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THE DISCHARGE MECHANISM OF ACONTIAL NEMATOCYTES INVOLVES THE RELEASE OF NITRIC OXIDE

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Summary

The events which trigger the activation of nematocytes are still poorly understood, and no evidence has been presented so far on either the nature of the activatory signal for the nematocyte or the transduction mechanism. In this paper, we present evidence for a role of NO in the discharge of acontial nematocytes. A citrulline-forming enzymatic activity, significantly decreased by the NO synthase inhibitor *N*$^{\omega}$-nitro-L-arginine (L-NNA) and by the Ca^{2+}-chelating agent EGTA, was found in the acontial tissue of *Aiptasia diaphana*. Staining for NADPH diaphorase suggested that NO synthase is localized in supporting cells surrounding the nematocytes. The ability of K^{+} to induce the discharge of nematocytes *in situ* could be abolished by preincubation of acontia with L-NNA and restored by addition of excess L-arginine. Direct measurements on K^{+}-induced discharging nematocytes *in situ* confirmed that NO was released by stimulated acontia. Both *in situ* and isolated acontial nematocytes promptly discharged when perfused with an aqueous solution of NO. The responsiveness to NO of isolated nematocytes was not abolished in Ca^{2+}-free medium or by treatment with La^{3+}, a well-known Ca^{2+} channel inhibitor. Since the discharge of *in situ* nematocytes is known to be Ca^{2+}-dependent, it is proposed that activation of *in situ* acontial nematocytes is triggered by a Ca^{2+}-dependent release of NO from supporting and/or sensory cells.

Key words: *Aiptasia diaphana*, isolated nematocytes, discharge, activation, nitric oxide, Ca^{2+}.

Introduction

Nematocytes (cnidocytes), the stinging cells of Cnidaria (coelenterates), are specialized cells, present in structures such as tentacles, acrorhagi and acontia, and characterized by a complex secretion product, termed the nematocyst, consisting of a capsule wall that contains the capsule fluid and an inverted tubule. When exposed to an appropriate stimulus, the tubule is explosively everted and releases the capsule fluid in which various toxins are stored. This extremely rapid process (Holstein and Tardent, 1984) is termed discharge. In Anthozoa, the nematocytes are considered to be effectors of a multicellular functional unit termed the cnidocyte-supporting cell complex (Watson and Hessinger, 1989). The triggering of discharge requires stimulation both of chemoreceptive sites situated on the supporting cells and of the ciliated mechanoreceptor situated on the nematocyte itself. The function attributed to the supporting cells is to modulate the sensitivity of the mechanoreceptor to frequencies matching those produced by the prey through elongation of the stereocilium bundle (Watson and Hessinger, 1991, 1992). Recently, it was suggested (Mire-Thibodeaux and Watson, 1994) that, following treatment with sensitizers, the kinocilium of sensory cells, rather than that of the nematocytes, elongates.

The discharge of *in situ* nematocytes has been recognized as a Ca^{2+}-dependent phenomenon involving Ca^{2+} channels (Gitter *et al.* 1994; Salleo *et al.* 1993; Santoro and Salleo, 1991*a*; Watson and Hessinger, 1994). Furthermore, a Ca^{2+}-dependent spreading discharge has been observed in acontia of *Calliactis parasitica* (Salleo *et al.* 1993, 1994; Santoro and Salleo, 1991*b*), suggesting the occurrence of cell-to-cell transmission of an unidentified activatory signal. The location of Ca^{2+} influx, although putatively attributed to cnidocytes (Gitter *et al.* 1994), is still uncertain since experiments on this aspect of nematocyte physiology have not so far been performed on isolated cells. Viable cnidocytes have been enzymatically isolated from the tentacles and acrorhagi, but not the acontia, of a number of species (McKay and Anderson, 1988*a*). In all cases, the isolated cells were induced to discharge neither by chemical agents nor by depolarization caused by either current injection or by high external [K^{+}] (Anderson and McKay, 1987; McKay and Anderson, 1988*b*), stimuli that are effective on *in situ* cnidocytes. Since cell

damage due to the isolation procedure was excluded (McKay and Anderson, 1988*b*), it is likely that these stimuli are not adequate for triggering the discharge process in isolated nematocytes. However, it is possible that the isolation procedure could damage some specific transduction mechanism, as suggested by Hidaka (1993). Acontial nematocytes can be isolated by treatment with SCN^- in the presence of a low Ca^{2+} concentration; however, these cells cannot be induced to discharge by depolarization (Barra *et al.* 1995). Owing to the lack of any method for activating isolated nematocytes, neither the nature of the triggering signals released by the supporting and/or nerve cells nor the transduction process occurring in the nematocytes has been identified so far.

Nitric oxide is a diffusible transmitter that plays an important role as a messenger molecule in several physiological systems (Moncada *et al.* 1991; Nathan, 1991; Schmidt and Walter, 1994). Many reports have now shown that NO is also produced in invertebrates (Elofsson *et al.* 1993; Elphick *et al.* 1995; Gelperin, 1994), and a recent study has indicated its involvement in the feeding response of the coelenterate *Hydra vulgaris* (Colasanti *et al.* 1995). In a preliminary communication, we reported that exogenous NO induces the discharge of isolated nematocytes (Barra *et al.* 1995). The present investigation was therefore undertaken to test a possible role of NO as a transmitter in the process of activation of anthozoan acontial nematocytes.

