0014-4754/94/020148-05$1.50 + 0.20

Hyposmotic shock-induced discharge in acontia of *Calliactis parasitica* is blocked by gadolinium

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Received 5 July 1993; accepted 25 November 1993

Abstract. On acontia of *Calliactis parasitica* it was observed that mechanical stimuli applied by a gelatin probe, a method effective in tentacles of Anthozoa, do not induce the discharge of nematocytes. Hyposmotic shock, performed by treatment with NaCl solution 35% hyposmotic with respect to sea water, induces, in the presence of Ca^{2+}, the discharge that spreads along the acontial filament, as previously observed following treatment with SCN^-. The hyposmotic shock-induced discharge is blocked by Gd^{3+} at a concentration of 1 μM. 10 μM Gd^{3+} prevents also the SCN^--induced discharge. These results suggest the presence of stretch activated cation channels either in nematocytes and/or in supporting cells as well as a possible effect of SCN^- on this class of ion channels.
Key words. Calliactis parasitica; nematocytes; discharge; hyposmotic shock; Gd^{3+}.

The nematocytes of Cnidaria (Coelenterates) control an organelle, the nematocyst, that everts explosively a tubule through which various toxins are injected into foreign tissues when the cell is properly stimulated. Both mechanical and chemical stimuli concur in inducing such a response[1], that is called discharge. More recent investigations revealed that the supporting cells, besides the nematocyte, are involved in the cell mechanisms that control the discharge, so that the cnidocyte-supporting cells complex (CSCC) should be considered as a functional unit. In particular, various classes of chemoreceptive sites, sensitive to molecules derived from prey integument, are placed on the apical membrane of the supporting cells which, once stimulated, modulate the mechanoreceptor responsiveness[2-5]. In any case, however, a mechanical stimulus is required for triggering the discharge. The above investigations were performed on fishing tentacles of Anthozoa, that are mainly used for prey capture, by testing the sensitizing effect of molecules likely to be present in prey integument. A number of species in the class of Anthozoa, grouped as acontiates, bear a very rich population of nematocytes also in long filamentous structures, termed acontia, that normally are not exposed, being contained in the mesenteric cavity and connected by one end to the mesenteric filaments. The acontia are extruded through the cinclides when the animal is disturbed. The function of acontia is still uncertain, mainly because quantitative and qualitative assay techniques performed in tentacles[2-5], have not been applied so far on acontial tissue. On the other hand, previous investigations concerning the activation mechanisms of the nematocytes showed that the discharge of acontial nematocytes can be experimentally induced by lyotropic anions, such as SCN^-, in the absence of any mechanical stimulus, and that such a response is Ca^{2+}-induced, since it lacks either in the absence of Ca^{2+} in the bathing medium or following treatment with Ca^{2+} channel blockers such as La^{3+}, Cd^{2+} and Co^{2+}[6,7,8]. Furthermore, it was observed[7,8] that, unlike tentacles, in acontia of *Calliactis parasitica* the discharge induced by treatment with SCN^- spreads sequentially through the entire length of the tissue.

Since in physiological conditions mechanoreception is expected to be involved in the control of discharge also in acontia, as in tentacles, the method[2] based on mechanical stimulation performed by contact with a gelatin bead was taken into account for investigating the discharge properties of acontial tissue. In such a method gelatin, besides stimulating the CSCC, holds the discharged nematocysts, thereby allowing a quantitative evaluation of the discharge response. We reasoned that an alternative method to induce experimentally membrane deformation is the treatment with hypotonic media that, by causing tissue swelling, is expected to act on stretch-activated (SA) ion channels as does mechanical deformation[9]. Recently, this class of ion channels received a great attention, with special regard to the cationic subtype, since they could be involved, in cell volume regulation[10-12]. The reasons for taking into account a possible role of SA cationic channels in the discharge of nematocytes are 1) mechanoreceptors must be stimulated for eliciting the discharge of tentacle nematocytes, and 2) Ca^{2+} inflow seems to be a necessary step in nematocyte discharge as well as in cell volume regulation, in which SA channels[11,13] are believed to play an essential role. A powerful blocker of SA cationic channels[10,14] recently proposed is the lanthanide gadolinium. Gd^{3+} is effective at micromolar concentration on SA channels and is impermeable, so that its action cannot be exerted on intracellular mechanisms.

