increases *in situ* cAMP level with a maximal response occuring at 1.8×10^{-5} M NANA. At higher NANA concentrations, the cAMP content decreases to that of controls. Because the cAMP dose-response to NANA coincides with the dose-responses of NANA-sensitized nematocyst discharge and nematocyst-mediated adhesive force, a role of cAMP in NANA- sensitized nematocyst discharge is strongly suggested. The addition of IBMX into the ambient medium of sea anemones enhances tissue cAMP levels. On the other hand, anesthetizing anemones in seawater containing high levels of Mg^{2+} blocks the NANA-stimulated cAMP response of the ectodermal layers to NANA. In addition,

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.

Introduction

Cnidarians capture swimming prey by discharging nematocysts located on their fishing tentacles. The explosive discharge of nematocysts is one of the most dramatic and rapid of single-cell events. Discharge is completed in less than 3 msec (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 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, 1988; Watson and Hessinger, 1989a, b). CSCCs consist of individual cnidocytes surrounded by two or more supporting cells. Two general classes of chemoreceptors detect substances derived from prey and predispose CSCCs to discharge nematocysts in response to suitable mechanical stimuli. One class of receptors 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 tentacle supporting cells (Watson and Hessinger, 1988) and, possibly also, on remote sensory cells (Thibodeaux and Watson, 1993).

Two classes of mechanoreceptors have been implicated in nematocyst discharge: contact-sensitive mechanoreceptors (CSM; Thorington and Hessinger, 1988a) that presumably trigger discharge; and vibration-sensitive mechanoreceptors (VSM; Watson and Hessinger, 1989a, b) that are frequency-tuned by chemoreceptors and presumably detect the swimming movements of nearby prey (Watson and Hessinger, 1991), thereby preparing a subpopulation of CSCCs for discharge.

Agents that increase intracellular cAMP levels (e.g. dibutyryl-cAMP, forskolin, and cholera toxin), sensitize CSMs and tune VSMs to lower frequencies, as does NANA. Furthermore, NANA stimulates adenylyl cyclase activity in supporting cells (Watson and Hessinger, 1992). In the present study, we demonstrate that NANA dose-dependently increases *in situ* cAMP levels in the ectodermal layer of tentacles from the sea anemone, *Aiptasia pallida*, while having no effect on tentacle endodermal cAMP content. 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

Materials

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

Maintenance of sea anemones

Monoclonal specimens of the sea anemone (A. *pallida*, Miami strain) were reared *en-masse* in flat-bottomed, glass trays and maintained individually in glass finger bowls containing natural seawater 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 light:12 h dark daily cycle, using white fluorescent lights at an intensity of 5.5 klux (66 $\mu Es^{-1}m^{-2}$; Hessinger and Hessinger, 1981).

Experimental anemones and test solutions

Anemones of the same size were selected and starved 72 h prior to experiments in order to maximize responsiveness (Thorington and Hessinger, 1988a). During each day of starvation, the natural seawater medium was replaced. During the last 48 h of starvation, anemones were kept under continuous fluorescent light at 4.5 klux

(54 $\mu Es^{-1}m^{-2}$). This provided optimal uniformity of anemone behavior (Thorington and Hessinger, 1988a, b).

Immediately before each experiment, sea anemones were gently rinsed with natural seawater to remove soluble wastes and then the medium was exchanged with the test solution. Test solutions of NANA in specified concentrations, containing 10^{-4} M IBMX, were prepared in natural, filtered (type 1, Whatman) seawater adjusted to pH 7.65 with 1 N HCl or 1 N NaOH. Artificial seawater (ASW) consisted of 423 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 24 mM $MgCl_2$, 25 mM $MgSO_4$, and 1.2 mM $NaHCO_3$, adjusted to pH 7.65. High magnesium/seawater (Mg-ASW) was prepared by dissolving 0.6 M $MgCl_2$ in distilled water and then diluting 1:1 with ASW.

Collection of tentacles

Individual anemones were chemosensitized with NANA solutions containing 10^{-4} M IBMX for 15 min after preincubating with 10^{-4} M IBMX alone for 20 min in filtered, natural seawater at room temperature. Individual primary tentacles were rapidly excised and transferred by fine forceps onto the polished ends of aluminum rods (2.2 cm diameter) standing in liquid nitrogen. The snap-frozen tentacles were then individually placed 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 individually extracted by adding 1 ml of ice-cold 1 N formic acid, pH 2.0 (Payne and Ames, 1982). After incubating 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 light microscopy and protein content of the supernatant medium. The endodermal layer remains intact and is surrounded by the sac-like mesogleal layer. To measure endodermal cAMP content, the endoderm plus mesoglea was homogenized in 1 ml of ice-cold 1 N formic acid using a pestle connected to a motor-driven mixer (Kontes Glass Co., Vineland, NJ). Formic acid was removed from the supernatant and homogenate samples by drying over night on a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). Each dried extract was then redissolved in 300 µl of 50 mM acetate buffer (pH 5.8). Individual aliquots of 100 µl were taken for separate measurements of cAMP and protein content.

The cAMP content of samples was measured by a commercial competitive radioimmunoassay kit (RPA 509, Amersham Life Sciences, Arlington Heights, IL) using 1.6 µCi adenosine 3', 5'-cyclic phosphoric acid 2'-0-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).

^{3}H-cAMP (adenosine-8-^{3}H 3', 5'-cyclic monophosphate; Sigma Chemical Co.; 1 mCi/ml) was used to measure efficiency of recovery of cAMP from tentacle extraction. ^{3}H-cAMP [0.1 µCi (≈ 2000 cpm)] was added to replicate single tentacles and extracted

normally and then counted by liquid scintillation. As a control for percent recovery [(extract cpm/total cpm) × 100], the same amount of ^{3}H-cAMP, without extraction and without tentacle, was counted by liquid scintillation. The percent recovery of ^{3}H-cAMP consistently measured between 90 and 95%. Assayed tentacle samples were corrected for recovery from extraction with each experiment.

Measurements of adhesive force and 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 by previously described methods (Geibel *et al.*, 1988; Thorington and Hessinger, 1988a, b). Briefly, test probes consisted of insect pins, the nylon heads (0.8 ± 0.01 mm diameter) of which were coated with 0.06 mm of 30% (w/v) gelatin. They were stored at 4°C under 100% humidity and used within 24 h. Single bowls containing individual sea anemones in various NANA concentrations in seawater 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) and a strip-chart recorder. The transducer was calibrated with gravimetric weights. The bowl was lowered gently and smoothly after 5 s of contact until tentacle separated from the probe. The force necessary to separate the probe from the tentacle was recorded and expressed in units of mg-force (mgf) since there is negligible acceleration (Miller, 1959).

After probes had been used to measure adhesive force, they were processed for counting nematocysts. Individual gelatin-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). After incubating the probes for 4 h at room temperature, the probes were removed and the nematocysts released from the hydrolyzed-gelatin were visually counted using an inverted light microscope at a final magnification of 512x.

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 broken cell preparations. 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 mM sodium phosphate buffer, pH 6.8, containing 5 mM EDTA, 0.5 mM IBMX, 75 mM NaCl, 10 mM $NaVO_4$, and 10 µM leupeptin). The sample was sonicated for 2-4 s in 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 with a pasteur pipette into a Dounce glass homogenizer. After removing most of the seawater, 100 µl of extraction buffer was added. Following gentle cell rupture by hand with ten strokes in ice, the total volume was raised to 2 ml. GTP and ATP at final concentrations of 0.1 mM were added to the preparation. The preparation was then divided to two 1 ml aliquots followed by adding 18 µl of 10^{-3} M NANA to one aliquot to a final concentration of 1.8×10^{-5} M, while the other aliquot received 18 µl of the

extraction buffer. Broken cell preparations with or without NANA were incubated in ice for 15 min, followed by taking aliquots for the assay of PKA.

Assay for PKA activity

PKA activity was measured according to modifications of the assay described by Cherrington *et al.* (1976) (Byus and Fletcher,1982). PKA activity was evaluated with kemptide as the substrate and assays were done under conditions of +/- cAMP, +/- PKA inhibitor (PKI), +/- kemptide, and various doses of kemptide. Positive and negative controls consisted of purified catalytic subunit from bovine heart and PKI purified from rabbit skeletal muscle, respectively, (Byus and Fletcher, 1982).

The assay mixture contained 35 μM kemptide, 0.2 mM ATP, gamma-[^{32}P]-ATP (total specific activity 200 μCi/pmol), and 200 mM MES buffer, pH 6.8, containing 40 mM $MgCl_2$ and 28 mM β-mercapto-ethanol. Aliqouts 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 otherwise indicated) at 30°C. The reaction was terminated by pipetting 40 μl of the reaction volume onto Whatman P81 filters (2 cm 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 15% acetic acid wash, 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 by the enhanced BCA protein assay (Wiechelman *et al.* 1988) using bovine serum albumin as the standard (Pierce Chemical Co., Rockford, IL).

Data analysis

Results of cAMP experiments were determined by using a standard curve of the assay kit generated with each experiment. Data was corrected for recovery from extraction of ^{3}H-cAMP, and then normalized as cAMP content per µg protein (fmol cAMP/µg protein). PKA activity was expressed as pmol PO_4 transferred per min. All results are given as mean ± SEM. 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). 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 4 and 20.

Results

Basal cyclic AMP contents of tentacle ectoderm and endoderm

The basal cyclic-AMP (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 M acetate buffer (pH 5.8) or 1 N 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-fold the cAMP content of the acetate-extracted tentacles (Fig. 1; $P < 0.0001$). The endodermal cAMP contents prepared by two extraction methods were indistinguishable ($P = 0.98$). In view of the higher cAMP recovery from the ectoderm extract and because the formic acid-extracted tentacles exhibited complete removal of the ectodermal layer as determined by light microscopic examination, we used 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 seawater containing 10^{-4} M IBMX, a potent phosphodiesterase inhibitor, were about 30% higher than those incubated in seawater alone (Fig. 2; $P < 0.01$). The cAMP content of tentacles from animals treated with 1.8×10^{-5} M NANA plus 10^{-4} M IBMX were about 35% higher than those incubated in seawater containing NANA alone (Fig. 2; $P < 0.002$). Because IBMX enhanced cAMP levels, we pre-incubated anemones in 10^{-4} M IBMX in all subsequent experiments.

Effect of Mg-ASW on NANA-induced cAMP content

In normal ASW, 1.8×10^{-5} M NANA stimulates almost a two-fold increase in *in situ* cAMP content of tentacle ectodermal layers (Fig. 3; $P < 0.0001$). Because high levels of Mg^{2+} are commonly used to anesthetize and immobilize spontaneously contracting excised tentacles in published experiments related to chemosensory signaling pathways, we tested the effects of anesthetizing levels of Mg^{2+} on NANA-stimulated

cAMP content. In Mg-ASW containing 324.5 mM $MgCl_2$, NANA-stimulated cAMP increase was prevented, with cAMP content not being significantly different than controls (Fig 3; $P = 0.17$).

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. 4): sensitization and desensitization. The sensitization region occurred at the lower tested concentrations of NANA. Half-maximal discharge (EC_{50}) occurred at approximately 10^{-7} M NANA. Maximum discharge was 3.4-fold that of the seawater controls and occurred at 1.8×10^{-5} M NANA (EC_{100}). The effect of NANA on adhesive force (Fig. 4) was also biphasic and coincided with the nematocyst discharge response curve. Maximum adhesive force was approximately 1.2-fold that of the seawater controls and occurred at 1.8×10^{-5} M NANA (EC_{100}).

NANA also dose-dependently increased the cAMP content of tentacle ectodermal layers (Fig. 4). 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 NANA-stimulated ectodermal layers occurred at 1.8×10^{-5} M NANA, approximately twice that of seawater-treated anemones ($P < 0.0001$). In contrast, endodermal layers showed no significant change in cAMP content over the tested range of NANA concentrations. The average cAMP content of endodermal layers from all NANA doses was 5.2 ± 0.5 fmol/μg protein (Fig. 4); a value about one-seventh that of ectodermal layers from control anemones and one-fourteenth that of ectodermal layers from anemones exposed to 1.8×10^{-5} M NANA.

Time-course of NANA-stimulated ectodermal cAMP

Ectodermal cAMP content was measured for individual tentacles from sea anemones incubated in 1.8×10^{-5} M NANA for different durations (Fig. 5). No significant increase in cAMP occurred in 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 mins the cAMP content did not change. The maximum increase in cAMP occurred at 15 min; approximately 1.8-fold that averaged for 0 to 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 ($P < 0.05$).

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 determined by measuring the activity both in the presence and absence of 0.1 mM cAMP (Fig. 6). 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 about 40% the undiluted supernatants.

To evaluate the effect of kemptide concentration on PKA activity, different concentrations of kemptide were tested with and without 0.1 mM cAMP. The enzyme activity increased with increasing substrate concentration both with or without added cAMP. Activities with added cAMP were consistently higher than activities without added cAMP at the same substrate concentrations (Fig. 7). The inhibitor PKI at 20 nM

concentration totally inhibited PKA activity both in the presence and absence of exogenously added cAMP (data not shown).

cAMP-dependent protein kinase activity in tentacle broken cells

Tentacles pooled together had their cells gently ruptured as described in the Materials and Methods section. Broken cell preparations were assayed for PKA activity. The enzyme activity in the absence of NANA was 14.08 ± 0.46 pmol of PO_4/min. In the presence of NANA, the activity averaged 15.57 ± 0.33 pmol of PO_4/min. The difference between the two groups was statistically significant ($P = 0.02$) (Fig. 8). 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, called 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 is 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, 1942a, b; Thorington and Hessinger, 1988a, b). Activated chemoreceptors are alleged to predispose contact-sensitive mechanoreceptors which

trigger discharge immediately upon 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 toward the mouth are controlled by asparagine 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 (CTX) (Awad and Anctil, 1993). Adenylyl cyclase activity is also found in membrane preparations of *Hydra*, where it is stimulated by Mg^{2+}, Mn^{2+}, NaF, and GTP (Venturini *et al*, 1984).