Materials and methods

The experiments were performed on acontia from specimens of *Aiptasia diaphana* (Rapp) collected in Lake Faro (Messina, Italy), maintained in a closed-circuit aquarium at 19–21 °C and fed weekly with shrimp meat. Acontia were extruded by gentle mechanical stimulation of the trunk. The excised acontia were placed in a depression slide and repeatedly rinsed in artificial sea water (ASW) having the following composition (in $mmol\,l^{-1}$): NaCl, 520; KCl, 9.7; $MgCl_2$, 24; $MgSO_4$, 28; $CaCl_2$, 10; imidazole, 10; pH 7.7. L-[^{3}H]arginine was purchased from Amersham. All other chemicals were from Sigma unless otherwise stated.

Measurement of nitric oxide synthase activity

The acontia excised from 20 specimens were homogenized within 2 h of collection by sonication in ice-cold 50 $mmol\,l^{-1}$ Tris/HCl buffer (pH 7.4) containing 1.15 % (w/v) KCl, 1 $mmol\,l^{-1}$ EDTA, 5 $mmol\,l^{-1}$ glucose, 0.1 $mmol\,l^{-1}$ DL-dithiothreitol (DTT), 2 $mg\,l^{-1}$ leupeptin, 2 $mg\,l^{-1}$ pepstatin A and 10 $mg\,l^{-1}$ phenylmethanesulphonyl fluoride. Nitric oxide synthase (NOS) activity was assessed by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Briefly, 50 μl samples of the homogenized tissue were incubated, in the presence or absence of 1 $mmol\,l^{-1}$ of the NOS inhibitors N^{ω}-nitro-L-arginine (L-NNA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME), for 30–60 min at 37 °C with 1–20 $\mu mol\,l^{-1}$ L-[^{3}H]arginine (3.7×10^5 Bq ml^{-1}), 1 $mmol\,l^{-1}$ NADPH, 15 $\mu mol\,l^{-1}$ 6*R*-tetrahydrobiopterin, 1 $\mu mol\,l^{-1}$ FAD and 1 $\mu mol\,l^{-1}$ calmodulin in 50 $mmol\,l^{-1}$ Hepes buffer (pH 7.4) containing 1 $mmol\,l^{-1}$ DTT, 18 $mmol\,l^{-1}$ valine, 1 $mmol\,l^{-1}$ EDTA and either 1.25 $mmol\,l^{-1}$ $CaCl_2$ or 6 $mmol\,l^{-1}$ EGTA (final volume 150 μl). The incubations were terminated by adding 500 μl of ice-cold 100 $mmol\,l^{-1}$ Hepes buffer (pH 5.5) containing 10 $mmol\,l^{-1}$ EDTA. The mixtures were then chromatographed on 2 ml of Dowex AG 50W-X8 (counter-ion Na^+) cation-exchange resin (Serva), and L-[^{3}H]citrulline concentration in the eluates was quantified by liquid scintillation counting with an LKB 1211 Rackbeta. NOS specific activity was calculated as the rate of L-NNA-sensitive formation of L-[^{3}H]citrulline per minute per milligram of protein. Protein concentrations were determined by the Biuret method according to Goa (1953).

Localisation of nitric oxide synthase

NOS was localised in cryostat sections of acontia by using NADPH diaphorase histochemistry according to Elphick *et al.* (1995).

Measurement of released nitric oxide

The release of NO during the discharge of *in situ* nematocytes was assessed by optically monitoring the NO-induced oxidation of oxyhaemoglobin to methaemoglobyn (Kelm *et al.* 1988) using a Perkin-Elmer 330 spectrophotometer. Samples of acontia removed from 30 specimens were treated with high-K^+ ASW, in which 200 $mmol\,l^{-1}$ NaCl had been substituted with 200 $mmol\,l^{-1}$ KCl, a well-known discharging agent of *in situ* nematocytes that acts through depolarization (McKay and Anderson, 1988*b*; Salleo *et al.* 1993; Santoro and Salleo, 1991*a*), in the presence of 5 $\mu mol\,l^{-1}$ oxyhaemoglobin. The amount of released NO was determined from the difference extinction coefficient between 401 and 411 nm (ε=38 000 $l^{-1}\,mol^{-1}\,cm^{-1}$). Acontia suspended in normal ASW plus oxyhaemoglobin, as well as ASW plus reagents in the absence of acontia, were used as controls. Alternatively, the acontia were preincubated with 100 $\mu mol\,l^{-1}$ L-[^{3}H]arginine (3.7×10^5 Bq ml^{-1}) in ASW for 60 min at 25 °C, and were then treated with normal or high-K^+ ASW. The supernatants were collected after 15 min, and the L-[^{3}H]citrulline formed was determined after cation-exchange chromatography as described above.

