

## THE INTRACELLULAR pH OF A MOLLUSCAN SMOOTH MUSCLE DURING A CONTRACTION–CATCH–RELAXATION CYCLE ESTIMATED BY THE DISTRIBUTION OF [<sup>14</sup>C]DMO AND BY <sup>31</sup>P-NMR SPECTROSCOPY

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### Summary

The intracellular pH (pHi) of the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L. was estimated during rest and during a contraction–catch–relaxation cycle by the distribution of [<sup>14</sup>C]DMO and by <sup>31</sup>P-NMR spectroscopy. The pHi of resting muscles was found to be nearly the same whether it was determined with DMO ( $7.41 \pm 0.06$ ,  $N=51$ ) or by <sup>31</sup>P-NMR spectroscopy ( $7.44 \pm 0.02$ ,  $N=5$ ). During catch the pHi was not significantly different from its value at rest. Serotonin ( $10^{-5}$  mol l<sup>-1</sup>) induced a rapid relaxation and a significant increase in pHi (DMO:  $7.56 \pm 0.05$ ,  $N=11$ ; <sup>31</sup>P-NMR:  $7.57 \pm 0.02$ ,  $N=5$ ). In resting ABRM serotonin ( $\geq 10^{-6}$  mol l<sup>-1</sup>) also induced an alkalosis. It was shown that cyclic AMP, the second messenger of serotonin, elicited an increase of pHi. Dopamine ( $10^{-5}$  mol l<sup>-1</sup>) did not cause an increase in either cyclic AMP levels or pHi. The rates by which tonically contracted ABRM loaded at 0.1 N were stretched were significantly increased by an alkalosis elicited by the addition of 10 or 20 mmol l<sup>-1</sup> methylamine. Acidosis elicited by 10 mmol l<sup>-1</sup> DMO caused a significant decrease in the rate of relaxation.

### Introduction

The anterior byssus retractor muscle (ABRM) of the common sea mussel *Mytilus edulis* L. is able to perform two different types of contraction distinguished by their velocity of relaxation. Phasic contraction is characterized by rapid relaxation, whereas tonically contracted muscles relax very slowly. The active phase of a contraction is elicited by acetylcholine. During a tonic contraction the active phase is followed by a passive phase called catch (for reviews, see Rüegg, 1971; Twarog, 1979). During catch the ABRM is able to maintain tension or

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shortening for long periods, although the metabolic rate is reduced almost to resting levels (Baguet and Gillis, 1968; Zange *et al.* 1989).

The molecular mechanism of catch is still unknown. Two main hypotheses are presently discussed. The cross-bridge hypothesis proposes that cross-bridge cycling is reduced to the resting value, but the phase of cross-bridge attachment is prolonged owing to an increased affinity of myosin for actin (Lowy *et al.* 1964; Takahashi *et al.* 1988). The paramyosin hypothesis proposes a cross-linking of the thick filaments, presumably through paramyosin (Rüegg, 1971; Hauck and Achazi, 1987).

Serotonin (Twarog, 1954), dopamine (Twarog and Cole, 1972) and electrical stimulation by alternating current pulses (Winton, 1937; Fletcher, 1937) abolish catch and lead to a rapid relaxation. Achazi *et al.* (1974) found that serotonin stimulates an adenylate cyclase system. They proposed that the increased levels of cyclic AMP finally lead to a phosphorylation or dephosphorylation of the paramyosin involved in catch performance. This mechanism of catch relaxation has been questioned, because serotonin affects catch at concentrations as low as  $10^{-9} \text{ mol l}^{-1}$  (Twarog, 1954), but  $10^{-6} \text{ mol l}^{-1}$  serotonin is needed to increase the content of cyclic AMP significantly (Köhler and Lindl, 1980). Furthermore, dopamine has little or no effect on cyclic AMP levels (Köhler and Lindl, 1980; Ishikawa *et al.* 1981).

Experiments with skinned fibres from ABRMs indicated that intracellular pH (pHi) may be involved in catch regulation. This preparation is able to enter a catch-like state when  $\text{Ca}^{2+}$  has been removed after an active contraction. The catch-like state of skinned fibres is sensitive to changes in the pH of the incubation medium. Between pH values of 6.2 and 6.7 skinned fibres relax slowly, whereas pH values higher than 7.0 cause a fast drop of the maintained tension or shortening (Johnson, 1959; Rüegg, 1971; Pfitzer and Rüegg, 1982). Another argument for an involvement of pHi in catch regulation is the observation that living ABRMs reduce their relaxation velocity during catch when they are acidified by incubation in a medium containing high concentrations of  $\text{CO}_2$  (Rüegg, 1964).