In the sea anemone, *H. luciae*, the membrane permeant cAMP analogue, dibutyryl-cAMP, biphasically sensitizes *in situ* nematocyst discharge as do forskolin, CTX, 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 findings, we directly demonstrate that the chemosensitizer, NANA, increases the *in situ* cAMP content of *A. pallida* tentacle ectoderm. The effect of NANA

concentration on cAMP content yields a biphasic dose-response that coincides with those of NANA-sensitized nematocyst discharge and nematocyst-mediated adhesive force (Fig.4). These dose-response curves have similar regions of sensitization, EC_{100} values, and regions of desensitization at yet higher concentrations. The relevance of NANA-stimulated increases in cAMP 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. The observation that NANA optimally increases cAMP levels of the ectoderm only 2-fold is likely due to the fact that, in addition to CSCCs, the tentacle ectoderm includes many cells which may not respond to externally applied NANA, including those of the neural plexus and the longitudinal muscle layer. Thus, our data strongly suggest that NANA-stimulated cAMP functions as a second messenger in NANA-sensitized nematocyst discharge.

High levels of Mg^{2+} are commonly used to anesthetize anemones as well as to immobilize excised tentacles (Thorington and Hessinger, 1988; Watson and Hessinger, 1991; 1992; 1994; Thibodeaux and Watson, 1993). High levels of Mg^{2+} in Mg-ASW also appear to block *in situ* NANA-stimulated cAMP increase in tentacle ectoderms (Fig. 3). The same high dose of Mg^{2+} also blocks *in situ* NANA-sensitized nematocyst discharge in *A. pallida* and *H. luciae* tentacles (unpublished data). However, in excised *H. luciae* tentacles anesthetized with Mg-ASW, stereociliary bundles (SBs), which compromise the vibration-sensitive mechanoreceptors (VSMs) of Type A CSCCs,

elongate in response to NANA and to agents that commonly increase intracellular cAMP levels (Watson and Hessinger, 1992). Thus, either NANA-induced SB elongation occurs by a cAMP-independent pathway or *A. pallida* and *H. luciae* differ with respect to the effects of high Mg-ASW on NANA-stimulated cAMP increases.

The time-course of NANA-stimulated cAMP shows that NANA increases cAMP levels above controls only after 3 min. On the other hand, unpublished experiments with gelatin-coated probes pre-equilibrated with solutions of various NANA concentrations indicate that the sensitizing effect of NANA on nematocyst discharge can occur within a few sec. In tentacles of *H. luciae*, chemosensitization of nematocyst discharge increases in 1 min, reaching the highest level at 5 min, followed by a gradual decline by 25 min. The 25 min minimum is then followed by an increase with smaller maximum at 35 min which is followed by a lower minimum at 45 min (Watson and Hessinger, 1989a). In experiments in which the whole anemone is bathed in medium containing NANA, specimens of *A. pallida*, which are found subtidally in nature, require 3-5 min to recover from the disturbance of changing medium, whereas *H. luciae*, which are found intertidally in nature, appear undisturbed by medium changes. 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.

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 portion of the basal cAMP content of 40 pmol/μg protein is maintained by contributions from the NANA-responsive CSCCs.

Our data also indicate the presence of endogenous protein kinase A activity in *A. pallida* tentacles. The monospecific protein kinase A inhibitor protein, PKI or Walsh protein, blocks 98% of this enzyme activity indicating that the activity is due to PKA (Fig. 8). This is further confirmed by the observation that added cAMP increases activity (Figs. 6 and 7). In broken cell preparations of tentacles to which ATP, but not cAMP, is added, NANA stimulates PKA activity by about 10% ($P < 0.02$). This suggests that stimulated NANA chemoreceptors activate adenylyl cyclase to synthesize enough additional cAMP to significantly stimulate PKA above untreated controls. That higher levels of NANA-stimulated PKA activity were not measured may be due to problems associated with diluting the broken cell preparations 40-fold for the PKA assay and with possible physical uncoupling of the membrane-bound chemosensory components of the signaling pathway during cell rupture.

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 seawater block NANA-stimulated cAMP increase is consistent with the fact that high Mg^{2+} blocks nematocyst discharge, but raises questions about the purported role of cAMP in NANA-induced

elongation of SBs of vibration-sensitive hair bundles. In conclusion, our findings are consistent with a second-messenger role for cAMP in NANA-sensitized discharge of nematocysts.

Figure 1. Effect of extraction buffer on measured cAMP content of untreated ecto- and endodermal layers from *A. pallida* tentacles. Tentacles of 72-h starved sea anemones were individually excised, snap-frozen, stored at -80°C, and later extracted in two different media. For measurement of ectodermal cAMP, either 1 ml of ice-cold 1 N formic acid, pH 2.0, (closed bars) or 1 ml of ice-cold 0.05 M acetate buffer, pH 5.8, (open bars) 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 either1 ml of 1 N formic acid or 1 ml of 0.05 M acetate buffer, pH 5.8, on ice, and then assayed for cAMP and protein content. Data were expressed as fmol cAMP per μg protein. A total of 28 sea anemones were used. Data are means ± SEM (N = 2; n = 17).

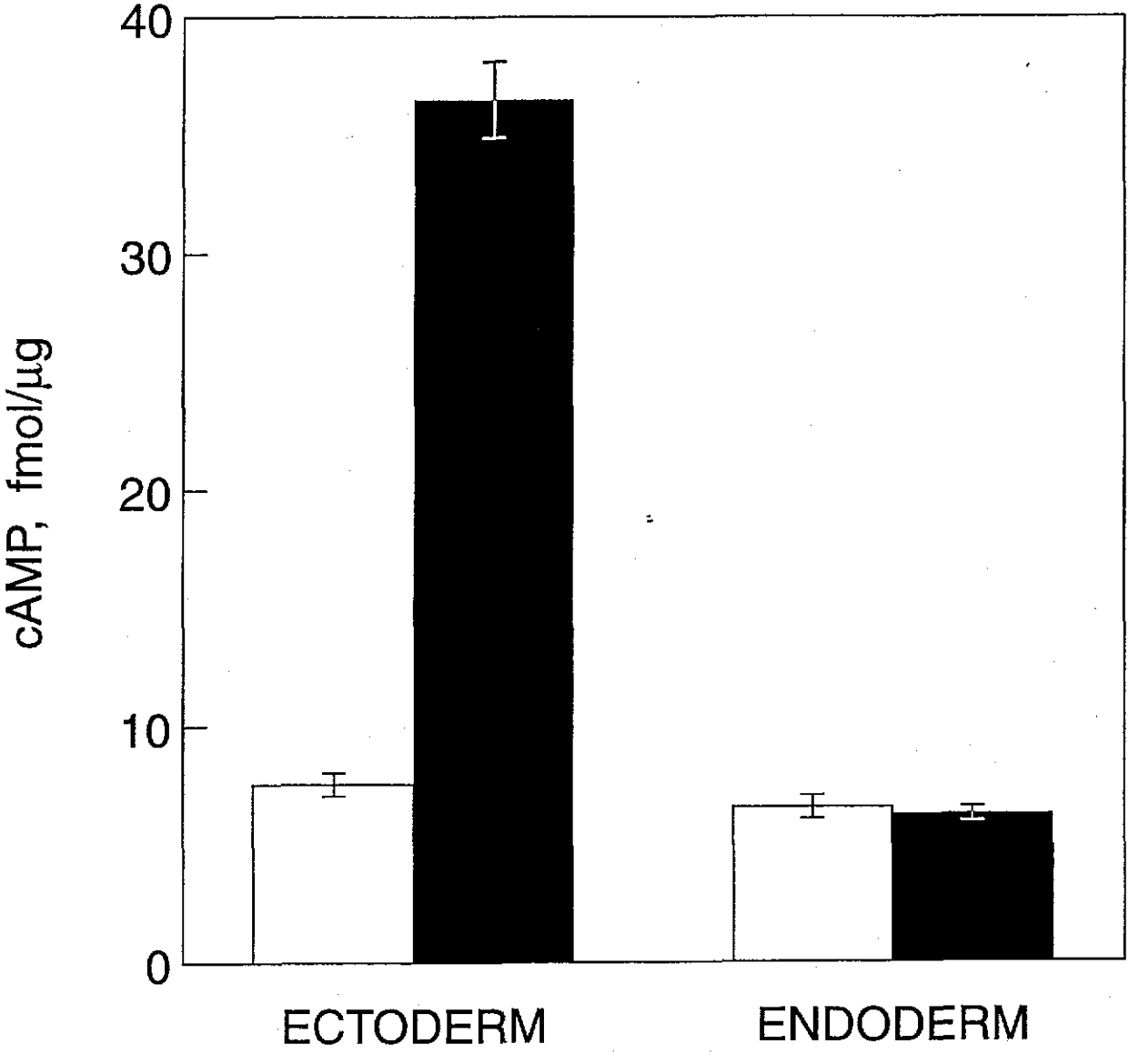

40
30
20
10
0
cAMP, fmol/µg
ECTODERM
ENDODERM

Figure 2. Effect of IBMX on tentacle ectodermal cAMP content following treatment with and without NANA. A group of 72-h starved sea anemones were incubated in filtered, natural seawater, pH 7.65 with or without 10^{-4} M IBMX for 20 min (closed bars). A second group of 72-h starved anemones were incubated in filtered, natural seawater, pH 7.65 containing 10^{-4} M IBMX with or without 1.8×10^{-5} M NANA for 20 min (open bars). Individual tentacles from each group were collected, snap-frozen, extracted, and assayed for ectodermal cAMP using 1 N formic acid. Data were expressed as fmol cAMP per μg protein. A total of 4 anemones were used. Data are means ± SEM (n = 8).

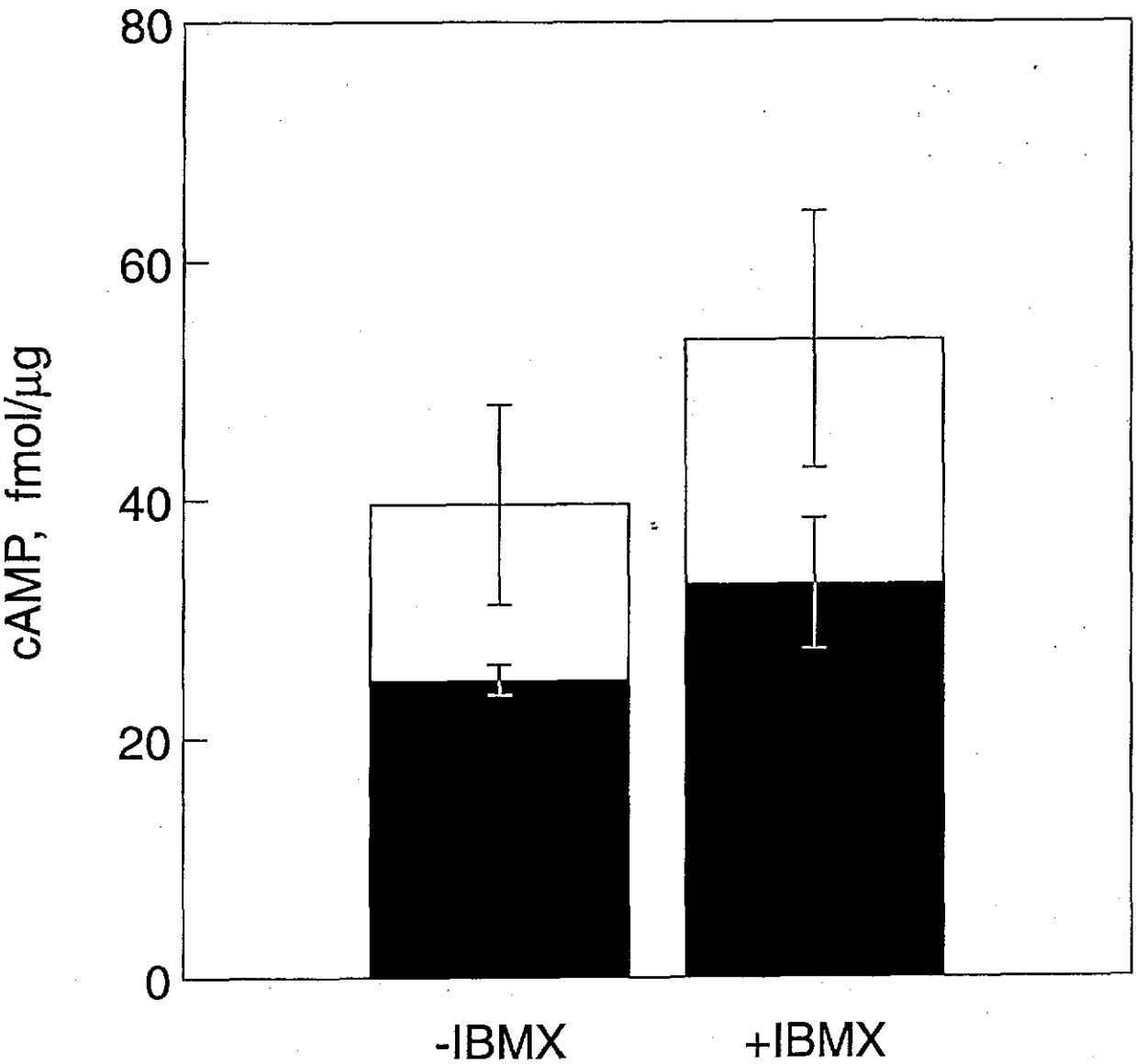

80
60
40
20
0
cAMP, fmol/µg
-IBMX
+IBMX

Figure 3. Effect of NANA and Mg-ASW on tentacle ectodermal cAMP content. Following 72-h starvation, one group of 4 anemones was pre-incubated in ASW containing 10^{-4} M IBMX for 10 min followed by incubation in ASW containing either 10^{-4} M IBMX and 1.8×10^{-5} M NANA (closed bars) or 10^{-4} M IBMX alone (open bars) for 15 min. Individual tentacles from each group were collected, snap-frozen, extracted, and assayed for ectodermal cAMP using 1 N formic acid. Data were expressed as fmol cAMP per μg protein. A second, similar group of 4 anemones were treated the same as above except Mg-ASW replaced ASW. A total of 8 anemones were used. Data are means ± SEM (n = 20).