Perfusion of in situ *or isolated nematocytes with exogenous NO*

Aqueous solutions of NO (NO-ASW) were prepared by equilibrating ASW with NO chemically generated as follows: 0.1 mol of ascorbic acid, dissolved in 0.1 $mol\,l^{-1}$ HCl, and 0.1 mol of sodium nitrite were reacted in a 1 l vacuum flask connected to a 0.5 l gas-tight flask containing 10 ml of ASW. Oxygen was completely removed before mixing the reactants. ASW was left to equilibrate under the resulting atmosphere of NO (approximately 1.5×10^{-5} Pa) for 2 h with constant stirring. The final solution had an NO concentration of approximately 0.4 $mmol\,l^{-1}$, as assessed by colorimetric

determination of nitrites using the Griess reagent after exposure to air (Tracey, 1992). To assess the effects of NO on *in situ* nematocytes, a glass cannula, with an inner tip diameter of approximately 30 μm, was connected by a three-way stopcock to a gas-tight syringe containing NO-ASW and to a nitrogen reservoir by an electrovalve. With the tip of the cannula, operated by a micromanipulator, placed close to the acontium suspended in 300 μl of ASW, less than 1 μl of test solution was ejected under microscopic observation. Observation was either by a videomicroscope (Hirox, model KH-2200-MD2) connected to a video recorder and a monitor or by a differential interference contrast (DIC) microscope (Zeiss, Axioscope) supplied with a video camera, for more accurate observation of morphological details.

Acontial nematocytes were isolated as follows: excised acontia were placed in a depression slide and repeatedly rinsed in low-Ca^{2+} ASW having the following composition (in $mmol\,l^{-1}$): NaCl, 550; KCl, 9.7; $MgCl_2$, 24; $MgSO_4$, 28; $CaCl_2$, 0.01 (instead of the 10 $mmol\,l^{-1}$ present in normal ASW), imidazole, 10; pH 7.7. They were then suspended in 605 $mmol\,l^{-1}$ NaSCN, also containing 0.01 $mmol\,l^{-1}$ $CaCl_2$, which induced acontial contraction and release of nematocytes of the microbasic mastigophore type within 1–5 min. As soon as the release of nematocytes occurred, the SCN^- solution was removed and substituted with Ca^{2+}-free ASW. Normal ASW, containing 10 $mmol\,l^{-1}$ Ca^{2+}, was then gradually substituted for Ca^{2+}-free ASW. The tissue contraction that occurred as the Ca^{2+} concentration increased induced the release of further nematocytes, mainly of the microbasic mastigophore type. The nematocytes could be easily distinguished from isolated nematocysts under the DIC microscope by the presence of a distinct girdle containing cytoplasm and organelles. The effect of NO was tested on 22 cell suspensions within 3 h of isolation. Under microscopic observation, a glass cannula was placed close to a group of cells and less than 1 μl of NO-ASW was ejected.

Since the NO solution could induce a local and transient lowering of pH, due to O_2 dissolved in the cell suspension medium, the effect of acidic pH on isolated nematocytes was tested. Cell suspensions were placed on a glass slide under a coverslip supported by two thin strips of adhesive tape. The suspending medium (ASW) was absorbed at one side of the coverslip and completely substituted at the opposite side with test solutions consisting of ASW whose pH had been lowered to 2–5 with nitric acid.

Effects of L*-NNA and* L*-arginine on K^+-induced discharge of* in situ *nematocytes*

Excised acontia were incubated in depression slides for 150 min in ASW containing either 200 or 500 $\mu mol\,l^{-1}$ L-NNA. High-K^+ ASW was then externally applied under microscopic observation using a glass cannula placed close to the acontium. Some acontia were subsequently treated with NO-ASW as described below. The specificity of L-NNA as a NOS inhibitor was tested by preincubating acontia for either 150 or 300 min in ASW containing 200 $\mu mol\,l^{-1}$ L-NNA plus 1 $mmol\,l^{-1}$ L-arginine before treatment with high-K^+ ASW. As a further control, high-K^+ ASW was applied to untreated acontia suspended in ASW.

Role of Ca^{2+} in the discharge of isolated nematocytes

To investigate the role of Ca^{2+} in the discharge of isolated nematocytes, the cells were repeatedly rinsed with Ca^{2+}-free ASW containing 0.2 $mmol\,l^{-1}$ EGTA, and NO dissolved in Ca^{2+}-free ASW was then applied (N=13). Furthermore, nematocytes suspended in ASW containing 1 $mmol\,l^{-1}$ $LaCl_3$, a well-known Ca^{2+} channel inhibitor (Hosey and Lazdunsky, 1988), were tested for NO-discharging effectiveness.

Results

Fig. 1 reports the results of measurements of the NOS activity in homogenates of acontia. Citrulline was produced at 0.51±0.02 $pmol\,min^{-1}\,mg^{-1}$ protein in control samples. In the presence of either 1 $mmol\,l^{-1}$ L-NNA or 1 $mmol\,l^{-1}$ L-NAME, a highly significant (P<0.001) decrease of the rate of citrulline formation was observed. Citrulline was produced at 0.19±0.013 and 0.17±0.007 $pmol\,min^{-1}\,mg^{-1}$ protein, respectively, confirming that the activity was due to the presence of NOS. When the Ca^{2+}-chelating agent EGTA was substituted for $CaCl_2$ in the assay mixture, a highly significant (P<0.001) decrease of the rate of citrulline formation was observed, so that citrulline was produced at 0.26±0.009 $pmol\,min^{-1}\,mg^{-1}$ protein, suggesting that acontia contain a Ca^{2+}-dependent NOS.

