Polar Biosci., 13, 74-86, 2000

Temperature-dependent tonic contraction of smooth muscle in Antarctic fishes *Notothenia neglecta* and *Chaenocephalus aceratus*—Role of calcium ions and responses to acetylcholine*

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Abstract: The isolated intestinal smooth muscle of Antarctic fishes *Notothenia neglecta* and the icefish *Chaenocephalus aceratus* displayed a sustained tonic contraction depending upon the temperature of the nutritive bath. The contraction was maximal at 2°C, relaxing proportionally with rise of the temperature, from 2°C to 18°C. The contractile responses to acetylcholine gradually increased in proportion to the rise of temperature. By chelating calcium ions, EDTA caused a reversible relaxation of the contracted muscle at 2.0°C. This effect was eliminated by washing out the drug from the muscle nutritive bath. Similar results were found with the administration of verapamil, a calcium channel blocker. However, after this drug, tonic contraction was not restored by washout. Such contraction was partially inhibited by the muscarinic blocking drug atropine. Results show that the tonic contraction is an active response, dependent on the entry of calcium into the cell and mediated by cholinergic receptors. The temperature-related tonic contraction is not a general event in poikilotherm animals, since it did not occur in the *Bufo marinus* intestinal smooth muscle, in similar experiments carried out as a control, in a tropical environment. The responsiveness of the smooth muscle to acetylcholine appeared to increase with temperature, suggesting lack of adaptation of the organ to low Antarctic temperature.

key words: Antarctic fish, intestinal smooth muscle, cryobiology, *Notothenia neglecta, Chaenocephalus aceratus*

Introduction

The Nototheniidae and Chaenichthyidae are benthic teleost widespread fishes in the cold waters of the Antarctic Continent. The species *Notothenia neglecta* is endemic in Admiralty Bay, where the Antarctic Brazilian Station is located, accounting for most of the fishes caught in this area. The icefish *Chaenocephalus aceratus* accounts for about 20% of the total. Antarctic fishes living at temperatures near the freezing temperature of water are assumed to have adaptative mechanisms and great specialization of

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biochemical and physiological characteristics. Studies on Antarctic fishes have been chiefly focused on the biochemical profile, metabolism, antifreezing glycoproteins, cardiovascular and respiratory characteristics, hematology, neurobiology and behavior, as well as anatomical/histological descriptions. Regardingig the digestive tract of the Antarctic fishes, few data are available. Anatomical and histological features of the intestine of the Nototheniidae *Patagonotothen ramayi* were described by Korovina (1986). Owed (1986) described morphological aspects of the alimentary tract of Antarctic fishes and its relation to feeding habits. The reactivity of the intestinal smooth muscle to physiological chemical mediators and antagonist drugs known to act upon smooth muscle of freshwater teleost fishes or mammals is unknown.

Tsai and Ochillo (1989) studied the influence of low temperature on the automaticity of isolated guinea pig smooth muscle using *in vitro* techniques. They found that lowering the temperature of the nutritive organ bath, from 37 to 22, 18 and 14℃ triggered automaticity of longitudinal muscle and that verapamil, a calcium channel blocker, which inhibits transmembrane Ca^{2+} influx during excitation, blocked such automaticity, suggesting that calcium transport plays a significant role in this physiological response, as well as mepenzolate, a specific antimuscarine drug that also blocked such responses. These authors investigated the effects of hypothermia on smooth muscle using temperatures beginning at 37 down to 9℃ (Ochillo, 1980; Ochillo *et al.*, 1981; Ochillo and Tsai, 1982; Tsai and Ochillo, 1983, 1989). They found (Tsai and Ochillo, 1986) that low temperature facilitated a parallel rightward shift of the dose-response curves of acetylcholine in isolated guinea pig ileum.

Regarding the influence of temperature on other kinds of smooth muscle, interest arises from the medical observation that during cardiovascular surgery, hypothermia can cause undesirable vascular spasm, making it important to elucidate the mechanism by which cold acts, in order to develop appropriate treatment. Fonteles and Karow (1977) demonstrated, in vascular smooth muscle of the isolated rabbit kidney, that cooling to 15℃ had an effect on the hypothermic automaticity elicited by α-adrenotropic drugs. Hypothermic research has been focused in order to elucidate how to manage accidental exposure to cold, such as fishermen working in frigid oceans or drunk people exposed to cold. How temperature can influence smooth muscle physiology or responses to drugs in fishes, whether freshwater or Antarctic, is not found in the literature.