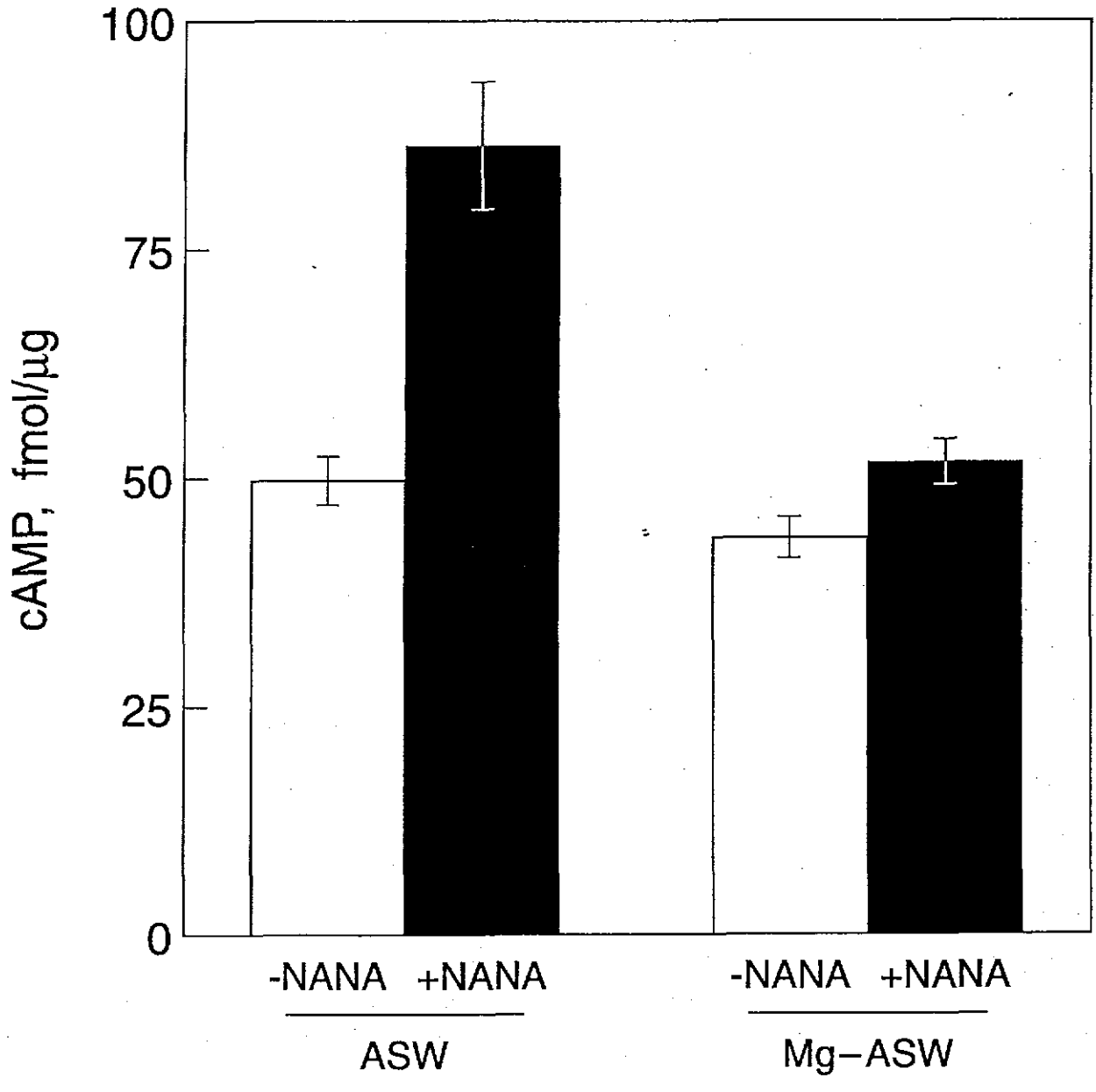

100
75
50
25
0
cAMP, fmol/µg
-NANA
+NANA
-NANA
+NANA
ASW
Mg−ASW

Figure 4. Effect of NANA on nematocyst discharge, adhesive force, and tentacle cAMP content. Pre-starved anemones were incubated in filtered, natural seawater containing the specified NANA concentrations for 10 min before measuring *in situ* adhesive force and nematocyst discharge. Nematocysts on six probes were counted for each dose tested and expressed as the mean of three experiments (□, N = 3; n = 18). Values of adhesive force measurements were expressed as the mean of three experiments (■, N = 3; n = 27). For measurement of cAMP, pre-starved anemones were pre-incubated in filtered, natural seawater containing 10^{-4} M IBMX for 20 min followed by incubation in seawater containing 10^{-4} M 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 N formic acid as in Figure 1. Results were expressed as fmol/μg protein. The number of anemones used in each experiment ranged between 16 and 20. Data are means ± SEM.

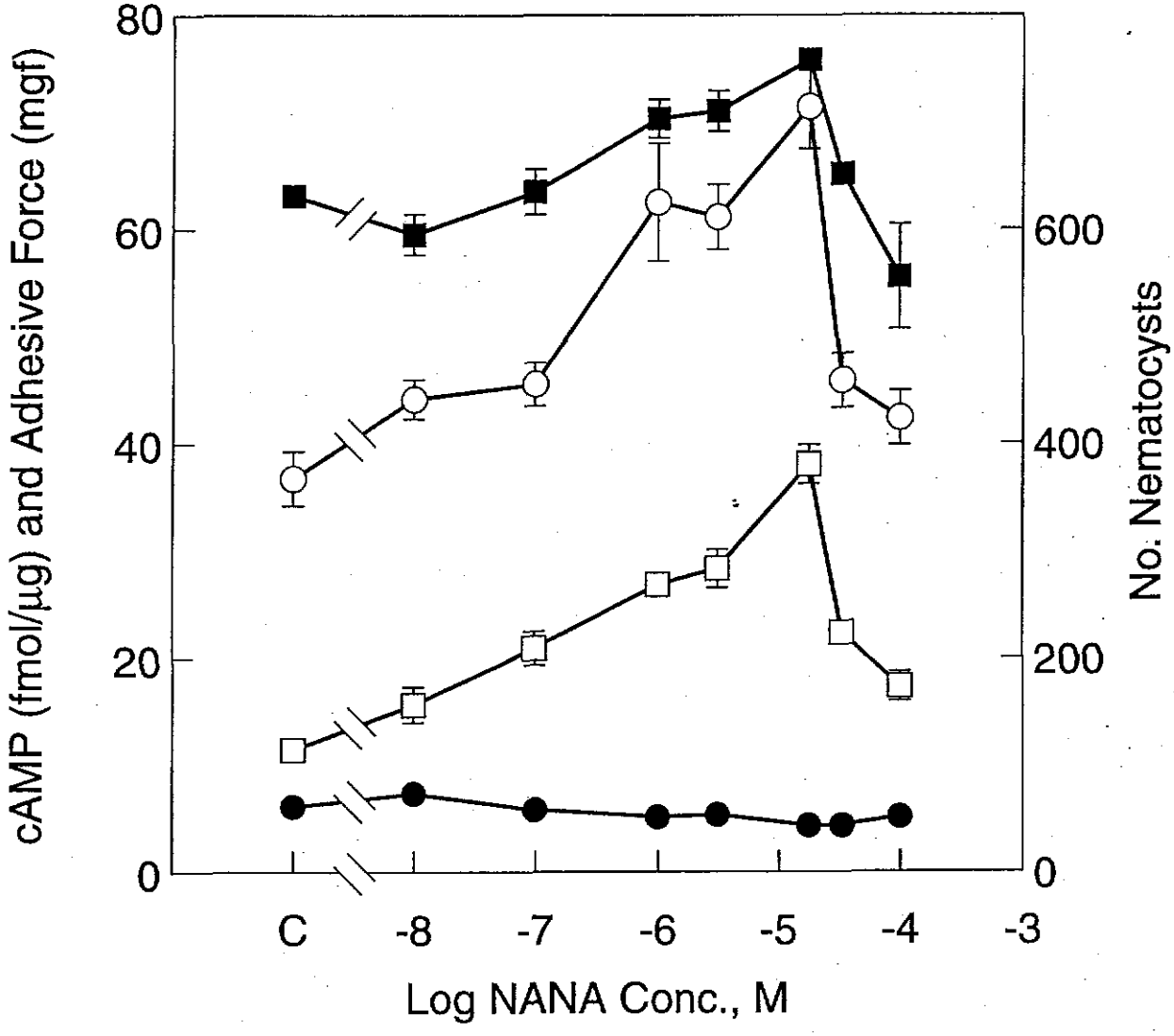

80
60
40
20
0
600
400
200
0
C
-8
-7
-6
-5
-4
-3
Log NANA Conc., M
cAMP (fmol/μg) and Adhesive Force (mgf)
No. Nematocysts

Figure 5. Time-course of NANA-stimulated cAMP content of tentacle ectoderm. Pre-starved anemones were pre-incubated in filtered, natural seawater containing 10^{-4} M IBMX for 20 min followed by incubating in filtered, natural seawater containing 10^{-4} M IBMX and 1.8×10^{-5} M NANA for specified durations followed by excising, snap-freezing, and assaying for ectodermal cAMP content as in Figure 4. Two sets of experiments were combined: one measuring the cAMP contents between 0 and 5 min of incubation with NANA (○, N = 3; n = 36) and the other between 5 and 45 min of incubation with NANA (●, N = 3; n = 22). The value for 5 min incubation was the average of means from the two sets of experiments. Data were expressed as fmol cAMP per μg protein. The number of sea anemones used in each experiment ranged between 18 and 20. Data points are means ± SEM.

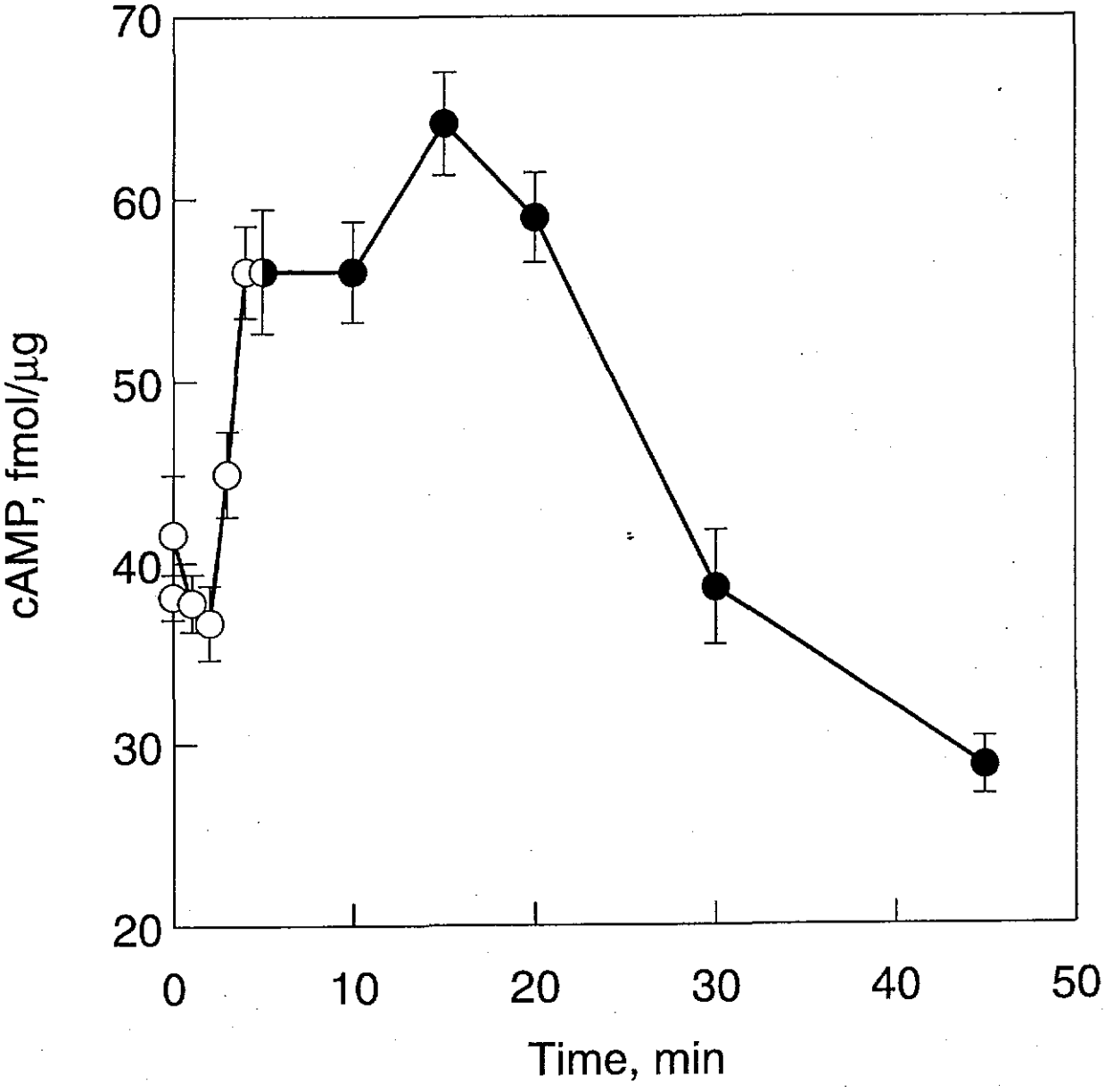

70
60
50
40
30
20
cAMP, fmol/µg
0
10
20
30
40
50
Time, min

Figure 6. 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 sample 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 μM kemptide in the presence and absence of 0.1 mM exogenous cAMP using undiluted (closed bars) and 1:1 diluted (open bars) supernatants. Protein content of undiluted supernatants was 176 μg/ml. Data were expressed as pmol phosphate transferred per min. Each data point represents the mean ± SEM (N = 2; n = 4).