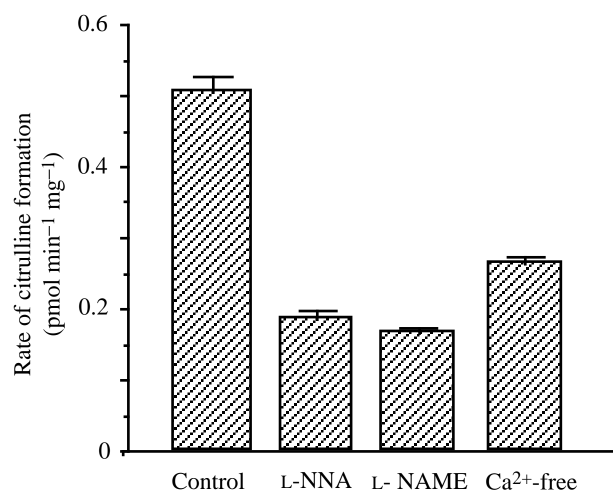

Fig. 1. Nitric oxide synthase activity represented by the rate of citrulline formation in homogenates of acontia (N=3). Column 1 represents the activity under control conditions. Columns 2 and 3 show the activity in the presence of the NOS inhibitors L-NNA or L-NAME, respectively. Column 4 shows the activity with EGTA added to chelate Ca^{2+}. The partial inhibition caused by EGTA addition could be due either to the presence of a Ca^{2+}-independent NOS or, more likely, to the extremely high concentration of Ca^{2+} in the capsule fluid of nematocysts, which could prevent EGTA from efficiently lowering the concentration of free Ca^{2+}. Values are means + S.E.M. The differences between the control value and the L-NNA, L-NAME and Ca^{2+}-free values were highly significant (P<0.001).

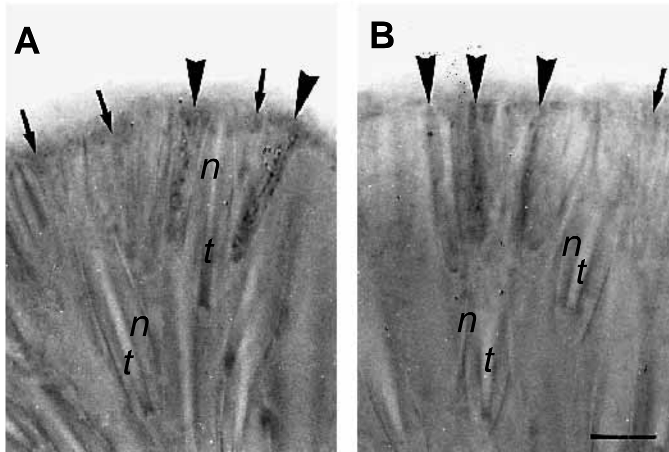

Fig. 2. NADPH-diaphorase staining in cryostat transverse sections of two acontia. Dense staining is observable (arrows) in both sections between the apical ends of the unstained nematocytes. (A) Two densely stained supporting cells (arrowheads) located on both sides of a nematocyte. (B) Three stained supporting cells surrounding a nematocyte. Staining was absent in control sections. *n*, nematocytes; *t*, inverted tubule. Scale bar, 10 μm.

NADPH diaphorase staining of cryostat sections of acontia revealed the presence of NOS in the spaces between the apical parts of adjacent nematocytes (Fig. 2), in which the supporting cells, which appear shorter than the nematocytes, are located. Thus, our results suggest that NOS is present in the supporting cells.

Acontia treated with high-K^+ ASW displayed, as expected, a massive nematocyst discharge. The phenomenon was accompanied by the release of NO, as monitored by the conversion of oxyhaemoglobin, present in the incubation medium, to methaemoglobin after the discharge. The shift of the Soret peak in the absorption spectrum of haemoglobin gave an estimate that approximately 0.5 nmol NO mg^{-1} protein was generated in this process (Fig. 3). Independent measurements of the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline (see Materials and methods) quantitatively confirmed that NOS was activated in the presence of high-K^+ ASW. Significantly ($P<0.01$) lower values of methaemoglobin and citrulline production were observed in samples lacking high-K^+ ASW or in the absence of acontia (Fig. 3).

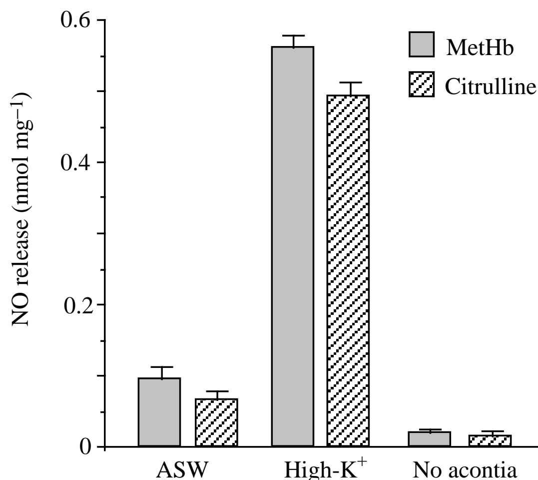

Fig. 3. NO release by high-K^+-stimulated acontia, determined from both methaemoglobin (MetHb; N=3) and citrulline (N=3) production. The release of NO by control acontia suspended in normal artificial sea water (ASW) was very low. The pair of columns on the right show the blank values in the absence of acontia. Values are means + S.E.M. For both methaemoglobin and citrulline production, the differences between the high-K^+ value and the values in normal ASW and in the absence of acontia were significant ($P<0.01$).