The role of calcium ions in contractile mechanisms in smooth muscle is well documented. As reviewed by Bolton (1979) and Bohr (1988), the tension developed by contractile proteins of smooth muscle cells is considered to be primarily dependent on the ionized calcium within the cytoplasm. As pointed out by Mangel and Taylor (1992) a rise in intracellular calcium is the predominant signal that leads to activation of the contractile machinery in gastrointestinal muscle. Smooth tissue can use two stores of Ca^{2+} in excitation-contraction coupling: one extracelular and the other consisting of one or more intracellular pools (Church and Zsoter, 1980). Lack of cytoplasmatic free Ca^{2+} leads to impairment of contractile mechanisms. This can be achieved by drugs such as the calcium-chelating agent EDTA (ethylenediaminetetracetic acid) or by inhibiting calcium transmembrane transport by calcium channel blockers such as the phenylalkylamine verapamil. EDTA, due to its ionic character, scarcely penetrates the cells; it is considered to be an extracellular calcium-

blocking agent (Brading and Jones, 1969; Keatinge, 1972). Verapamil inhibits the transmembrane entry of calcium in muscular excitable cells by blocking the voltage-sensitive Ca^{2+} channels of L-type (Nowycky *et al.*, 1985; Hille, 1992). Its action on cholinergic contractions of isolated gastric muscle of *Bufo marinus* smooth muscle was described by Ochillo and Tsai (1982). Van Nueten and Vanhoutte (1981) and Bolton *et al.* (1988) reviewed the role of calcium blockers on smooth muscle.

The purpose of the present research is to study the influence of temperature, from 2°C to 18°C, on the isolated intestinal smooth muscle of Antarctic fish, the role of calcium ions and the effects of these temperatures on the acetylcholine responses.

The present experiments grew from casual observations, while we were studying the role of autonomic drugs on the isolated *N. neglecta* smooth muscle. On that occasion, we remarked that minor changes of the nutritive bath temperature significantly modified responses to drugs, besides inducing, by itself, important contraction of the organ.

Material and methods

Notothenia neglecta and *Chaenocephalus aceratus* were caught in Admiralty Bay, King George Island, South Shetlands, Antarctica, near the Antarctic Brazilian Station "Comandante Ferraz". The fishes were caught with a special tri-mesh bottom net at 80–100 m depth. Ten adult *N. neglecta*, 35–40 cm length and two juvenile, 12–15 cm length and two *C. aceratus* were used. *N. neglecta* was the most frequently caught species while *C. aceratus* were difficult to catch in good condition. They did not survive or were often found dead in the net, devoured by the amphipoda *Waldechia obesa*. The fishes were kept in indoor tanks with aerated marine water, at temperatures of 0–1.5°C, acclimated for at least 10 days prior to the experiments. Water pH and salinity were monitored. The fishes were offered fresh fish slices every week.

For the experiments, the fishes were spinalated. The abdominal cavity was opened and the intestine lifted forward, removed and placed in a dish containing aerated nutritive solution at 1.0–1.5°C. The proximal end of the anterior intestine (so named by Bullock, 1963, for salmonid fishes) was tied off with a thread to serve as a marker. The organ lumen was washed through with physiological salt solution to remove alimentary residues and parasites. Severely parasited intestines were discarded. Great care was taken in order to not damage the muscle. The organ was placed in the aerated physiological salt solution for about 12 hours, at 0–1.0°C. All the experiments were run in a cold room with air temperature no higher than 5°C.