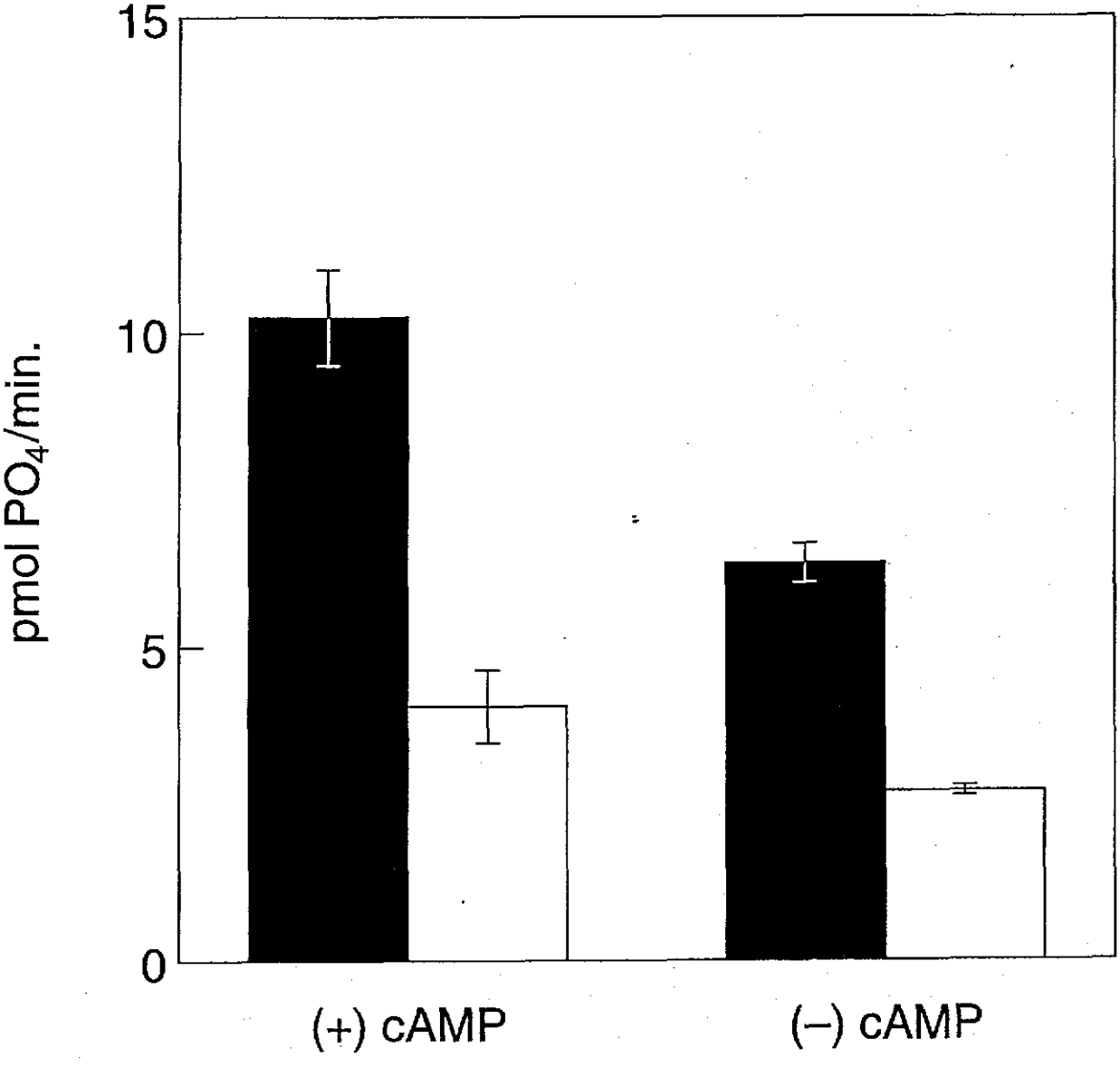

15
10
5
0
pmol PO_4/min.
(+) cAMP
(–) cAMP

Figure 7. Effect of substrate concentration on PKA activity in cell-free extracts of tentacles. Tentacles were collected, pooled, and processed for the assay of PKA activity as in Figure 6. The supernatant extract was immediately mixed with different concentrations of kemptide in the reaction mixture in the presence (●) or absence (○) of 0.1 mM cAMP. Protein content of supernatants was 140 μg/ml. Data were expressed as pmol phosphate transferred per min. Each data point represents the mean ± SEM (N = 2; n = 4).

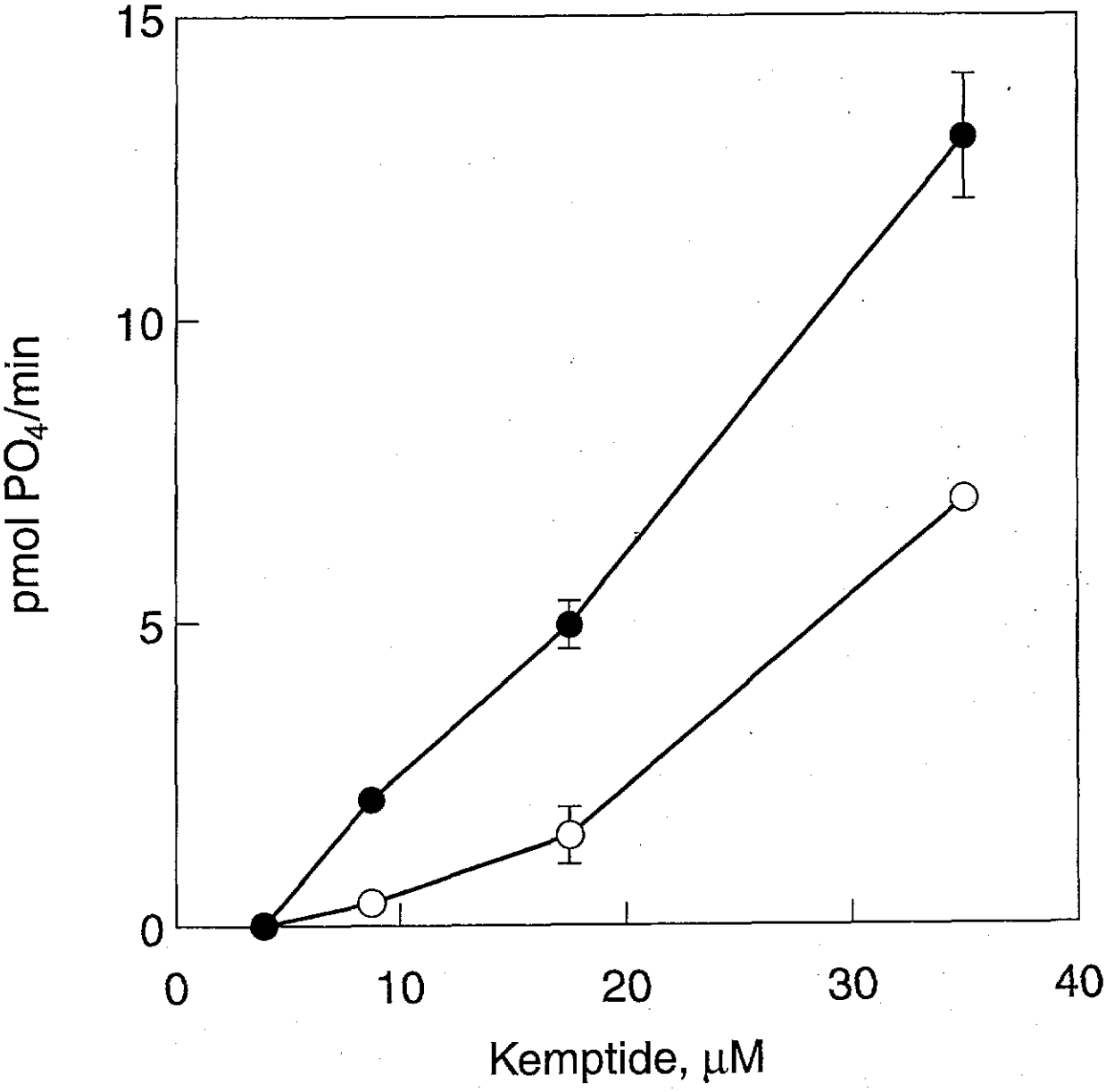
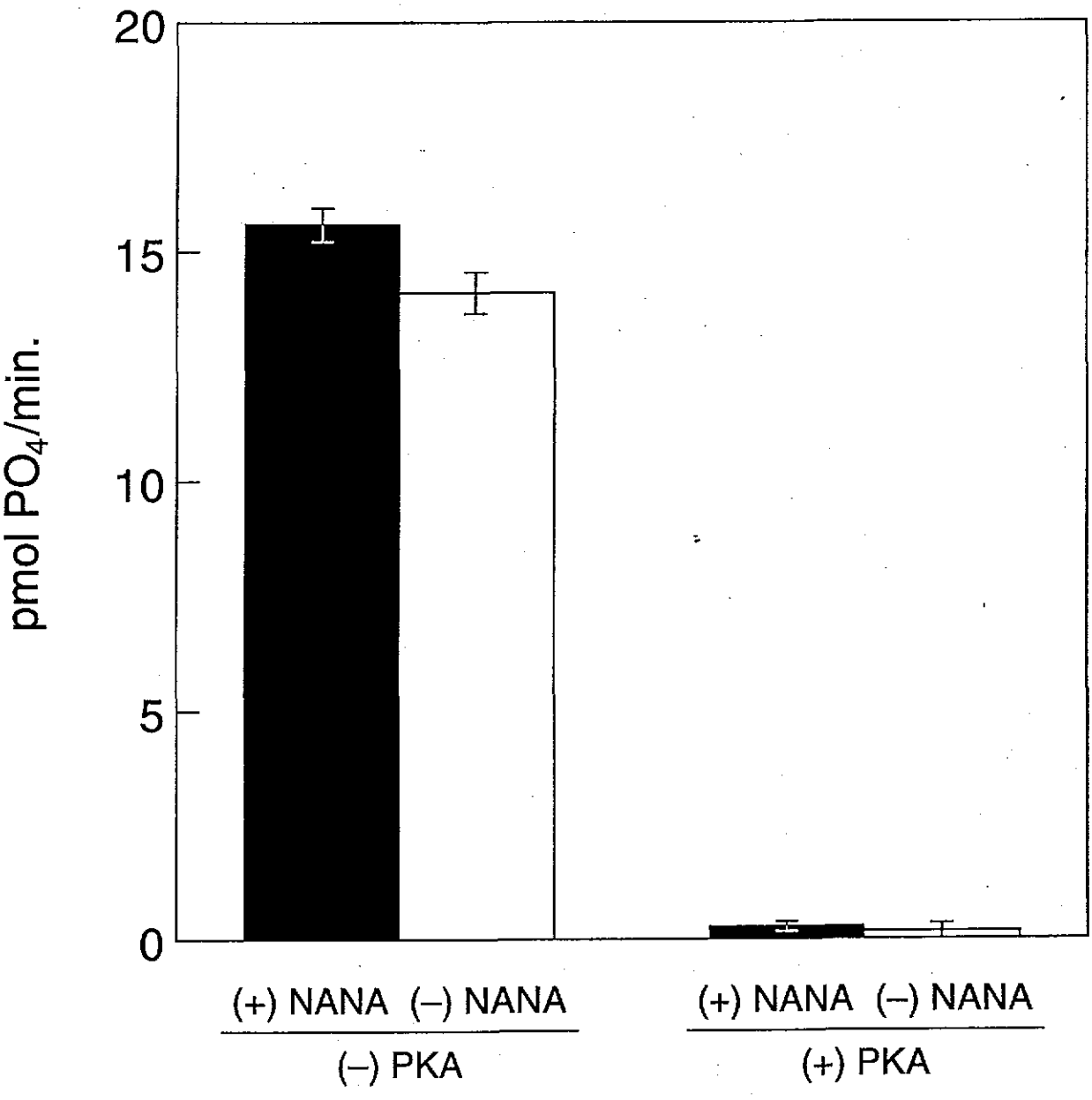

15
10
5
0
pmol PO4/min
0
10
20
30
40
Kemptide, μM

Figure 8. Effect of NANA on PKA activity in whole tentacle homogenate. Tentacles were harvested, pooled, and gently homogenized on ice in extraction buffer, pH 6.8, with ten strokes of a dounce, all-glass homogenizer. Homogenates including 0.1 mM GTP and 0.1 mM ATP were stored on ice for 15 min in the presence (closed bars) and absence (open bars) of 1.8×10^{-5} M NANA after which 40 µl aliquots were taken for PKA assay at 30°C for 10 min. The addition of 20 nM of PKI completely blocked the PKA activity both in the presence and absence of NANA. Protein concentration of the homogenate was 528 µg/ml. Data were expressed as pmol phosphate transferred per min. Each data point represents the mean ± SEM (n = 4).