If SA cationic channels are involved in the process of discharge, it is expected that hyposmotic shock elicits the discharge, and that the discharge induced either by mechanical stimulus or by hyposmotic shock is Ca^{2+}-dependent and Gd^{3+} sensitive. Two main problems are frequently encountered in physiological investigations on acontial nematocytes: 1) the size of these cells, which in *Calliactis parasitica* have a diameter of about 3 µm, and 2) the scarce responsiveness of isolated nematocytes observed on various species of Cnidaria[15,16]. Therefore, the present investigation was undertaken to test the responsiveness of in situ acontial nematocytes of *Calliactis parasitica* to mechanical stimuli and to hyposmotic swelling, and the effects of Ca^{2+} and Gd^{3+} on both experimental conditions.

Materials and methods

The experiments were performed on specimens of *Calliactis parasitica* collected in the Straits of Messina (Italy), maintained in closed circuit aquaria at 18–21 °C and fed weekly with shrimp meat. The acontia were obtained by mechanically stimulating the trunk. Segments of acontia were excised, collected by fire-polished silicon-coated Pasteur pipettes and placed in a Sylgard-coated glass channel connected to a constant flow pump that allows the complete substitution of bathing media within 5 sec, as described elsewhere[7]. The acontial segment was fixed, neither stretched nor slackened, at both ends by two *Opuntia* spines, and observed with an inverted microscope (Cambridge Instruments, Photozoom) equipped with a video camera (JVC mod.) or with a conventional camera. Osmolalities of all solutions as well as that of sea water in aquaria were measured with an osmometer and expressed in mosmol/kgH_2O. The standard artificial sea water (ASW) had the following composition (in mM): NaCl 520, KCl 9.7, $CaCl_2$ 10, $MgCl_2$ 24, $MgSO_4$ 28. The osmolality of all isotonic solutions was the same as that of sea water (SW) in acquaria, that averaged 1081 ± 7.7 mosmol/kgH_2O.

Mechanical stimulation. The acontium was fixed in the Sylgard channel through which was flowed throughout each test artificial sea water (ASW). Since mucin, albumin and cAMP[3,5] were found to increase the discharge response of tentacles in various species of Anthozoa to mechanical stimuli, the following bathing media were tested: ASW (n = 8), ASW plus 10^{-7} M (n = 3), 10^{-6} M (n = 6) or 10^{-5} M (n = 5) bovine submaxillary mucin (Sigman type I S), ASW plus either 10^{-4} M (n = 3) or 10^{-3} M (n = 3) dibutirryl-cAMP, a permeable analogue of cAMP.

The mechanical stimulation was performed as described by Giebel et al.[2]. The test probe was prepared by coating one end of a nylon wire with gelatin (Sigma, Type B) previously dissolved in ASW (30% w/v). The gelatin formed a bead about 200 µm in diameter. In some experiments the gelatin bead was coated with albumin. To contact the acontial surface, the probe was operated through a micromanipulator under a stereo microscope. The acontium was touched once for about 2 sec with the test probe. Each gelatin bead was then placed in a microtiter well containing 80 µl of 1% Trizyme (Amway Products), a mixture of detergent and proteolytic enzyme. After 4 h, once the gelatin was completely hydrolized, the wells were observed at an inverted microscope for counting the discharged nematocysts released from gelatin.

Hyposmotic shock. The acontia were fixed as described above. Isosmotic ASW (1081 ± 8 mosmol/kgH_2O), was flowed for 10 min before each test to remove the SW. Then the test solution was flowed for 15 min or until the discharge occurred. The hyposmotic shock was applied by flowing an hyposmotic solution of NaCl whose Ca^{2+} concentrated varied as follows: 0, 0.01, 0.1, 1 or 10 mM. In Ca^{2+} free solutions 0.2 mmol l^{-1} EGTA was added to prevent Ca^{2+} contamination. An osmolality of 697 ± 5 mosmol/kgH_2O for hyposmotic solutions was chosen for applying an osmotic shock of about 35% with respect to standard ASW. The Ca^{2+} concentration of isosmotic ASW flowed before each test was the same as that of each test solution. Osmolality of all solutions was adjusted by changing the NaCl concentration. The effect of an isosmotic NaCl solution containing 1 mM Ca^{2+} was also checked as a control.