Setting up the preparation by the method of Magnus

The isolated intestine was put in a Petri dish with aerated nutritive solution and the adherent tissue carefully cut off. A piece of about 1.5 cm (corresponding to about 2.0–3.0 cm when relaxed) was cut off. A thread was tied at each end by inserting a needle from the inside of the gut outward, avoiding the lumen to be closed off. One thread was tied to a fixed pin inside the organ chamber and the other to a frontal-writing lever for an isotonic recording. The lever had a 0.5 g load and 6 times magnification. The preparation was suspended in a 10 m*l* organ bath with aerated physiological salt solution and left for about 45–60 minutes at 2°C before the experiment started, in order to come

to a balance between the organ and the nutritive solution. The time to balance varied from one preparation to another and it was considered satisfactory after a steady writing of the isotonic lever on the recording paper. The physiological salt nutritive solution composition (in mEq/L) was: Na^+ 113.7; K^+ 1.9; Ca^{2+} 2.2; Cl^- 115.3; $H_2PO_4^-$ 0.1; HCO_3^- 2.4. Glucose 2.0 g/L. It was used Ringer-frog nutritive solution (Gaddum, 1953). The organ could survive up to 48 h in this nutritive solution, if provided continuous aeration and temperature of 0–2.0°C. The composition of this solution was established after initial trials. Recording of the muscle contractions was carried out in a rotating drum, 0.7 cm min^{-1}.

In order to control whether the responses of smooth muscle of Antarctic fish to temperatures were an unspecific event, but a common one to poikilotherms animals, experiments using toads (*Bufo marinus*), intestinal smooth muscle were carried out in our home laboratories in Brazil, using a comparable experimental model.

Temperature control

The temperature of the bath was increase during the experiments from 2°C to 18°C (stepped 2.0–6.0–10.0–14.0–18°C) and the response to acetylcholine assayed at each temperature. Convenient and reproducible control of temperature was achieved by surrounding the glass organ chamber with sliced ice to achieve the temperature of 2°C. The gradual rise of temperature was achieved by controlled removal of the cooling ice. This method, although simple, allowed very good control of temperature, as each experiment lasted no more than 10 min. It had the advantage of using the same organ for the whole experiment. Another method tried was similar to the one described by Tsai and Ochillo (1989) consisting of a double walled glass apparatus. The experimental chamber temperature was increased from 2.0°C to 18°C by gradually pumping warm water into the outer chamber. The apparatus was built with the 10 m*l* organ bath on the inside and the outer wall forming an exterior space through which water at various temperatures could circulate. In order to avoid freezing of the circulating water, a solution of 30 per cent propylene glycol was used. This method was abandoned because the temperature lasted too long at each level, and was not easy to control within our laboratory conditions, making difficult an intended short term experiment.

Drugs: acetylcholine (ACh), EDTA, barium chloride, verapamil and atropine

When the muscle bath reached the desired temperature and the corresponding sustained tonic contraction of the organ was maintained, ACh was administered (5×10^{-4}M). At the start of the experiment, at least two doses of ACh were used, in order to establish reproducible responses. ACh was administered after initial testing of the contractile capacity of the organ by barium chloride (0.2–0.4 m*l* of a 10^{-2}M solution). In experiments with ETDA (as dissodium salt) or verapamil, the drugs were added to the bath during the sustained contraction of the muscle at 2.0°C and then relaxation was observed. Final concentration of EDTA in the organ nutritive solution was 4×10^{-4}M and that of verapamil was 1.0×10^{-5}M. Atropine (3×10^{-6}M) was in some experiments administered to the organ bath during the sustained maximal contraction. Barium chloride was administered whenever it was desired to check the maximal responsiveness of the organ, usually at the start and always at the end of an experiment.

After the response to any drug the organ was washed out three to five times to assure complete removal of the chemical. Then, a resting time of 10–15 min was allowed. The term "contraction" is understood as used by Bolton (1979).

Fish visceral temperature

Just as the fish was spinalated, the visceral intestinal temperature was checked by inserting a thermometer into the abdominal cavity, through a small muscle incision.

Control experiments in intestinal smooth muscle of *Bufo marinus*-(5 experiments)

The animal was spinalated, the intestine excised, the adherent mucus gently cleared off, and the organ was put in aerated nutritive Ringer-frog solution at 2.0°C for 5 hours. For the experiment, the identical method described for Antarctic fish was used. The temperature of the bath was gradually increased, using the method previously described. After reaching the desired reaction temperature, the response of the organ to acetylcholine (5×10^{-4}M) was tested. Temperatures used were: 9°C, 15°C, 25°C and 35°C.

Results

Osmotic and ionic balance between smooth muscle and nutritive solution

The fish's smooth muscle contractile response to the agonist acetylcholine was higher after incubation in the nutritive solution for at least 12 hours. Fresh muscle often failed, or presented a poor response to acetylcholine, barium or potassium chloride. The preparation survived up to 48 hours in the aerated nutritive solution, at temperatures from 0–1.0°C.