20
15
10
5
0
pmol PO_4/min.
(+) NANA
(–) NANA
(–) PKA
(+) NANA
(–) NANA
(+) PKA

CHAPTER THREE

N-ACETYLNEURAMINIC ACID (NANA)-INDUCED $^{45}Ca^{2+}$ UPTAKE BY ISOLATED ECTODERMAL CELLS FROM TENTACLES OF SEA ANEMONE, *Aiptasia pallida*: POSSIBLE ROLE IN DESENSITIZATION OF NANA CHEMOSENSORY PATHWAY

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N-ACETYLNEURAMINIC ACID (NANA)-INDUCED $^{45}Ca^{2+}$ UPTAKE BY ISOLATED ECTODERMAL CELLS FROM TENTACLES OF SEA ANEMONE, *Aiptasia pallida*: POSSIBLE ROLE IN DESENSITIZATION OF NANA CHEMOSENSORY PATHWAY

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Key words: Sea anemone, *Aiptasia pallida*, nematocyst discharge, NANA, $^{45}Ca^{2+}$ uptake, L-type calcium channels, dihydropyridine.

Summary

In fishing tentacles of sea anemones, nematocyst discharge is regulated and triggered by stimulation of chemo- and mechanoreceptors. Exogenous *N*-acetylated sugars (e.g. *N*-acetylneuraminic acid, NANA) bind to chemoreceptors and sensitize *in situ* nematocysts to discharge in response to triggering physical contact. Micromolar NANA sensitizes discharge, while at higher concentrations, desensitization occurs, causing a decrease in discharge. We previously reported indirect pharmacological evidence suggesting a modulatory role for dihydropyridine (DHP)-sensitive calcium channels in the NANA chemosensory signaling pathway that regulates *in situ* discharge in the sea anemones, *Aiptasia pallida* and *Haliplanella luciae*. The present findings relate to the possible role of DHP-sensitive calcium channels in desensitization of the NANA signaling pathway. We find that NANA increases calcium influx into isolated tentacle ectodermal cells from *A. pallida*, but only at concentrations that desensitize. The dose-dependent, NANA-stimulated increase in calcium influx is completely blocked by nifedipine, diltiazem, methoxy verapamil (D-600), and Cd^{2+}. The dihydropyridine (DHP) calcium channel activators, (-) and (+) enantiomers of Bay K-8644, stimulate calcium influx into ectodermal cells, as does the calcium ionophore, A23187. High levels of extracellular KCl dose-dependently stimulate DHP-sensitive calcium influx, suggesting that NANA-stimulated calcium influx is mediated by voltage-gated calcium channels. Our findings indicate that among tentacle ectodermal cells, at least two types of voltage-sensitive calcium channels exist: DHP-sensitive and DHP-insensitive. Our

findings document the existence of NANA-stimulated, DHP-sensitive calcium channels in the anemone tentacle ectoderm and implicate these channels in the desensitization of the NANA signaling pathway.

Introduction

Sea anemones and other cnidarians, such as jellyfish, hydra, and corals, are the simplest metazoans having a nervous system. Cnidarians employ complex secretory products, called nematocysts, for such functions as prey capture, defense, and aggression. The fishing tentacles of sea anemones are armed with these stinging organelles. Being contained within specialized cells called cnidocytes, 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 is conveyed, is called nematocyst discharge. In this manner the discharging nematocyst may inject potent toxins into the prey (Hessinger, 1988).

Nematocyst discharge is initiated by appropriate stimulation of chemo- and mechanoreceptors by prey (Pantin, 1942a, b; Thorington and Hessinger, 1988a, b). Activated chemoreceptors predispose contact-sensitive mechanoreceptors which trigger discharge immediately upon prey contact with the tentacle. Chemical and mechanical stimuli originating from prey also regulate feeding behavior and ingestion of prey by sea anemones. In the sea anemone, *Anthopleura elegantissima*, concerted tentacle movements toward the mouth is controlled by asparagine presumed to leak from

nematocyst-inflicted wounds, while reduced glutathione controls the ingestion of food into the mouth (Lindstedt, 1971).

On the tentacles of the sea anemones, *Haliplanella luciae* and *Aiptasia pallida*, nematocyst discharge is controlled by cnidocyte/supporting cell complexes (CSCCs; Watson and Hessinger, 1989a; Thorington and Hessinger, 1990). In *A. pallida*, at least two classes of chemoreceptors have been identified: one sensitive to free and conjugated *N*-acetylated sugars (e.g. *N*-acetylneuraminic acid, NANA); the other to low molecular weight amino compounds (Thorington and Hessinger, 1988a). Chemoreceptors for NANA are located on the supporting cells rather than on the cnidocytes (Watson and Hessinger, 1989a). At micromolar concentrations, NANA increases discharge of nematocysts, with maximal discharge occuring at 1.8×10^{-5} M. However, at higher concentrations of NANA, discharge decreases to control levels due to dose-dependent desensitization (Thorington and Hessinger, 1988a; Watson and Hessinger, 1989b).

Knowledge about the role of calcium in sensitization and desensitization of nematocyst discharge in the NANA signaling pathway is relatively limited. While a role for calcium in the *in situ* discharge of nematocysts is now certain, its mechanism is yet vaguely understood. In this study, we demonstrate that the N-acetylated sugar, NANA, dose-dependently stimulates a dihydropyridine (DHP)-sensitive calcium influx into isolated tentacle ectodermal cells from *A. pallida* in a manner consistent with the calcium influx being involved in desensitization. These findings support the model of Thorington and Hessinger (2001) that DHP-sensitive calcium channels play a role in desensitization of the NANA chemoreceptor signaling pathway.

Materials and methods

Reagents and experimental solutions

Nifedipine, diltiazem, methoxy verapamil (D-600), A23187 (free acid form), N-acetyl neuraminic acid (NANA; type VI), and $CdCl_2$ were obtained from Sigma Chemical (St. Louis, MO). Both (-) and (+) enantiomers of Bay K-8644 were obtained from Research Biochemicals Inc. (Natick, MA). Radioactive $^{45}CaCl_2$ (specific activity = 12.8 mCi/mg) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Nifedipine, A23187, and (+) Bay K-8644 were dissolved in dimethyl sulfoxide (DMSO) with the final DMSO concentration less than 1.0%. All other compounds were soluble in water.

Artificial seawater (ASW) was prepared as a solution of 423 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 24 mM $MgCl_2$, 25 mM $MgSO_4$, and 1.2 mM $NaHCO_3$. Freshly prepared ASW for each experiment was adjusted to pH 8.0 with 1 N NaOH immediately before use. Divalent cation-free artificial seawater (df-ASW) was prepared with the same components as ASW except that $CaCl_2$, $MgCl_2$, and $MgSO_4$ were omitted and the NaCl concentration was increased to 500 mM. EDTA (ethylenediamine tetraacetic acid; Sigma) was added to a final concentration of 11 mM and the final pH was adjusted to 8.0. Magnesium/seawater (MgSW) was prepared by dissolving 0.6 M $MgCl_2$ in distilled water and then diluting 1:1 with filtered, natural seawater. Filtered, natural seawater was obtained from the Kerckoff Marine Laboratory of California Institute of Technology (Corona del Mar, CA). Aqueous solutions were prepared in Millipore-filtered (Bedford, MA) distilled water (Milli-Q grade).

Maintenance of sea anemones

Monoclonal sea anemones (*A. pallida*; Miami strain) were reared en-masse in flat-bottomed, glass trays and then maintained individually in glass finger bowls containing filtered natural seawater at 23 ± 1 °C. The animals were fed daily with freshly hatched brine shrimp nauplii (*Artemia salina*) and washed 4-6 hours after feeding. Animals were kept on a 12 h light:12 h dark daily cycle using white fluorescent lights at an intensity of 5.5 klux ($66\mu Es^{-1}m^{-2}$; Hessinger and Hessinger, 1981). Experimental anemones were used approximately 72 h after the last feeding in order to maximize responses (Thorington and Hessinger, 1988b).

Counting discharged nematocysts

The number of nematocysts discharged in response to NANA sensitization was determined by methods previously described (Geibel *et al.*, 1988; Thorington and Hessinger, 1988a, b). Briefly, test probes consisted of insect pins, the nylon heads (0.8 ± 0.01 mm diameter) of which were coated with 0.06 mm of 30% (w/v) gelatin. They were stored at 4°C and used within 24 h. Single bowls containing individual sea anemones in various NANA concentrations in seawater were raised by hand until the tip of a tentacle contacted the probe attached to a ring stand. The bowl was lowered gently and smoothly after 5 s of contact until the separation of the tentacle from the probe occured. Then the individual gelatin-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). After incubating the probes for 4 h in the solution at room temperature, the probes were removed and the

nematocysts released from the hydrolyzed-gelatin were visually counted using an inverted light microscope at a final magnification of 512x.

Harvesting ectodermal cells

Two, pre-starved anemones of the same size were prepared for collecting tentacles by anesthesizing them for 5 min in MgSW. Ten large, outer tentacles from each anemone were rapidly excised and rinsed with df-ASW followed by incubation in the same medium for 1 h at 23 ± 1 °C. At the end of the incubation, the 20 tentacles were transferred to a 1-ml eppendorf tube in which they were gently triturated (20-30 times) through a siliconized (Sigmacote; Sigma Chemicals), fire-polished pasteur pipette. Residual tentacles consisting of intact mesoglea and the contained gastrodermal cell layers were removed with fine forceps. The medium containing the cells of dissociated ectodermal layer was immediately centrifuged at 600×g for 5 min (Beckman GS-15R) at room temperature to collect the cells. The supernatant was discarded and the pellet was resuspended in 0.5 ml of ASW. Using a hemocytometer, a small aliquot was differentially counted for cells, algae, and undischarged nematocysts. On average, the total cellular count included 9.46 (±1.25) % algae and 1.27(±0.27) % undischarged nematocysts. For standardizing the cell counts, the volume of the cell suspension was adjusted to provide a final cell density of 15×10^6 cell/ml. Cell preparations were also analysed for protein content. The protein content was measured spectrophotometrically by the enhanced BCA protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the standard (Wiechelman *et al.*, 1988).

$^{45}Ca^{2+}$ uptake into the cells

Aliquots (50 µl) of cell suspension containing 750,000 cells each were incubated in a $^{45}Ca^{2+}$- ASW solution (4.5 µCi/ml) for 10 min with or without *N*-acetyl neuraminic acid (NANA) in the presence or absence of a channel inhibitor/activator at room temperature. Final volume of the incubation mixture was 200 µl. In the presence of an inhibitor/activator, cells were pretreated with the inhibitor/activator for 5 min before adding NANA. Aliquots (50 µl) of the mixture were then pipetted onto Whatman GF/C glass microfiber filters (2.4 cm diameter, 1.2 µm pore size), which had each been prewashed with 5 ml of ASW. Filters were then washed 10 times with 5 ml ice-cold ASW on a sampling manifold providing simultaneous vacuum filtration (Millipore 1225 sampling manifold, Millipore Corp., Bedford, MA). Since a water content in excess of 100 µg per filter caused quenching, filters were dried by placing them into individual, 20 ml polyethylene scintillation vials (Fisher Scientific, Tustin, CA) on a hot plate at 40°C for 20 min. A total of 10 ml of scintillation cocktail (Fisher Scientific, SX20-5) was added into each vial and the radioactivity was counted in a liquid scintillation counter (Beckman LS 5801, Beckman Instruments, Irvine, CA).

Data analysis

All experiments designed for $^{45}Ca^{2+}$ uptake employed negative (ASW-treated) and positive (NANA-treated) controls in addition to blanks (no cells) and the various experimentally treated samples. Results were corrected by blank subtraction. Raw data in the form of counts per min (cpm) were converted to nmol calcium, then normalized as uptake per mg protein (nmol/mg protein). In some cases, data was normalized to ASW-

treated controls and expressed as percent of control. All results are given as mean ± SEM. Differences between groups were compared by two-way analysis of variance (ANOVA) with repeated measures and *post-hoc* Fisher PLSD tests. The differences were considered to be significant at $P < 0.05$. Replicate samples (n) and replicate experiments (N) are indicated in the figure legends.

Results

Effects of NANA on nematocyst discharge and calcium uptake

The effect of NANA concentration on *in situ* nematocyst discharge was biphasic and consisted of two regions (Fig. 1): sensitization and desensitization. The sensitization region occurred at low concentrations of NANA and was marked by half-maximum discharge (EC_{50}) at approximately 10^{-7} M NANA. Maximum discharge was 3.3-fold that of the seawater controls. Maximum discharge (EC_{100}) occurred at 1.8×10^{-5} M NANA and marked the biphasic transition from sensitization at lower concentrations to desensitization at higher concentrations. Half-maximal desensitization (DC_{50}) occurred at approximately 10^{-4} M NANA.

NANA also dose-dependently increased the extent of calcium uptake into isolated ectodermal tentacle cells. The dose-response curve of NANA-induced calcium uptake appeared to be sigmoidal, although a definitive plateau was not clearly evident at the highest tested NANA concentrations (Fig. 1). Significant stimulation of calcium uptake was observed at concentrations as low as 10^{-6} M NANA ($P = 0.04$). Maximum

calcium uptake in NANA-stimulated cells occurred at 10^{-3} M NANA and was approximately 2 nmol Ca^{2+} / mg protein; 5.7 times that of ASW control-treated cells ($P < 0.001$). Half-maximum uptake occured at approximately 10^{-5} M NANA.

Effects of calcium channel inhibitors on calcium uptake

Preincubation of the ectodermal cells with organic L-type calcium channel blockers, such as nifedipine, diltiazem, methoxy verapamil, or with the inorganic calcium channel blocker, Cd^{2+}, followed by the addition of 10^{-5} M NANA dramatically diminished NANA-induced calcium uptake (Fig. 2). Nifedipine at 10^{-8} M completely blocked calcium influx induced by 10^{-5} M NANA. In the presence of NANA alone, calcium influx was approximately 3-fold that of the ASW controls (Fig. 2A). Nifedipine by itself had neither stimulatory nor inhibitory effects. Diltiazem at 10^{-6} M completely blocked NANA-induced calcium influx, while diltiazem by itself had negligible effect (Fig. 2B). Similarly, 10^{-9} M methoxy verapamil (D-600) and 10^{-4} M $CdCl_2$ each blocked NANA-induced calcium influx, giving values of calcium uptake comparable to ASW controls (Fig. 2C and D, respectively).