The effectiveness of the following ion channel blockers in preventing the discharge induced by hyposmotic NaCl plus 1 mM Ca^{2+} solution was tested: 100 µM $LaCl_3$(n = 5); 1, 5 and 10 µM $GdCl_3$(n = 5); 20, 50, 75 and 150 µM Verapamil (n = 5); 100 µM Nifedipine (n = 5). To test the reversibility of the blocking action of Gd^{3+}, the acontium treated for 10 min with 1 µM Gd^{3+} was rinsed with ASW for 5 min and the hyposmotic NaCl solution was applied again (n = 5). Each blocker was added to both ASW and hyposmotic test solution. Nifedipine was dissolved in a stock ethanol solution at a concentration of 13.8 mg per ml and added to the experimental solution before each test. The final ethanol concentration was <0.1%. Nifedipine and Verapamil solutions were prepared and used in a dark room at low intensity red lighting.

The inhibitory effect on discharge of channel blockers was also tested with isosmotic solutions of NaSCN containing 10 mM Ca^{2+}, whose discharging effectiveness had been already assessed[6–8]. In particular, Verapamil was tested at a concentration of 150 µM, Nifedipine at 100 µM and Gd^{3+} at either 1, 5 and 10 µM.

The time interval between the start of test solution flow and the occurrence of the discharge was measured.

To evaluate the degree of swelling of the tissue following treatment with the hypotonic solutions the acontium

was photographed twice while ASW was flowing and subsequently at 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 260, 300, 420, 540, 660 and 780 sec after applying the test solution or until the onset of discharge. The cross sectional diameter of the acontial filament was measured at 5 random points in each photograph. The data were averaged and expressed as relative volume:

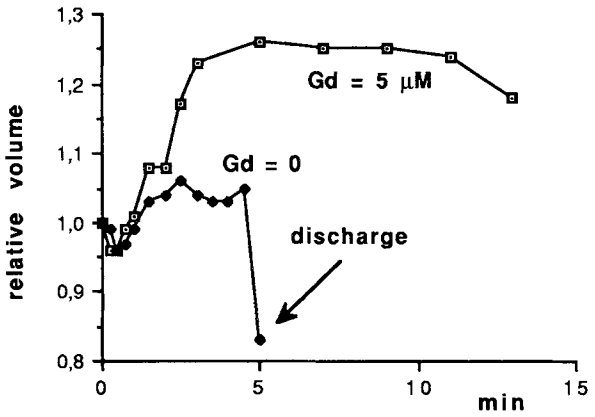
$$V/V_0 = \pi r_t^2 L/\pi r_0^2 L$$

where L is an arbitrary length of acontium[10].

Results

Surprisingly, the contact with the test probe did not elicit any discharge in the acontium. In fact, when the probe was removed from the acontial surface no adhesion of the tissue to the probe was observed. After the gelatin was completely hydrolized in the titration wells no discharged nematocysts could be found. Neither the presence of the sensitizers, namely mucin or db-cAMP, in the bathing solution nor albumin coating the probe were effective in promoting the discharge during touch with the gelatin probe.