Temperature and tonic contraction

When the temperature of the organ bath was 2.0°C, a sustained long lasting contraction was observed. Increase of temperature to 18°C caused proportional relaxation of the muscle. The same preparation, however, after lowering of the temperature from 18°C to 2.0°C, displayed a gradual contraction.

Results of six experiments with *N. neglecta* and two with *C. aceratus* are shown in Fig. 1B and 1D, respectively. There were no significant differences in the responses between adult and juvenile fish.

The values were plotted as percentage of the maximum contraction (elicited by barium chloride): at 2°C, it was considered 100 per cent and at 18°C, as zero. The recording of a typical experiment, showing the sustained contraction/relaxation according to the temperature, the contractions elicited by acetylcholine and relaxing response to EDTA during sustained contraction is shown in Fig. 2.

Tonic contraction and response to acetylcholine

The amplitude of the contractile response to acetylcholine was proportional to the temperature of the organ bath. It was minimum at 2°C and maximum at 18°C. A delay was observed also in the onset of the responses, which were larger at 2°C and shorter at 18°C. The delays of responses after the administration of acetylcholine were:

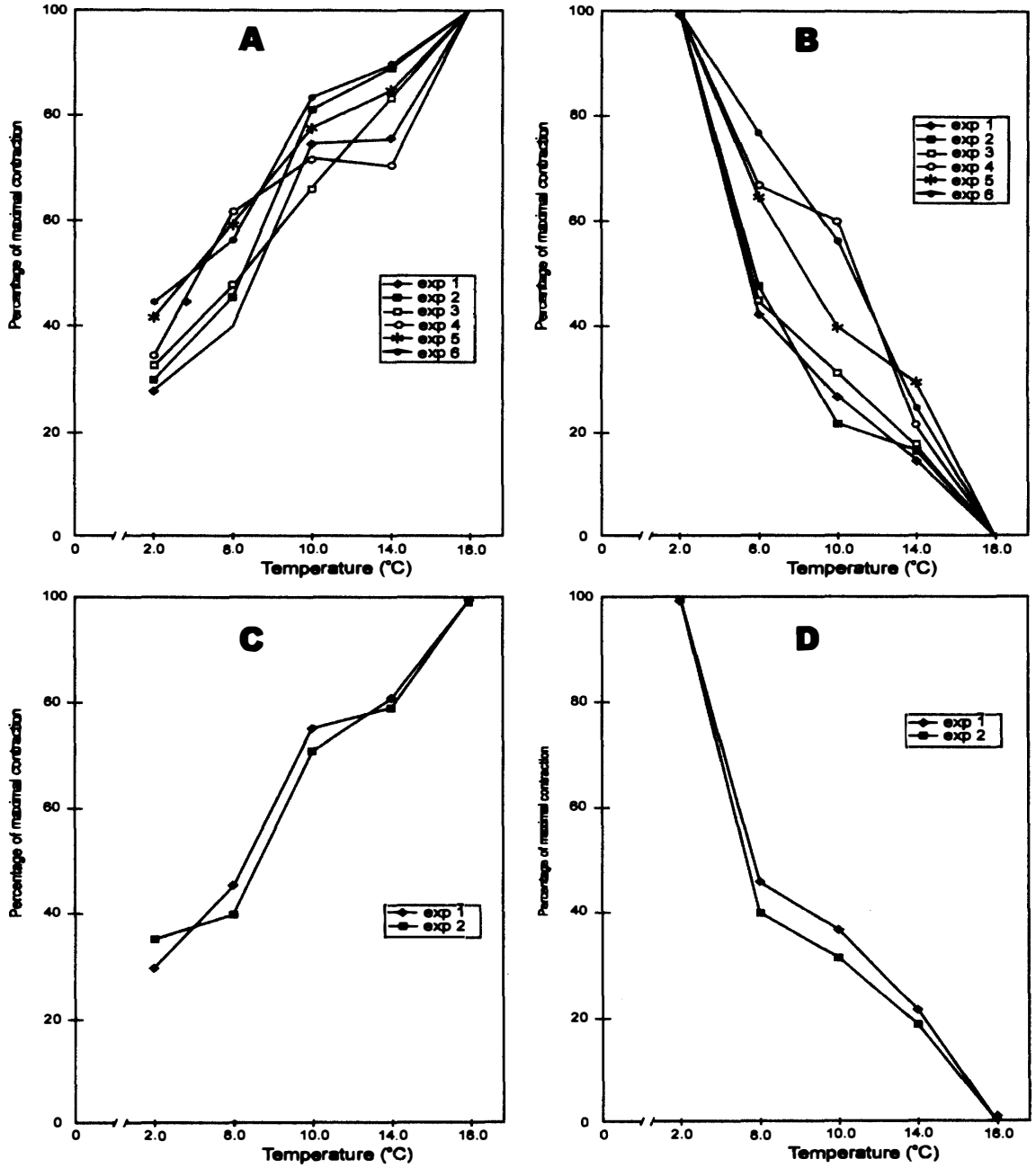

Fig. 1. *Notothenia neglecta* (A, B) and *Chaenocephalus aceratus* (C, D) intestinal smooth muscle responses towards temperature and acetilcholine (ACh). Results are plotted as percentage of maximal response of the organ to the temperature and to the agonist Ach.

A, C—ACh response: at 2°C the contractile response to ACh was minimal, increasing progressively up to maximal contraction (100%) at 18°C.