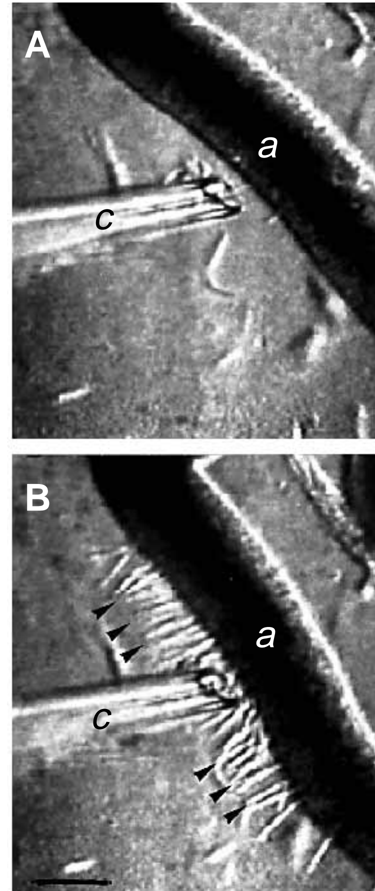

Fig. 4. Excised acontium (*a*) freely floating in artificial sea water (ASW). (A) The glass cannula (*c*) was placed close to the acontial surface. (B) Following perfusion of NO solution, the nematocytes in the acontial segment in front of the cannula (*c*) discharged massively (small arrowheads). Discharge did not occur when either normal ASW or NO solution previously exposed to air was perfused. Scale bar, 100 μm.

Discharge of *in situ* nematocytes localized in the proximity of the cannula was observed a few seconds after perfusion with NO (Fig. 4). This effect could be reproduced by repeating the local perfusion of NO-ASW in different regions of the same acontium.

The effects of L-NNA on the K^+-induced discharge of *in situ* nematocytes are summarized in Table 1. Acontia treated with this NOS inhibitor did not discharge in high-K^+ ASW. The inhibitory effect of L-NNA could be prevented by co-

Table 1. *The effect of the NOS inhibitor L-NNA on the K^+- and NO-induced discharge of* in situ *nematocytes*

Treatment	Incubation time (min)	K^+-induced discharge		NO-induced discharge	
		Yes *N*	No *N*	Yes *N*	No *N*
200 μmol l^{-1} L-NNA	150	2*	12	7	1
500 μmol l^{-1} L-NNA	150	0	15	9	0
200 μmol l^{-1} L-NNA +1 mmol l^{-1} L-arginine	150	8	0	ND	ND
1 mmol l^{-1} L-arginine	300	9	2	ND	ND
ASW	300	10	0	12	0

N = number of responses.

*Only a few sparse nematocytes discharged.

L-NNA-treated acontia that did not respond to K^+ were subsequently stimulated with NO. Untreated acontia suspended in artificial sea water (ASW) were stimulated either with K^+ or with NO.

ND, not done.

incubation with 1 mmol l^{-1} L-arginine. However, the same acontia discharged promptly following treatment with NO-ASW. These results demonstrate that depolarization is ineffective in promoting discharge when NO release is prevented by inhibition of NOS, suggesting a role for NO in the K^+-induced discharge of nematocytes.

Isolated nematocytes, clearly identifiable by the presence of a distinct, centrally placed girdle, promptly discharged upon addition of NO solution (Fig. 5). In contrast, isolated nematocysts, a minority in nematocyte suspensions, never discharged under NO perfusion. Membrane remnants were generally observed on discharged capsules, since the plasma membrane is perforated by the everting tubule. The discharge of isolated nematocytes started within 1 s of NO perfusion and proceeded for about 10 s. In some cases, a brief interval separated the eversion of the shaft from complete discharge (Fig. 6). Any possible discharging effect of pH changes could be safely excluded. The suspending medium, when made

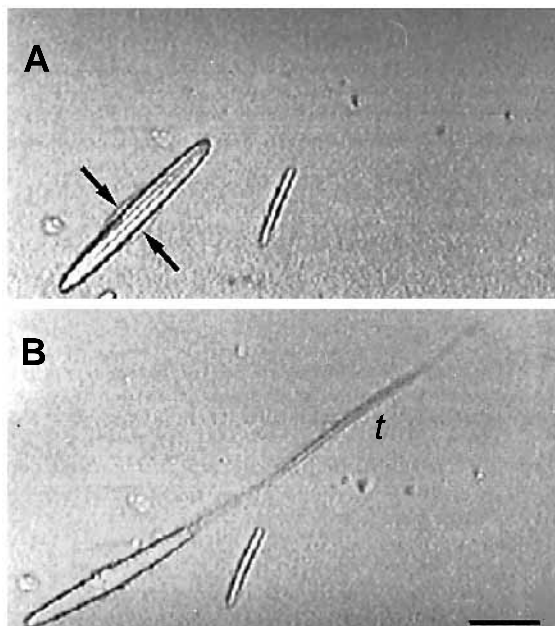

Fig. 5. (A) Isolated nematocyte. The cell volume is almost completely occupied by the nematocyst containing the inverted tubule, except in the centrally placed girdle (arrows). (B) 1 s after the addition NO solution, the nematocyte discharged, as shown by the complete eversion of the tubule (*t*). Scale bar, 20 μm.