Ectodermal tentacle cells were also preincubated with dihydropyridine activators of L-type calcium channels. Both the (-) and (+) enantiomers of Bay K-8644 at 10^{-8} M stimulated calcium influx to levels comparable to that of 10^{-5} M NANA (Fig. 3). Average calcium uptake among Bay K-8644-treated groups was about 4-fold above ASW controls. The (-) enantiomer appeared to slightly enhance NANA-stimulated calcium influx, but not significantly.

Effect of A23187 on calcium uptake

Ectodermal cells isolated from tentacles were treated with the divalent cation ionophore, A23187. As shown in Figure 4, 7.5×10^{-7} M A23187 stimulated calcium influx to a level similar to that of 10^{-5} M NANA. Thus, A23187 stimulated calcium uptake to approximately 4-times that of untreated ASW control cells.

Effects of high extracellular potassium on calcium uptake

The KCl concentration in normal ASW was 10 mM. Ectodermal cells were incubated separately with different artificial seawater solutions containing various elevated concentrations of KCl. The osmolality of the different solutions was kept the same as normal ASW by proportionally decreasing the concentration of NaCl. Increasing the extracellular KCl concentrations dose-dependently increased calcium uptake in a hyperbolic manner (Fig. 5A). Calcium uptake appeared to reach a plateau at 80 mM KCl. That 80 mM KCl maximally stimulated calcium uptake was confirmed in Figure 5B in which all the high K^+ -treated samples dramatically stimulated calcium influx into ectodermal cells to the same extent. The extent of uptake induced by high levels of extracellular KCl was greater than that induced by 10^{-5} M NANA as indicated by two-way ANOVA [80 mM ($P = 0.05$); 100 mM ($P = 0.01$); 120 mM ($P = 0.02$)]. In addition, 10^{-8} M nifedipine caused a pronounced decline in high K^+-induced calcium influx ($P = 0.02$), but did not lower calcium influx to the level of ASW controls as it did in cells treated with 10^{-5} M NANA (Fig. 2A).

Discussion

Cnidarians capture swimming prey by discharging nematocysts on their fishing tentacles (Ewer, 1947). The explosive discharge of cnidarian nematocysts is one of the most dramatic of single-cell events. Discharge is completed in less than 3 milliseconds (Holstein and Tardent, 1984). Despite a large number of studies on nematocyst discharge, the mechanism and control of the discharge is still poorly understood. *In situ* nematocyst discharge involves sensory stimulation and consequent intracellular events leading to the rapid eversion of the nematocyst tubule and penetration of prey. In general, a combination of appropriate prey-derived chemical and mechanical stimuli initiate discharge (Wagner, 1905; Pantin, 1942a, b; Jones, 1947).

Extracellular calcium is necessary for nematocyst discharge in hydra (Lenhoff and Bovaird, 1959; Lenhoff, 1968). Calcium and magnesium inhibit discharge of isolated acontial nematocysts from the sea anemone, *A. pallida* (Blanquet, 1970). In the nematocysts of the sea anemone, *Anthopleura elegantissima*, both partially discharged and undischarged nematocyst capsules contain high concentrations of calcium (Lubbock and Amos, 1981). At the onset of discharge, intracapsular calcium is released and influx of water occurs. Free calcium is released from the discharging nematocysts of *Pelagia noctiluca* before the eversion of the nematocyst tubule occurs (Salleo *et al.*, 1983).

Reduced glutathione, an activator of the feeding response in the sea anemone, *A. elegantissima* (Lindstedt, 1971), stimulates adenylyl cyclase activity in the oral disc and pharynx and also increases calcium binding to membrane preparations of these tissues (Gentelman and Mansour, 1974). In the feeding tentacles of the sea anemones, *A.*

pallida and *H. luciae*, *in situ* Type B and Type A CSCCs are sensitized to triggering mechanical stimuli by *N*-acetylated sugars, such as *N*-acetyl neuraminic acid (NANA) (Thorington and Hessinger, 1988a; Watson and Hessinger, 1989b), after binding to chemoreceptors located on supporting cells (Watson and Hessinger, 1988). Various stimulators of adenylyl cyclase and membrane permeant analogues of cAMP also sensitize anemone tentacles (Watson and Hessinger, 1992). Furthermore, NANA stimulates adenylyl cyclase activity in the apical membranes of supporting cells.

Watson and Hessinger (1994) implicated DHP-sensitive calcium channels in nematocyst discharge from Type B CSCCs in *H. luciae*, but not from Type A or C CSCCs, although extracellular calcium was needed for discharge from all three Types. Microfluorometric measurements of fluo-3 in excised sea anemone (*H. luciae*) tentacles showed that NANA increased intracellular calcium levels two- to three-fold, although it was not ascertained whether the increased calcium signal originated from calcium influx or mobilization of intracellular stores (Thibodeaux and Watson, 1993). Thorington and Hessinger (2001) demonstrated that calcium channel blockers and activators, as well as changes in extracellular calcium levels, modulate *in situ* sensitivity of nematocyst discharge to NANA. In normal seawater containing 10 mM calcium, the NANA dose-response of discharging nematocysts is biphasic with a peak response occuring at 1.8×10^{-5} M NANA (i.e. EC_{100}). In seawater containing lower levels of calcium, sensitivity to NANA is positively modulated. At 1 mM calcium, the EC_{100} is approximately 10^{-12} M NANA. On the other hand, increasing the calcium concentration from 10 to 11 mM completely blocks NANA-sensitized nematocyst

discharge. In the presence of the calcium channel blockers, nifedipine, diltiazem, and Cd^{2+}, the NANA dose-response of nematocyst discharge shifts to the left, while in the presence of the calcium channel activator, (-) Bay K 8644, as with elevated extracellular calcium, the response to NANA is totally blocked.

The preceding studies implicate DHP-sensitive calcium channels in NANA-sensitized discharge. In our present findings, we demonstrated that NANA stimulates calcium influx into isolated tentacle ectodermal cells (Fig. 1) and that this influx is DHP-sensitive (Fig. 2). The calcium channel blockers used by us did not affect calcium influx by themselves. These same calcium channel blockers left-shifted the in situ NANA dose-response of nematocyst discharge (Thorington and Hessinger, 2001). Furthermore, calcium channel activators, such as the (-) and (+) enantiomers of Bay K 8644, stimulated calcium influx (Fig. 3), while completely blocking the in situ NANA dose-response of nematocyst discharge (Thorington and Hessinger, 2001). Our findings both confirm the previous pharmacological findings of Watson and Hessinger (1994) and Thorington and Hessinger (2001) of a cellular DHP-sensitive process. Furthermore, for the first time, we directly demonstrate that a DHP-sensitive calcium influx occurs in tentacle ectodermal cells. Finally, we extend previous findings based upon pharmacological effects on NANA-sensitized nematocyst discharge to show that the DHP-sensitive calcium influx is stimulated by physiologically relevant concentrations of NANA. In addition, we showed that increasing levels of extracellular KCl dose-dependently stimulate calcium uptake (Fig. 5A). This suggests that calcium influx can be induced by graded depolarizations of the cell membrane resting potential.

Furthermore, high K^+-induced calcium influx was partially blocked by 10^{-8} M nifedipine. The fact that nifedipine lowered high K^+-induced calcium uptake by an average of 52% indicates that at least two types of voltage-sensitive calcium channels are present in tentacle ectodermal cells: DHP-sensitive and DHP-insensitive. In the preceding study, Thorington and Hessinger (2001) presented indirect evidence for the involvement of both DHP-sensitive and DHP-insensitive calcium channels in aspects of in situ nematocyst discharge. Since NANA-stimulated calcium influx is also DHP-sensitive (Fig. 2A), we infer that the NANA-stimulated calcium influx is also voltage-sensitive.

Regarding the role of calcium channels in the NANA signaling pathway, two opposing models have been proposed. Watson and Hessinger (1994) proposed that elevated intracellular calcium would be required to predispose contact-sensitive mechanoreceptors to trigger discharge. This positive modulatory model proposed that increased intracellular calcium had a sensitizing effect on NANA-sensitized discharge. On the other hand, Thorington and Hessinger (2001) provided compelling indirect evidence that increased intracellular calcium negatively modulates NANA-sensitized discharge and proposed that calcium entry via DHP-sensitive calcium channels plays a role in desensitization. Our present observation that the desensitization region of the nematocyst discharge curve coincides with those NANA concentrations that stimulate the greatest calcium influx (Fig. 1) support the role of calcium influx in desensitization. Half-maximum uptake occured at approximately 10^{-5} M NANA, the approximate concentration of NANA that marked the onset of dose-dependent desensitization of NANA-induced nematocyst discharge (Fig. 1). Thus, our results are consistent with the

Thorington and Hessinger model that DHP-sensitive calcium channels negatively modulate NANA chemosensory control of nematocyst discharge and may play a role in dose-dependent desensitization.

Based upon current and published findings, we propose a modified model for the role of DHP-sensitive calcium channels in desensitization of the NANA signaling pathway. We propose that high levels of NANA (i.e. $> 1.8 \times 10^{-5}$ M) cause desensitization by stimulating DHP-sensitive calcium channels. The resulting increase in intracellular calcium activates calmodulin. In *H. luciae* (Thibodeaux and Watson, 1994) and in *A. pallida* (our unpublished findings), the calmodulin inhibitor, W-7, sensitizes nematocyst discharge, a result consistent with the proposed role for calmodulin in desensitization. Our model predicts that increased levels of activated calmodulin will either inhibit adenylyl cyclase (Yu *et al.*, 1993) or activate cAMP-specific phosphodiesterase (Sharma *et al.*, 1997), thereby lowering intracellular cAMP levels produced from stimulating NANA chemoreceptors.

Figure 1. *N*-acetyl neuraminic acid (NANA) dose-response curves for nematocyst discharge (○) and calcium uptake (●). *In situ* discharge of nematocysts was tested after anemones were incubated in ASW containing the specified NANA concentrations for 10 min. Nematocysts on six probes were counted for each dose tested on each of 3 days (N = 3; n = 18). Calcium uptake was measured in isolated tentacle ectodermal cells incubated with ASW (pH 8.0) containing $^{45}Ca^{2+}$ and the specified NANA concentrations for 10 min, then rapidly collected on filters, washed, and counted in a scintillation counter (N = 13; n = 39). Data in the form of cpm were converted to nmol calcium, then normalized as calcium uptake per mg protein (nmol/mg). All data points represent the mean ± SEM.

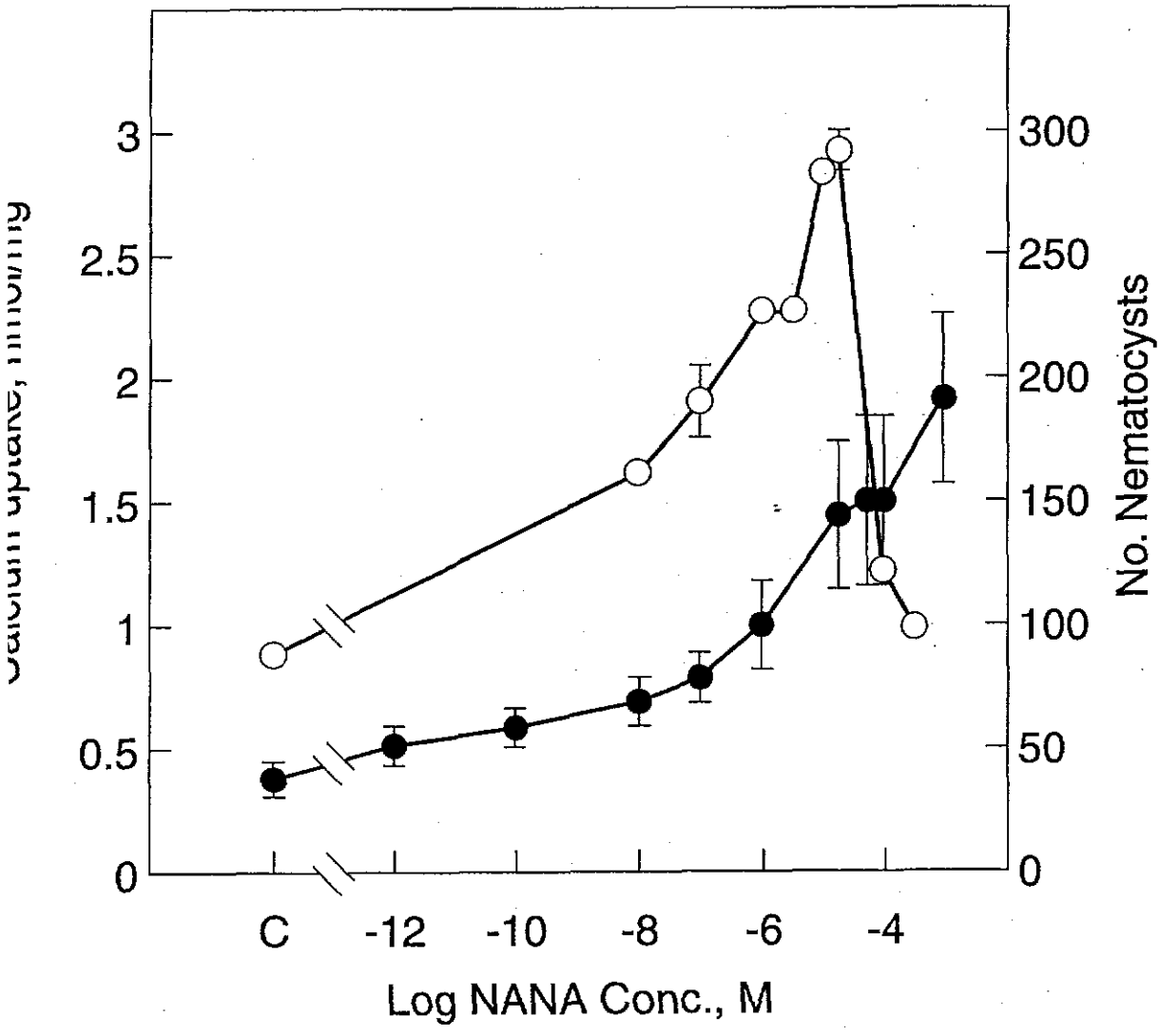

Calcium Uptake, nmol/mg
3
2.5
2
1.5
1
0.5
0
300
250
200
150
100
50
0
No. Nematocysts
C
-12
-10
-8
-6
-4
Log NANA Conc., M

Figure 2. Effects of calcium channel blockers on NANA-induced calcium uptake into isolated tentacle ectodermal cells. Cells were preincubated in ASW (pH 8.0) containing either 10^{-8} M nifedipine (A), 10^{-6} M diltiazem (B), 10^{-9} M verapamil (D-600) (C), or 10^{-4} M $CdCl_2$ (D) for 5-10 min, followed by incubation with $^{45}Ca^{2+}$ in the presence or absence of 10^{-5} M NANA in ASW, then collected on filters and counted as in Figure 1. For the sake of comparison, calcium influx for individual experiments were normalized as percent of artificial seawater (ASW) controls. Data points are means ± SEM (N = 15; n = 54 for nifedipine; N = 7; n = 34 for diltiazem; N = 3; n = 18 for D-600; and N = 10; n = 20 for $CdCl_2$).