B, D—Temperature response: at 2°C the sustained tonic contraction was 100%, decreasing proportionally to the rising of the temperature. At 18°C, it was zero, meaning maximal relaxation.

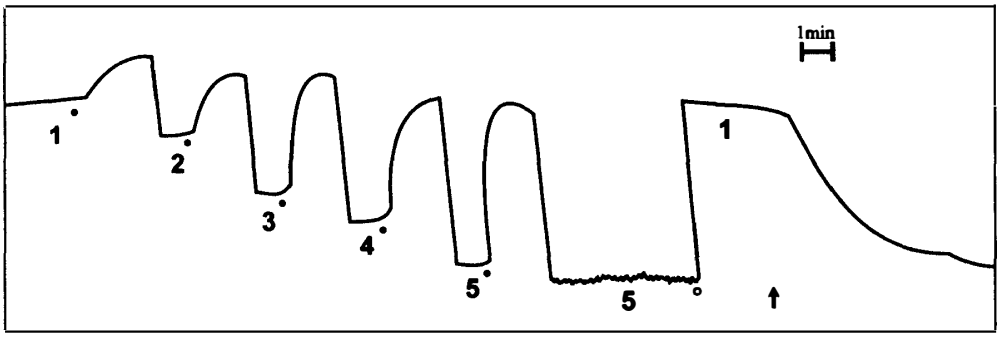

Fig 2. Recording of isotonic responses by isolated intestinal smooth muscle of *Notothenia neglecta*, in nutritive aerated bath.
• : administration of acetylcholine 5×10^{-4} M.
1, 2, 3, 4 and 5: baseline showing the level of smooth muscle sustained contraction according to the temperature: 1 = 2°C; 2 = 6°C; 3 = 10°C; 4 = 14°C; 5 = 18°C.
○ : return of the bath temperature, from 18°C to 2°C (recording stopped).
↑ : administration of EDTA (4×10^{-4}).
The organ was washed out 3–4 times after the recording of contraction caused by acetylcholine.

at 2°C, 20–33 s; at 6°C, 12–16 s; at 10°C, 6–10 s; at 14°C, 8.0–10.0 s; and at 18°C, less than 2.0 s. Results of experiments with *N. neglecta* and *C. aceratus* are shown in Fig. 1A and 1C respectively. Contractions of *N. neglecta* smooth muscle elicited by barium chloride and acetylcholine during the sustained contraction of the muscle at 2°C temperature are shown in Fig. 3A.

Inhibition of sustained contraction by sodium EDTA

During maximal sustained contraction at 2.0°C, administration of sodium EDTA caused gradual relaxation of the smooth muscle. After washout, when the normal calcium supply was replenished, contractions returned to initial values.

Inhibition of sustained contraction by verapamil

Verapamil added to the bath during maximal contraction of the smooth muscle, at 2°C, caused progressive relaxation, not reversible by washing out and returning the calcium supply to the organ bath. Results of experiments with EDTA and verapamil are given in Table 1.