Isotonic NaCl plus 1 mM Ca^{2+} did not elicit any discharging response. The discharge induced by treatment with hyposmotic NaCl solutions spread sequentially along the entire acontial tissue starting most frequently at one cut end of the acontium. In few experiments the discharge started in the middle of the tissue and spread towards both ends. The time interval between the beginning of the treatment and the onset of discharge is shown in table 1. Such a discharge was elicited in all tests in the presence of Ca^{2+} at a concentration of either 0.1 or 1 mM, while it was completely lacking in Ca^{2+} free hyposmotic solution. At a Ca^{2+} concentration of 10 mM the discharge was observed only in 2 tests out of 5, and it started with a delay significantly longer with respect to solutions containing Ca^{2+} at a lower concentration. At a Ca^{2+} concentration of 0.01 mM the discharge was elicited, but the usual spread was observed in 2 tests out of 5, while in 3 tests only short tracts of the acontium discharged. The treatment with the hyposmotic solution induced a slow swelling of the acontial filament. In control tests, a sudden decrease in volume was observed concomitantly to the discharge. On the other hand, when the discharge was prevented by Gd^{3+}, the volume of the filament increased progressively reaching its maximum by about 5 min (fig.).



Swelling of acontial filaments from the same specimen treated with hyposmotic NaCl solution plus 1 mM Ca^{2+}. In the presence of Gd^{3+} although a higher degree of swelling occurred, no discharge was elicited. Note the decrease in volume associated with the discharge.

Gd^{3+} revealed to be a powerful inhibitor of hyposmotic shock-induced discharge. In fact, at 10 and 5 µM the discharge was prevented. Even at a Gd^{3+} concentration as low as 1 µM the hyposmotic shock did not elicit any discharge. Only at the latter concentration the blocking effect of Gd^{3+} was reversible. In fact, after rinsing of the acontial segment with ASW for 5 min, a second treatment with the hyposmotic solution not containing Gd^{3+} induced a delayed discharge that occurred within a time interval of 364 sec ($\pm$46). Verapamil was completely

Table 1. Effectiveness of Ca^{2+}, Gd^{2+}, Verapamil and Nifedipine in blocking the discharge induced by hyposmotic NaCl solution

Osmolality (mosmol/kgH_2O)	Ca^{2+} (mM)	Treatment	Discharge yes	no	Delay (sec)	n.
1081 $\pm$ 1	1			5		5
679 $\pm$ 5	10		2	3	704 $\pm$ 16	5
	1		19		234 $\pm$ 10	19
	0.1		5		192 $\pm$ 24	5
	0.01		5*		301 $\pm$ 36	5
	0			5		5
	1	10 µM Gd^{3+}		5		5
	1	5 µM Gd^{3+}		5		5
	1	1 µM Gd^{3+}		5		5
	1	1 µM Gd^{3+} rinsed	5		364 $\pm$ 46	5
	1	150 µM Verapamil		5		5
	1	100 µM Nifedipine	5		221 $\pm$ 64	5
	1	100 µM La^{3+}		5		5

*The discharge occurred only in short tracts of acontia.

Table 2. Effectiveness of Gd^{3+}, Verapamil and Nifedipine in blocking the discharge induced by isosmotic NaSCN plus 10 mM Ca^{2+}

Osmolality (mosmol/kgH_2O)	Treatment	Discharge yes	Discharge no	Delay (sec)	n.
1081 ± 8		7		230 ± 29	7
	5 μM Gd^{3+}	5		214 ± 9	5
	10 μM Gd^{3+}		5		5
	150 μM Verapamil	5		246 ± 36	5
	100 μM Nifedipine	5	2*	237 ± 16	7

*Tissue damage was observed.

ineffective in blocking the hyposmotic shock-induced discharge at 20 μM, since the discharge occurred in all tests after a time interval similar to that observed in control tests. At concentrations of 50, 75 and 100 μM Verapamil had a partial effectiveness, while at a concentration of 150 μM Verapamil exerted a full blocking effect. 100 μM La^{3+} blocked in all tests the hyposmotic shock-induced discharge. Unlike Verapamil, and La^{3+}, 100 μM Nifedipine neither prevented the hyposmotic discharge nor delayed significantly its onset.

The response induced by the isosmotic solutions of NaSCN (table 2) in the presence of 10 mM Ca^{2+} occurred as previously described[7], consisting of a spreading discharge. The response was inhibited by 10 μM Gd^{+}· 5 μM Gd^{3+} was ineffective in preventing the effect of SCN^-. Both Verapamil and Nifedipine were ineffective in preventing the SCN^--induced discharge.