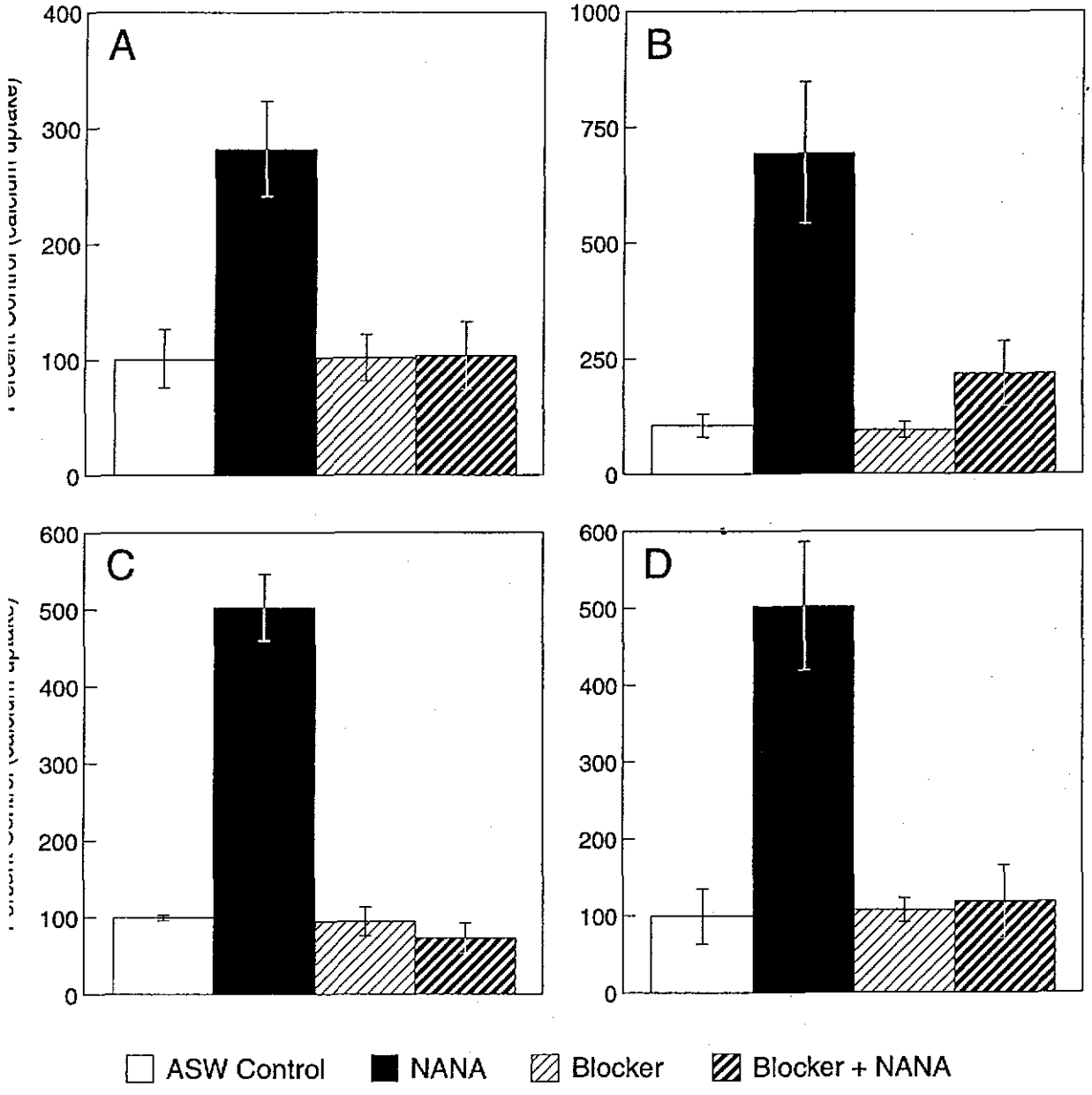

A
B
C
D
400
300
200
100
0
1000
750
500
250
600
ASW Control
NANA
Blocker
Blocker + NANA

Figure 3. Effects of (-) and (+) enantiomers of Bay K-8644 on NANA-induced calcium uptake into isolated tentacle ectodermal cells. Cells were treated with Bay K-8644 enantiomers (10^{-8} M) and then collected and counted as in Figure 1. Data were expressed as nmol calcium per mg protein. Each data point represents the mean ± SEM (N = 10; n = 20).

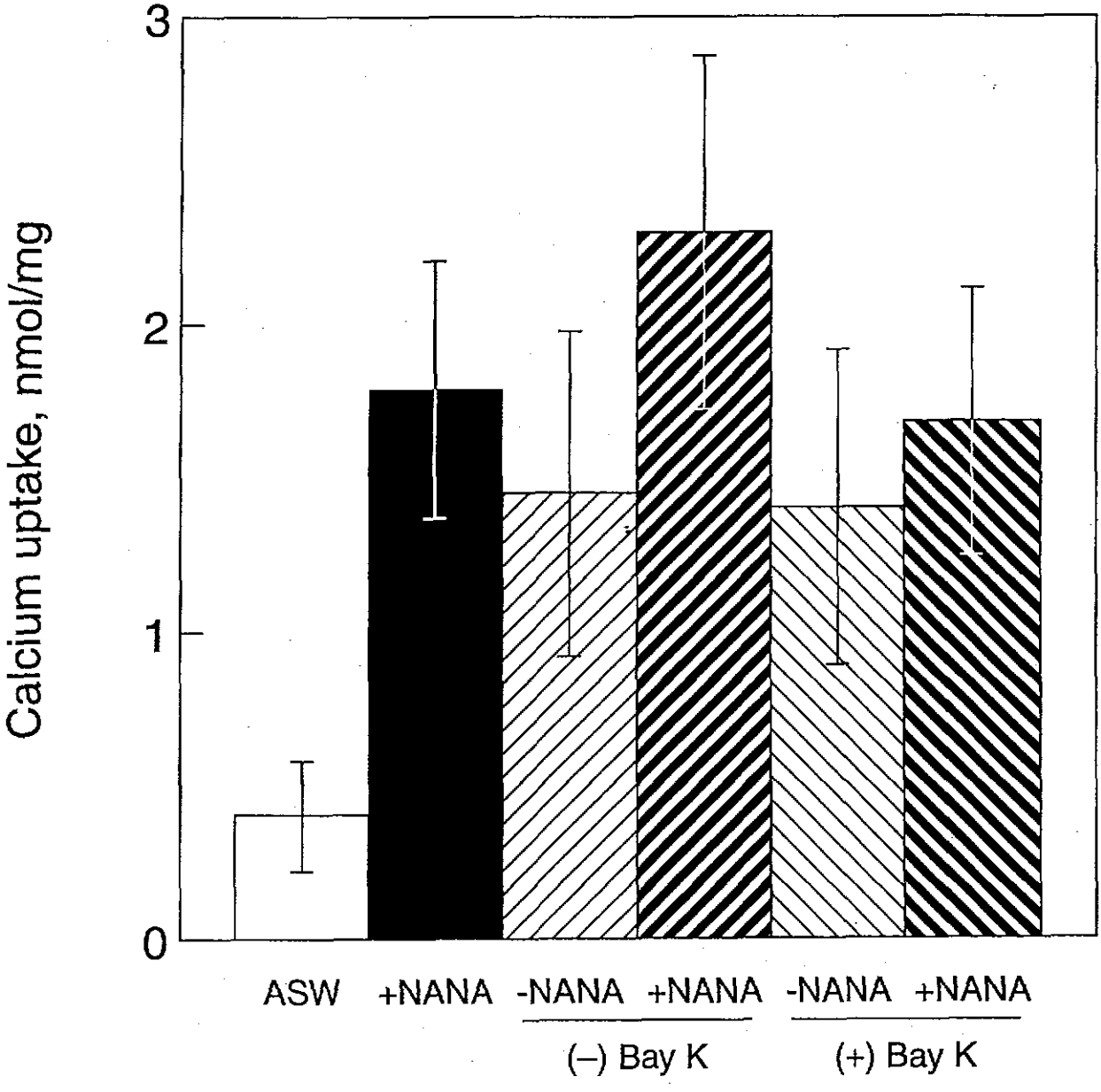

Calcium uptake, nmol/mg
3
2
1
0
ASW
+NANA
-NANA
+NANA
-NANA
+NANA
(–) Bay K
(+) Bay K

Figure 4. Effect of A23187 on calcium uptake into isolated tentacle ectodermal cells. Cells were incubated with ASW (pH 8.0) containing $^{45}Ca^{2+}$ and 7.5×10^{-7} M A23187 for 10 min and then collected and counted as in Figure 1. Positive (10^{-5} M NANA) and negative (ASW) controls were included. Data were expressed as nmol calcium per mg protein. Each data point represents the mean ± SEM (N = 5; n = 15).

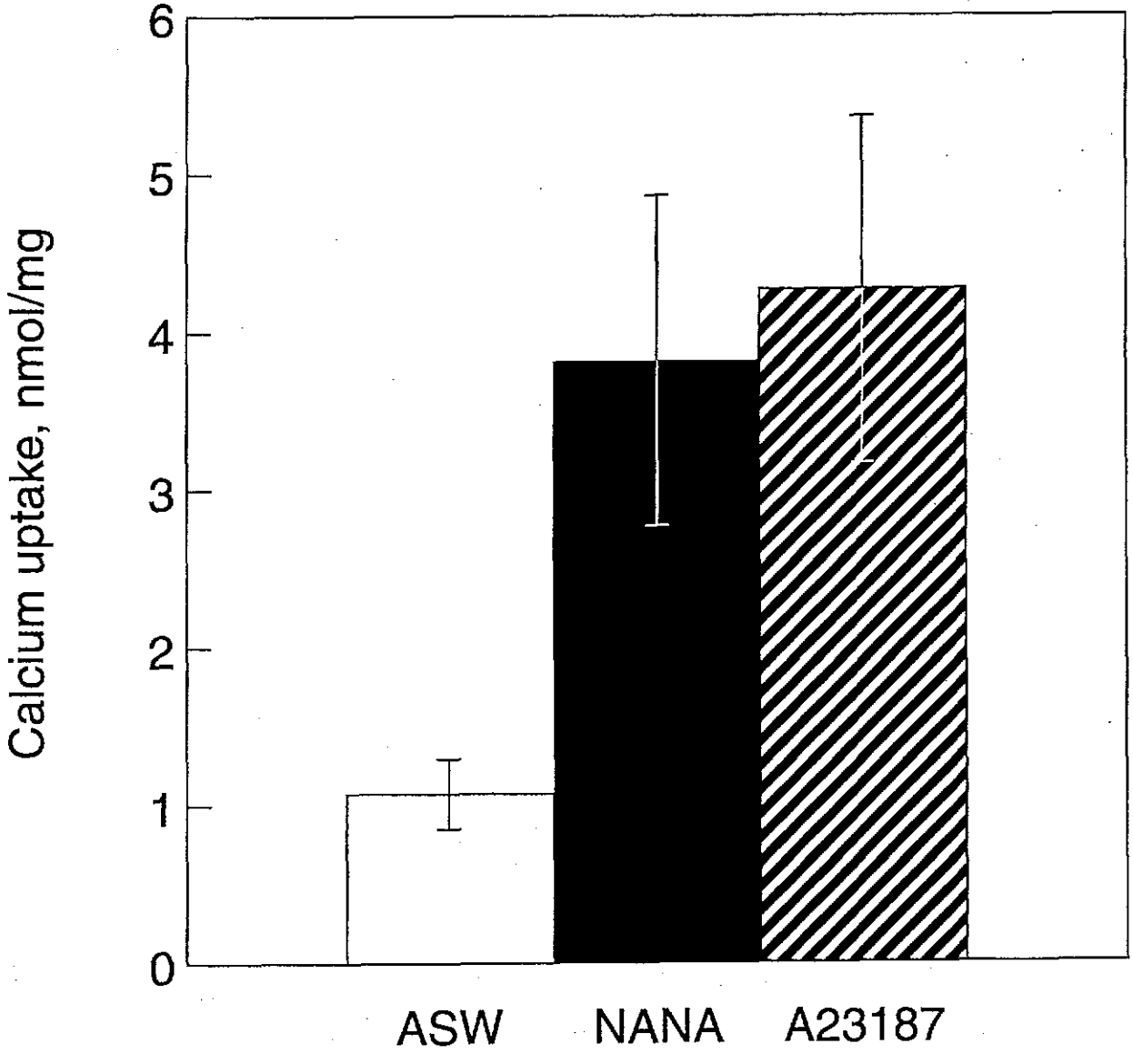

Calcium uptake, nmol/mg
6
5
4
3
2
1
0
ASW
NANA
A23187

Figure 5. Effect of extracellular potassium levels on calcium uptake into isolated tentacle ectodermal cells. (A) Potassium dose-response curve. Cells were incubated in ASW (pH 8.0) containing $^{45}Ca^{2+}$ and different concentrations of KCl for 10 min and then collected and counted as in Figure 1 (N = 7; n = 21). (B) Effect of nifedipine on high K^+-induced calcium uptake. Cells were incubated in ASW (pH 8.0) containing $^{45}Ca^{2+}$ and either 80, 100, or 120 mM KCl in the presence and absence of 10^{-8} M nifedipine and then collected and counted as in Figure 1 (N = 6; n = 18). Negative (C or ASW) and positive (N or NANA) controls were included. Data were normalized as nmol calcium per mg protein. Each data point represents the mean ± SEM.