Inhibition of sustained contraction by atropine

During the maximum contraction, at 2°C, atropine caused relaxation of the muscle, not reversible by washout. An experiment of this group is shown in Fig. 3B.

Internal temperature of fishes

The temperature of internal viscera, taken just when the fish was spinalated, was always 1.0 to 1.5°C higher than the aquarium water.

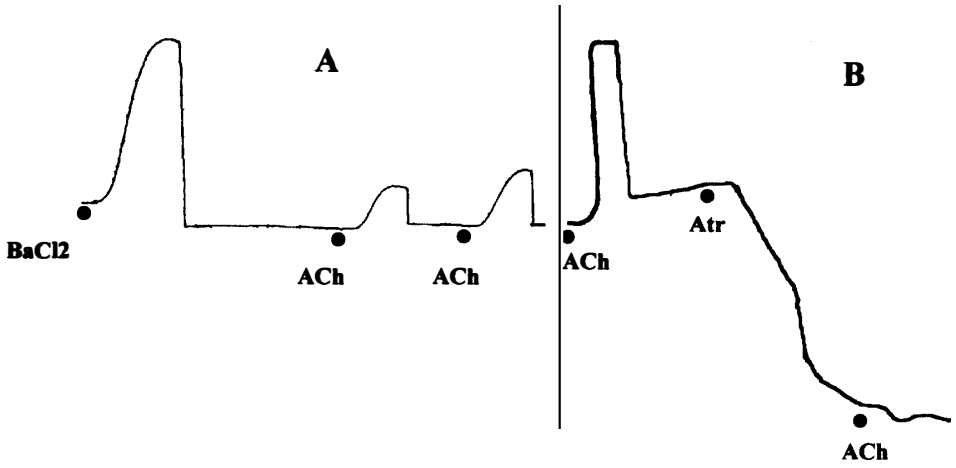

Fig. 3. Recording of isotonic responses by isolated intestinal smooth muscle of *Notothenia neglecta*, in nutritive aerated bath at temperature of 2.0°C.
A—Barium chloride-BaCl2-(0.2 m*l* of a 10^{-2} M sol.) and Acetylcholine-ACh (5×10^{-4} M).
B—Acetylcholine-ACh (5×10^{-3} M) before and after atropine-Atr (3×10^{-6} M).

Table 1. Intestinal smooth muscle of *Notothenia neglecta* in nutritive solution, at 2°C. Relaxation (in mm) of the muscle after EDTA (4×10^{-4} M) and verapamil (1.0×10^{-5} M). Recovery (%) after return of calcium by washout with the nutritive solution.

EDTA		Verapamil	
Relaxation	Recovery	Relaxation	Recovery
30	100	42	0
32	78	39	0
20	47	22	0
32	100	22	0
38	100	40	0
25	100	22	0
18	100	24	0
16	100		
47	85		

Comparison of responses of intestinal smooth muscle of Antarctic fish and the muscle of poikilotherm *Bufo marinus*

Intestinal smooth muscle of the toad failed to respond with sustained contraction when the temperature of the bath was decreased to 9.0°C. On the contrary, some relaxation was observed at lower temperatures. The muscle displays spontaneous motility, more intense in frequency and amplitude at higher temperatures. At 9.0°C the spontaneous motility almost disappeared. Responses to acetylcholine increased with rising temperature (see Fig. 4).