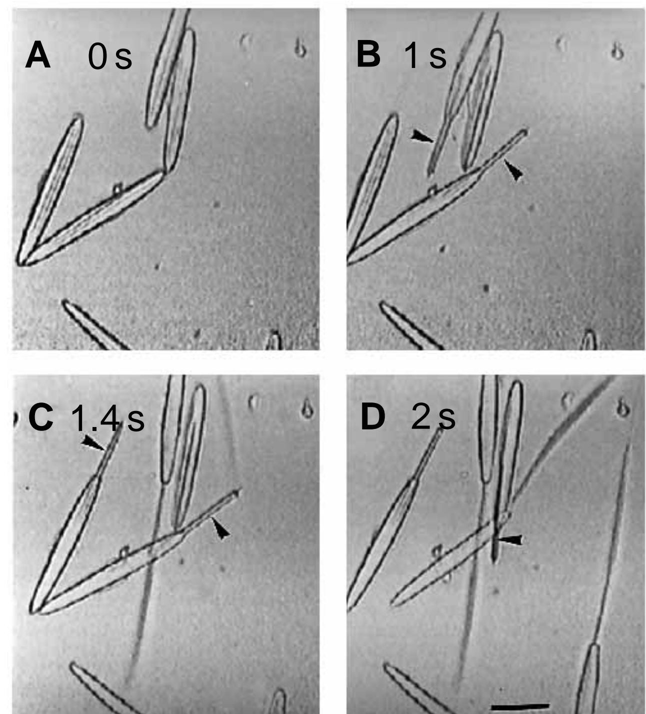

Fig. 6. Sequence of events following perfusion of isolated nematocytes with NO. The time elapsed after NO perfusion is shown in each micrograph. In some cases, the time of eversion of the shaft (arrowheads) was separated from the complete discharge by a brief interval. (A) Undischarged nematocytes immediately before NO perfusion. (B) 1 s after the addition of the NO solution, the shafts (arrowheads) of two nematocysts were everted. The complete discharge of these cells is shown in C and D. (C) One further cell now has an exposed nematocyst shaft. (D) Further progress of nematocyte activation. Scale bar, 20 μm.

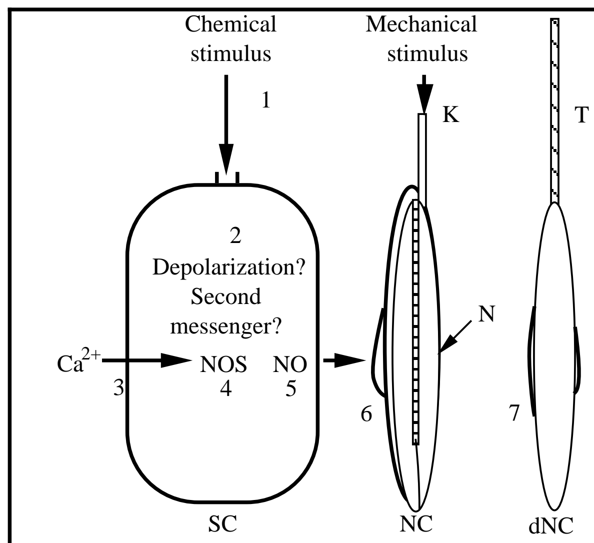

Fig. 7. Diagram of the proposed mechanism for nematocyte discharge in physiological conditions. A sensitizer (1) acts on the receptor on the supporting or sensory cell (SC). Either depolarization or a second messenger (2) induces Ca^{2+} influx (3) into the supporting (or sensory) cell that activates nitric oxide synthase (NOS) (4). The NO released (5) reaches the resting nematocyte (NC). The transduction process (6) increases its excitability to mechanical stimuli applied to the ciliary receptor (K) so inducing the discharge (7). dNC, discharged nematocyte; N, nematocyst; T, tubule.

acidic by addition of nitric acid, proved effective in discharging isolated nematocytes only at pH 2, in agreement with previous results (McKay and Anderson, 1988*b*). The medium at pH 3, although not inducing discharge, caused cell damage as revealed by bulging and deformation of the central girdle containing the nucleus and organelles. Once damaged, the cells did not discharge when pH was lowered to 2. The NO solutions, once exposed to air, had a pH of 4–5 and were not effective in inducing discharge.

Isolated nematocytes suspended in Ca^{2+}-free ASW discharged following perfusion with NO-ASW with no noticeable difference from the cells suspended in the Ca^{2+}-containing medium. Previous incubation of isolated nematocytes in ASW containing La^{3+} did not prevent NO-induced discharge (results not shown).