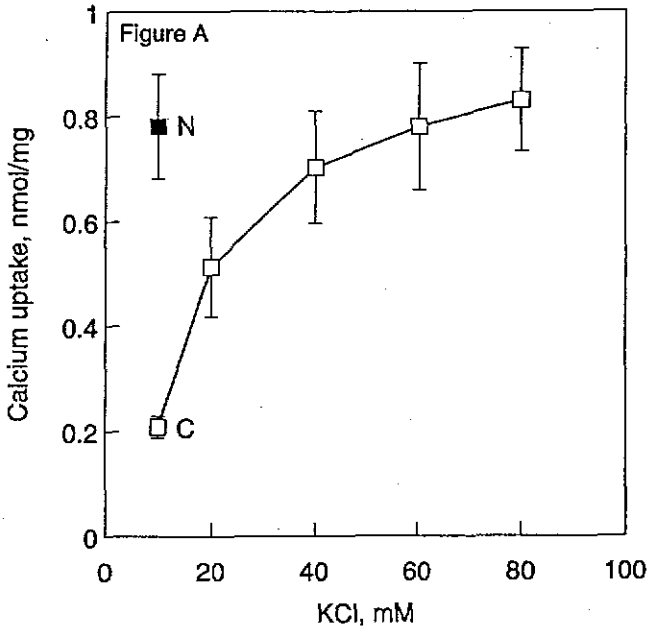


Figure A
Calcium uptake, nmol/mg
1
0.8
0.6
0.4
0.2
0
N
C
0
20
40
60
80
100
KCl, mM

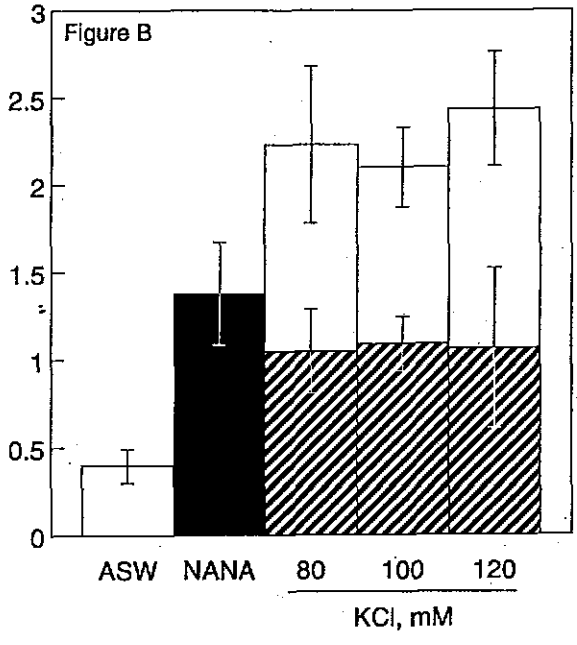


Figure B
3
2.5
2
1.5
1
0.5
0
ASW
NANA
80
100
120
KCl, mM

CHAPTER FOUR

CONCLUSIONS AND DISCUSSION

The primary objective of this project was to investigate the roles of cyclic-AMP and calcium in the *N*-acetyl neuraminic acid (NANA) chemosensory pathway leading to nematocyst discharge from the tentacles of the sea anemone, *Aiptasia pallida*.

In the first chapter, we recognized the complexity of nematocyst discharge, unique to all metazoans, and the medical importance of cnidarian stings. We also recognized that *N*-acetylated sugars, which are present on the surfaces of aquatic prey, sensitize contact-sensitive mechanoreceptors (CSMs) to trigger nematocyst discharge upon contact with prey. Previous studies suggested that cAMP and dihydropyridine (DHP)-sensitive calcium channels were involved in the NANA chemosensory pathway.

In the second chapter, we established that NANA dose-dependently increases *in situ* cAMP levels in the ectodermal layers of tentacles, while having no detectable effect on cAMP levels in the endodermal layers. The dose-response of NANA-stimulated cAMP coincided with the dose-response of NANA-sensitized nematocyst discharge. Furthermore, our results suggested that NANA-stimulated cAMP activates cAMP-dependent protein kinase in whole tentacle homogenates.

In the third chapter, we established the possible role of calcium in desensitization of the NANA signaling pathway. NANA stimulates calcium influx into isolated tentacle ectodermal cells from *A. pallida*, but does so most effectively at desensitizing concentrations. NANA-stimulated calcium influx appeared to involve DHP-sensitive calcium channels since it is blocked by nifedipine, diltiazem, methoxy verapamil, and

Cd^{2+}, while the DHP-calcium channel activator, Bay K-8644, stimulates the influx. Furthermore, we demonstrated that high levels of extracellular KCl dose-dependently stimulate DHP-sensitive calcium influx, suggesting that NANA-stimulated calcium influx is mediated by voltage-gated calcium channels.

Based on the previous findings and the results of the present project, we propose the following model for the control of NANA-sensitized nematocyst discharge (Fig. 1):

1. Sensitization. Stimulation of NANA chemoreceptors, located on the apical surfaces of supporting cells (Watson and Hessinger, 1988), activates adenylyl cyclase via a cholera toxin-sensitive G-protein (Watson and Hessinger, 1992). The resulting increased levels of cAMP (Fig. 4, chapter II) either directly or indirectly via cAMP-dependent protein kinase (PKA) (Fig. 8, chapter II) change a contact-sensitive mechanoreceptor (CSM) from an inactive to an excitable state. We speculate that the CSM is a stretch-activated ion channel. Initiation of nematocyst discharge results from mechanical contact and stimulation of the CSM.

2. Desensitization. Stimulation of NANA chemoreceptors by concentrations of NANA greater than 1.8×10^{-5} M (Fig. 1, chapter III) causes calcium entry through dihydropyridine (DHP)-sensitive calcium channels (Figs. 2 and 3, chapter III). NANA-stimulated calcium influx may occur by either NANA-stimulated cAMP acting directly on cyclic nucleotide-gated calcium channels or indirectly via PKA or by means of direct G_s-protein-channel interaction. Increases in intracellular calcium activate calmodulin (CaM). In *H. luciae* (Thibadoeux and Watson, 1994) and in *A. pallida* (unpublished findings), the calmodulin inhibitor, W-7, sensitizes nematocyst discharge, a result

consistent with the proposed role for CaM in desensitization. Activated CaM which would either activate a cAMP-specific phosphodiesterase (PDE) (Sharma *et al.*, 1997) or inhibit adenylyl cyclase (Yu *et al.*, 1993), thereby lowering intracellular cAMP levels produced from stimulating NANA chemoreceptors. Therefore, elevated intracellular calcium offsets the effects of chemoreceptor activation, resulting in inhibition of sensitization (i.e. desensitization). In addition, the NANA-stimulated calcium influx may also cause calcium-induced calcium release, thereby further augmenting the intracellular effects of calcium influx.

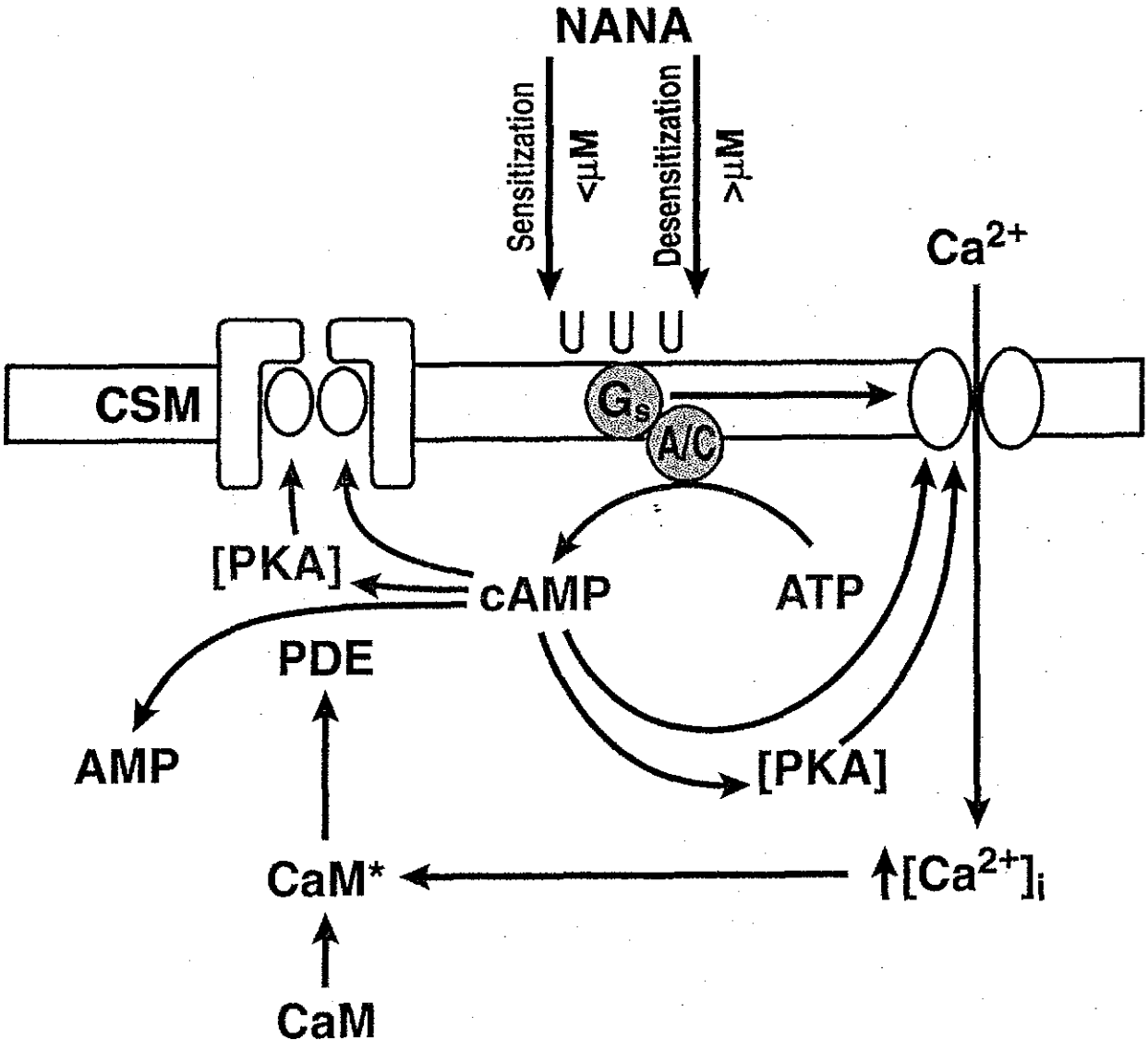
Further experiments are needed to elucidate the mechanism by which nematocyst discharge is regulated by NANA chemosensory pathway.

To determine the level in the NANA signaling pathway at which NANA-stimulated calcium influx is regulated, $^{45}Ca^{2+}$-influx into isolated tentacle ectodermal cells should be monitored in the presence of membrane-permeant cAMP (e.g. 8-Br-cAMP), cholera toxin, forskolin, and PKA inhibitors (e.g. RP-cAMP-S, H-7 or H-8). If calcium influx is mediated by a cAMP-gated calcium channel, then the agonists will stimulate influx, while the inhibitors will have no effect on NANA-stimulated influx. If calcium influx is activated by interaction with activated G_s protein, then only cholera toxin will stimulate the influx.

According to our model, the NANA-signaling pathway is negatively autoregulated by feedback from NANA-stimulated calcium influx via activated CaM. To test this aspect of the model, the following experiments are proposed while measuring the levels of cAMP in isolated tentacle ectodermal cells exposed to desensitizing

concentrations of NANA (i.e. > EC_{100} NANA). Our model predicts that the following agents would increase cAMP levels: CaM inhibitors (e.g. W-7, compound 48/80, calmidazolium); PDE inhibitors (e.g. IBMX, rolipram); and inhibitors of L-type calcium channels (e.g. nifedipine, diltiazem, verapamil, and Cd^{2+}). On the other hand, our model predicts that the following agents would decrease cAMP levels in isolated ectodermal cells exposed to EC_{100} NANA: (-) Bay K-8644; A23187; and 100 mM extracellular KCl.

Figure 1. A partial mechanistic model for the control of NANA-sensitized nematocyst discharge. Green arrows indicate NANA sensitization pathway, while desensitization pathways are shown by red arrows.


NANA
Sensitization
<μM
Desensitization
>μM
Ca2+
U U U
CSM
Gs
A/C
[PKA]
cAMP
ATP
PDE
AMP
[PKA]
CaM*
↑[Ca2+]i
CaM

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