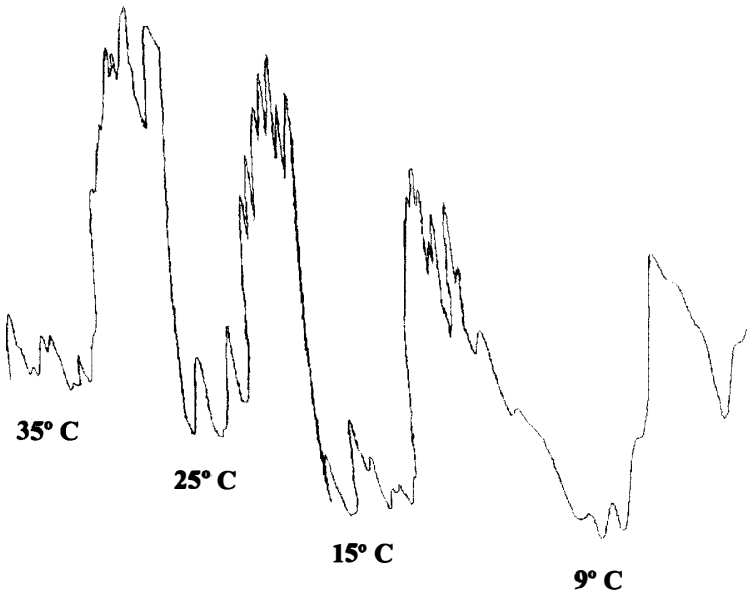

Fig. 4. Recording of isotonic responses by isolated intestinal smooth muscle of *Bufo marinus* in nutritive muscle bath, at temperatures of 35, 25, 15 and 9℃. Responses to acetylcholine (5×10^{-4} M).

Discussion

Results show that in Antarctic fishes, the intestinal smooth muscle undergoes a state of sustained contraction at lower temperatures, and relaxation as the temperature rises. It is difficult to establish if this phenomenon is a natural physiological state, within the natural environmental temperature in which the fishes live, because, when the animal is sacrificed, the gastric cavity was opened and the intestine immediately observed, it was uncertain whether it was contracted or not. The intestinal length of any animal is always quite difficult to establish, due to its high sensitivity to several stimuli, including manipulation. The wide range of temperatures used in our experiments, from 2°C to 18°C, had just an experimental purpose, and was not intended to demonstrate the physiological status of the fish in his natural cold environment. The isolated organ is an interesting preparation, because it can be studied without humoral and nervous influence other than the local neural plexuses. On the other hand, it may not display an exact physiological pattern.

Sustained intestinal smooth muscle contraction to cold from adult and juvenile *N. neglecta* was also observed in the icefish *C. aceratus*. This leads to the supposition that it is common in fishes adapted to live in cold Antarctic conditions. On the other hand, this phenomenon could be common to poikilotherm animals also, which could respond with contraction when submitted to low environmental temperatures. However, this supposition was not corroborated by our experiments with toads (*Bufo marinus*). Experiments in similar conditions to those used with Antarctic fish showed that the lowering of temperature did not cause sustained contraction of the intestinal toad

muscle, but relaxed the muscle and decreased the spontaneous peristaltic activity.

For the *in vitro* experiments, the composition of the physiological nutritive solution used in the muscle bath was an initial problem. Saline or marine water was not suitable; we did not find a proper application of physiological salines described by Wolf (1963) for freshwater teleosts. After initial trials, we established a chemical composition for the solution that permitted the tissue to live longer. We cannot assure that it was really the best one (an extensive trial would be necessary) but it was unquestionably appropriate, since the organ was alive and perfectly responsive after 48 hours. Longer times were not attempted.

We concluded that several hours are necessary to balance the fish organ with the nutritive solution. It was observed that when the organ was fresh, within the first 1 to 3 hours of being excised from the fish, the responses to acetylcholine were poor, although sustained contraction at low (2°C) temperature was observed. The responses of three adjacent segments were compared, one within the first hour of being excised, and the second and third, 12 hours and 30 hours later. A much better response of the two latter to acetylcholine indicated that this time or maybe a little longer should be chosen for all experiments. One can suppose that this is due to the low metabolic rate of the organ, delaying the balance between the muscle and the nutritive solution.

The internal body temperature of the fishes was always 1 to 1.5°C higher than the temperature of the surrounding aquarium water. This suggests that a calorigenic metabolic process is present. We observed smaller sensitivity of the fish smooth muscle to drugs, as compared to guinea pig ileum. This could be due to low metabolic rate and/or the low temperature of the organ nutritive bath, influencing absorption or drug dissociation.