Discussion

Nematocytes of the microbasic mastigophore type isolated from the acontia of *Aiptasia diaphana* using the SCN^{-} extrusion method promptly discharge under treatment with NO. Isolated cnidocytes from a number of species, such as *Cladonema* sp., *Chrysaora quinquecyrrha* (Anderson and McKay, 1987) and *Anthopleura elegantissima* (MacKay and Anderson, 1988*b*), have previously been obtained by enzymatic dissociation. Although the integrity of these isolated cells was determined by ultrastructural and electrophysiological techniques, a reproducible discharge could not be obtained upon application of various different stimuli. On the basis of this low incidence of discharge as well as its lack of correlation with electrophysiological events, Anderson and McKay (1987) concluded that either voltage-insensitive ion channels or a second messenger could be involved in the transduction of adequate stimuli for discharge. McKay and Anderson (1988*b*) confirmed, on isolated spirocytes from *Anthopleura elegantissima*, the lack of discharge in response to depolarization as well as following application of various chemical stimuli. They proposed that the lack of an adequate mechanical stimulus associated with the chemical one could possibly explain the failure to induce the discharge. Alternatively, the difference between the responses of *in situ* and isolated cells could depend on a regulatory role of adjacent cells and nerves.

In acontia, the situation is complicated further by the lack of responsiveness of *in situ* nematocytes to sensitizers and mechanical stimuli that are highly effective on tentacle cells (Salleo *et al.* 1994). In the present study, anatomically intact nematocytes isolated from acontia could be discharged, in the absence of any mechanical stimulus, by treatment with NO, a transmitter not considered so far as a possible physiological signal for triggering the discharge of cnidocytes. The unresponsiveness of isolated nematocytes to acidic media, except at pH 2 (which was never reached in NO-ASW), is in agreement with the results obtained by McKay and Anderson (1988*b*) and allows us to exclude a pH-dependent effect. The lack of involvement of mechanoreception in the NO-induced discharge that we observed could be due either to the dose of exogeous NO used or to a discharge control system different from that operating in tentacles. In addition to the responsiveness of isolated nematocytes to exogenous NO, the possible role of NO as the physiological activatory transmitter for inducing the discharge of *in situ* nematocytes is supported by (i) the presence of a Ca^{2+}-dependent NOS in acontia, (ii) the release of NO from discharging acontia, and (iii) the inhibitory effect of L-NNA on the high-K^{+}-induced discharge of *in situ* nematocytes. Damage to acontial tissue due to treatment with L-NNA can be excluded, since discharge could subsequently be induced in the same acontia by exogenous NO application. It is known that, while depolarization induces the discharge of *in situ* nematocytes, it is ineffective on isolated ones. The inhibitory effect of L-NNA on depolarization-induced discharge of *in situ* nematocytes therefore strongly suggests that depolarizing stimuli do not act directly on nematocytes, but rather on other tissue components such as supporting and/or sensory cells that, in turn, activate the nematocytes through the release of NO.

The NO-induced discharge of isolated nematocytes is not Ca^{2+}-dependent, as was shown by their responsiveness in Ca^{2+}-free medium as well as following treatment with La^{3+}. Ca^{2+}-dependence of discharge of *in situ* nematocytes has been reported for acontia (Santoro and Salleo, 1991*a*,*b*) and confirmed for anthozoan (Watson and Hessinger, 1994) and hydrozoan (Gitter *et al.* 1994) tentacles. Nevertheless, since the latter experiments were performed *in situ*, it was not possible to state whether Ca^{2+} elicited discharge by acting on the

nematocytes themselves or, instead, on the supporting cells. The present results demonstrating the presence of a Ca^{2+}-dependent NOS in acontia and on the localisation of NOS outside the nematocytes strongly suggest that the Ca^{2+}-dependence of *in situ* discharge is not located in the nematocytes but rather in the supporting cells. However, a recent study (Mire-Thibodeaux and Watson, 1993) showed that treatment of anthozoan tentacle with sensitizers induced a two- to threefold increase in the number of rare epidermal cells, possibly sensory cells, characterized by high internal [Ca^{2+}]. Therefore, we propose that the activation mechanism of nematocytes is based on the sequence of events shown in Fig. 7. The depolarization of the supporting and/or sensory cell caused by a chemical stimulus induces a Ca^{2+} influx into that cell which, in turn, results in activation of NOS and, according to our experimental evidence, appears to be selectively localized to the supporting cells. No conclusion can be reached at this stage as to the presence of NOS in the sensory cells, since these cells are widely spread over the entire acontial tissue and are therefore difficult to identify in our sections. The NO released reaches the nematocytes and induces activation, triggering discharge, under physiological conditions, by a mechanical stimulus.

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References

ANDERSON, P. A. V. AND MCKAY, M. C. (1987). The electrophysiology of cnidocytes. *J. exp. Biol.* **133**, 215–230.

BARRA, P. F. A., MUSCI, G. AND SALLEO, A. (1995). Control of discharge of acontial nematocytes. In *Sixth International Conference on Coelenterate Biology, 16–21 July 1995.* Noordvijkerhout, The Netherlands.