Discussion

The mechanical stimulus applied by contact with the test probe did not induce any response in the acontia. Such a result was unexpected. The discharging response to contact stimuli with protein probes has been observed in tentacles of various sea anemones such as *Anemonia sulcata*[1], *Aiptasia pallida*[4], *Haliplanella luciae*[5], *Stichodactyla haddonni*[18] and either in situ and excised tentacles of *Aiptasia mutabilis* (Salleo, unpublished). The observed lack of responsiveness of acontia to touch with the gelatin test probe suggests that in this tissue the receptive system has different properties compared with tentacles. The receptors do not seem to be simply insensitive to gelatin, since no discharge was observed when the probe was coated with albumin. At present, an interpretation of the lack of responsiveness of the acontia to the gelatin probe is hard. Possible function either in digestion or in defense have been tentatively ascribed to acontia[19]. The extrusion of acontia through the cinclides does not seem in agreement with the digestive function of acontia. Harris[21] reports that *Metridium* uses acontia for interspecific aggression against other Anthozoa. It is therefore possible that for acontia, unlike feeding tentacles, food stimuli are not adequate. In this case, the mechanical stimulus not being associated with the chemical one, would be ineffective in activating the CSCC. On the other hand, membrane deformation induced by hyposmotic swelling, by-passing the sensory mechanisms, could directly open stretch-activated ion channels. Further investigation is required about the nature of adequate chemical stimuli for acontia.

The hyposmotic shock induced a spreading discharge in acontial tissue similar to that elicited by SCN^-[7]. In both experimental conditions a time interval of about 4 min elapsed between the start of treatment and the onset of discharge. Such a delay could be ascribed to the mucus coating the acontia that could slow the diffusion of test media towards the apical surface of nematocytes. The hyposmotic shock-induced discharge is Ca^{2+}-dependent as shown by its lack either in Ca^{2+} free solutions or in the presence of La^{3+}. Gd^{3+} revealed a powerful inhibitory effect on hyposmotic shock-induced discharge, being fully effective even at a concentration of 1 μM. It is therefore likely that hyposmotic shock-induced discharge is caused by a Ca^{2+} inflow through SA channels made permeable by swelling-induced membrane deformation. Obviously, the presence of these channels in either the nematocytes and/or the supporting cells of acontia is putative until investigation will be performed on single channels. Nifedipine, that was found ineffective in preventing the hyposmotic shock-induced discharge, prevents hyposmotic cell volume regulation in rat proximal tubule, that is a Ca^{2+} controlled phenomenon involving SA channels[11,17]. Nevertheless, it is worthy to point out that Ca^{2+} antagonists of the dihydropyridine group are ineffective on Ca^{2+} channels of Coelenterates[20].

A comparison between the characteristics of SCN^--induced discharge described elsewhere[7,8] and hyposmotic shock-induced one shows that in the former a Ca^{2+} concentration of 10–1 mM is fully effective, while at 0.1 mM Ca^{2+} the discharge spreads along short segments and at 0.01 as well as at 50 mM it does not occur. In the hyposmotic shock-induced discharge the effective Ca^{2+} concentration is 1–0.1 mM. At 0.01 mM Ca^{2+} the discharge still occurs with a significant delay invading short segments of acontia. 10 mM Ca^{2+} can prevent the discharge. Besides these analogies, the time intervals between treatment and discharge response are similar in the two experimental conditions and, finally, the response to both conditions is blocked by micromolar concentration of Gd^{3+}. The only difference was the effectiveness of Verapamil at high concentration on the hyposmotic shock-induced discharge. The response to hyposmotic shock seems to have the same properties as the SCN^--induced one, with a higher sensitivity to blockers. The similarities suggest that SCN^- could act on the same structures involved in hyposmotic swelling, putatively SA channels, possibly by exerting its

chaotropic properties on channel proteins. The ascertained effectiveness of lyotropic anions on other ion channels such as Ca-release channels of muscle fibers[22], adds support to this hypothesis.

Acknowledgments. Dr. D. A. Hessinger, Dept. of Physiology and Pharmacology, Loma Linda University, USA, is warmly thanked for generous supply of Trizyme.

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