The temperature-dependent tonic contraction of the smooth muscle was closely reproducible in all of the experiments (see Fig. 1B and 1D). This phenomenon is not a simple mechanical response to cold, but an active process, possibly with physiological significance, since it is calcium dependent and is acted upon by muscarinic cholinergic receptors. The importance of calcium ions was evidenced by the immediate relaxation response after administration of the calcium chelating agent EDTA and calcium channel blocker verapamil. An interesting observation is that after addition of EDTA, contraction was re-established by washing out the preparation, but after the administration of verapamil, the relaxation response was permanent (see Table 1). It is known that EDTA scarcely penetrates the cell, due to its ionic character. Figure 2 shows the relaxing curve after the administration of EDTA. The relaxing response is progressive, indicating that the muscle is using intracellular calcium up to total depletion, since the muscle remains relaxed until replenishment of the external calcium. The muscle did not respond to acetylcholine after relaxation by EDTA. However, when the relaxation response, to a comparable extent, was elicited just by raising the temperature to 18°C, this agonist displayed its maximal response. Then, the relaxation response to temperature must be a physiologically active state, keeping the muscle reactive to muscarinic receptor activation.

The role of calcium ions in the response of the fish smooth muscle to cold is reinforced by the fact that the tonic sustained contraction was inhibited by calcium channel blocker verapamil. This drug is known to inhibit the transmembrane calcium

influx during excitation (Church and Zsoter, 1980; Van Nueten and Vanhoutte, 1981), blocking the tonic contraction due to cold. As it happened after the administration of EDTA, the drug did not immediately block the contraction. There was a lag period, during which the relaxation of the muscle was gradual and progressive, suggesting that the cells were using their intracellular calcium stocks. However, in contrast to what happened with EDTA, washing out of the preparation, up to 10 times, failed to restore any contractile response (see Table 1). Maybe the interaction of verapamil with calcium channel molecular structures is long lasting or, after the closure of the calcium channels, a period of time is required to reopen the channel. One can speculate that the tonic contraction observed in our experiments is somewhat related to the findings of Tsai and Ochillo (1989): these authors noted that lowering the temperature of guinea pig isolated ileum triggered automaticity and that such a response was blocked by inhibition of muscarinic receptors and by the calcium channel blocker verapamil.

The tonic contraction of the isolated smooth muscle was partially dependent on the cholinergic muscarinic receptor. Atropine, in doses that completely blocked the responses to ACh, caused partial inhibition of the tonic contraction (Fig. 3B). One can suppose that the contractile response to cold is completely dependent upon the entry of calcium into the muscle cells, and is influenced, at least partially, by activation of muscarinic receptors.

Responses to acetylcholine were smaller at 2°C, and increased proportionally with rising temperature, being highest at 18°C (Fig. 1A and 1C). A lag of 20 to 33 s at 2°C was observed, but it was about zero at 18°C. These findings suggest that the acetylcholine-receptor binding follows Van't Hoff's law, since the rising energy of the system (rising temperature) leads to increased responses. These results suggest that the agonist-receptor binding is similar to that displayed by animals adapted to a temperate environment, temperature being the determinant factor in the observed differences. However, the same relationship was not observed in the smooth muscle of the toad (*Bufo marinus*), in equivalent conditions. The depressed response of the smooth muscle to acetylcholine in lower temperatures could be attributed to inability of the organ to undergo further contraction in a currently contracted organ. This supposition can be discarded because barium chloride, used to test the capacity of the organ to contract, elicited, at the lower temperature- 2.0°C, a much higher contraction as compared to acetylcholine. An experiment showing this is shown in Fig. 3A. However, one cannot exclude the possibility that the contracted state could exert some influence in the responses of acetylcholine. Simultaneous recording of membrane potentials is desirable.

The results presented in this paper are consistent with those found by Tsai and Ochillo (1989). These authors noted that in isolated guinea pig ileum, muscarinic receptor responsiveness to acetylcholine was reduced by hypothermia. The responsiveness of the fish muscle was in general quantitatively different as compared to classical guinea pig smooth muscle responses.

From the present results, one could tentatively speculate that, although the physiological and biochemical efficacy of the digestive apparatus of the Antarctic fishes, carrying out digestive absorption of nutrients and undergoing chemical biotransformations, the physiological adaptation of the intestinal muscle is only partially adapted to

low Antarctic temperatures, despite 25 million years of acclimatization.

Acknowledgments

The authors are grateful to SECIRM, to the Marine Group of the Brazilian Antarctic Station and in particular to Sergeant Nilo Dias dos Santos, for their support. We thank Mr. Anselmo Facundin for his help in English revision.

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(Received February 8, 1999; Revised manuscript accepted July 12, 1999)