COLASANTI, M., LAURO, G. M. AND VENTURINI, G. (1995). NO in hydra feeding response. *Nature* **374**, 505.

ELOFSSON, R., CARLBERG, M., MOROZ, L., NEZLIN, L. AND SAKHAROV, D. (1993). Is nitric oxide (NO) produced by invertebrate neurons? *NeuroReport* **4**, 279–282.

ELPHICK, M. R., RAYNE, R. C., RIVEROS-MORENO, V., MONCADA, S. AND O'SHEA, M. (1995). Nitric oxide synthesis in locust olfactory interneurones. *J. exp. Biol*. **198**, 821–829.

GELPERIN, A. (1994). Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusc. *Nature* **369**, 61–63.

GITTER, A. H., OLIVER, D. AND THURM, U. (1994). Calcium- and voltage-dependence of nematocyst discharge in *Hydra vulgaris*. *J. comp. Physiol.* A **175**, 115–122.

GOA, J. (1953). A micro biuret method for protein determination. Determination of total protein in cerebrospinal fluid. *J. clin. Lab. Invest.* **5**, 218–223.

HIDAKA, M. (1993). Mechanism of nematocyst discharge and its cellular control. *Adv. comp. env. Physiol*. **15**, 45–76.

HOLSTEIN, T. W. AND TARDENT, P. (1984). An ultrahigh-speed analysis of exocytosis: nematocyst discharge. *Science* **223**, 830–833.

HOSEY, M. M. AND LAZDUNSKI, M. (1988). Calcium channels: molecular pharmacology, structure and regulation. *J. Membr. Biol.* **104**, 81–105.

KELM, M., FEELISCH, M., SPAHR, R., PIPER, H.-M., NOACK, E. AND SCHRADER, J. (1988). Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem. biophys. Res. Commun.* **154**, 236–244.

MCKAY, M. C. AND ANDERSON, P. A. V. (1988*a*). On the preparation and properties of isolated cnidocytes and cnidae. In *The Biology of Nematocysts* (ed. D. A. Hessinger and H. M. Lenhoff), pp. 273–293. San Diego: Academic Press.

MCKAY, M. C. AND ANDERSON, P. A. V. (1988*b*). Preparation and properties of cnidocytes from the sea anemone *Anthopleura elegantissima*. *Biol. Bull. mar. biol. Lab., Woods Hole* **174**, 47–53.

MIRE-THIBODEAUX, P. AND WATSON, G. M. (1993). Direct monitoring of intracellular calcium ions in sea anemone tentacles suggests regulation of nematocyst discharge by remote epidermal cells. *Biol. Bull. mar. biol. Lab., Woods Hole* **185**, 335–345.

MIRE-THIBODEAUX, P. AND WATSON, G. M. (1994). Morphodynamic hair bundles arising from sensory cell/supporting cell complexes frequency-tune nematocyst discharge in sea anemones. *J. exp. Zool*. **278**, 282–292.

MONCADA, S. J., PALMER, R. M. AND HIGGS, E. A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmac. Rev.* **43**, 109–142.

NATHAN, C. (1991). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051–3064.

SALLEO, A., LA SPADA, M., DRAGO, M. AND CURCIO, G. (1994). Hypoosmotic shock-induced discharge in acontia of *Calliactis parasitica* is blocked by gadolinium. *Experientia* **50**, 148–152.

SALLEO, A., SANTORO, G. AND BARRA, P. (1993). Spread of experimentally induced discharge in acontia of *Calliactis parasitica*. *Comp. Biochem. Physiol*. **104**A, 565–574.

SANTORO, G. AND SALLEO, A. (1991*a*). The discharge of *in situ* nematocysts of the acontia of *Aiptasia mutabilis* is a Ca^{2+}-induced response. *J. exp. Biol.* **156**, 173–185.

SANTORO, G. AND SALLEO, A. (1991*b*). Cell-to-cell transmission in the activation of *in situ* nematocytes in acontia of *Calliactis parasitica*. *Experientia* **47**, 701–703.

SCHMIDT, H. H. W. AND WALTER, U. (1994). NO at work. *Cell* **78**, 919–925.

TRACEY, W. R. (1992). Spectrophotometric detection of nitrogen oxides using azo dyes. *Neuroprotocols* **1**, 125–131.

WATSON, G. M. AND HESSINGER, D. A. (1989). Cnidocyte mechanoreceptors are tuned to the movements of swimming prey by chemoreceptors. *Science* **243**, 1589–1591.

WATSON, G. M. AND HESSINGER, D. A. (1991). Chemoreceptor-mediated elongation of stereocilium bundles tunes vibration-sensitive mechanoreceptors on cnidocyte-supporting cell complexes to lower frequencies. *J. Cell Sci*. **99**, 307–316.

WATSON, G. M. AND HESSINGER, D. A. (1992). Receptors for N-acetylated sugars may stimulate adenylate cyclase to sensitize and tune mechanoreceptors involved in triggering nematocysts discharge. *Expl Cell Res*. **198**, 8–16.

WATSON, G. M. AND HESSINGER, D. A. (1994). Evidence for calcium channels involved in regulating nematocyst discharge. *Comp. Biochem. Physiol.* **107**A, 473–481.