The present study was designed to estimate the pHi of the ABRM during rest and during a contraction-catch-relaxation cycle. pHi was measured either by the distribution of [ $^{14}\text{C}$ ]dimethylloxazolidine-2,4-dione (DMO; Waddell and Butler, 1959) or by  $^{31}\text{P}$ -NMR spectroscopy (Moon and Richards, 1973). Further DMO studies focused on the relationship between cyclic AMP levels and pHi. Lastly, the effect of changes in pHi, elicited by the application of either a weak base or a weak acid, on relaxation velocity of the ABRM during catch was investigated.

## Materials and methods

### *Animals*

Mussels *Mytilus edulis* L. were obtained from the Nederlands Instituut voor Onderzoek der Zee, Den Helder, The Netherlands. The animals were kept in

recirculating, filtered artificial sea water (32‰) for 3–12 weeks at  $15 \pm 1^\circ\text{C}$  until they were used in the experiments.

#### *Preparation and incubation*

When pHi was estimated using the DMO method, the isolation of the ABRM followed the procedure of Hoyle and Lowy (1956). Intact muscles were attached to a piece of shell and held by a silk thread tied to the byssal end of a muscle. The mussel contains a pair of identically shaped ABRMs. For each experiment both muscles were taken. For contraction experiments, one muscle of a pair was taken as a resting control and the other was used in the experiment.

The muscles were mounted in incubation chambers filled with a seawater medium equilibrated with air at  $15 \pm 1^\circ\text{C}$  and pH 7.65. Resting and contracting muscles were loaded with a weight of 0.1 N. Since freshly prepared ABRMs were usually contracted, isolated muscles were subjected to electrical stimulation with rectangular pulses applied by platinum electrodes (2 Hz, 100 ms, 10 V; Winton, 1937). Muscles were then incubated for 4 h before the experiment was started.

When pHi was monitored using  $^{31}\text{P}$ -NMR spectroscopy silk threads were tied to both ends of the muscles prior to separation of the ABRMs from the byssus and the shell. For each series of experiments four or five ABRMs with a total wet mass of 100–150 mg were used. The muscles were tied at their shell ends to a muscle holder placed at the bottom of a 10 mm NMR tube. Using the threads tied to the byssal ends of the muscles, the ABRMs were arranged vertically within the NMR tube. The tube was filled with 4 ml of air-equilibrated seawater medium at  $15 \pm 1^\circ\text{C}$  and a pH of  $7.65 \pm 0.02$ . A gas-inlet capillary was placed 0.5 cm above the sensitive range of the receiver coil.

#### *Recording of muscle movements*

Changes in muscle length were followed by means of an isotonic transducer connected to an HF modem and an amplifier (Hugo Sachs Electronic, Freiburg, FRG) and were displayed on a chart recorder.

#### *Tonic contractions*

In cases where DMO was used to estimate pHi, tonic contractions under isotonic conditions (0.1 N) were elicited by the application of acetylcholine ( $0.5 \text{ mmol l}^{-1}$ ; Nauss and Davies, 1966) to a single muscle. The ABRM contracted maximally within 1 min. Two minutes after the addition of acetylcholine, the transmitter substance was washed out using two washes of acetylcholine-free medium. The second medium contained [ $^{14}\text{C}$ ]DMO and [ $^3\text{H}$ ]inulin.

During NMR experiments, the pHi of four simultaneously contracting ABRMs was monitored. The transducer was placed 5 m away from the spectrometer to avoid damage by the magnetic field. The average isotonic shortenings of these four muscles, which were each loaded with 0.1 N, were transmitted to the transducer using an arrangement of three plastic rings working like a tackle block. Contraction was again elicited by the application of acetylcholine ( $0.5 \text{ mmol l}^{-1}$ ).

Acetylcholine was washed out after 3 min by the use of a glass capillary placed on the muscle holder.

#### *Determination of pHi by the distribution of DMO*

Intracellular pH was determined by application of the DMO distribution method (Waddell and Butler, 1959) to an ABRM incubated in a medium containing [ $^{14}\text{C}$ ]DMO ( $1.5 \times 10^5$  disints  $\text{min}^{-1} \text{ml}^{-1}$ ,  $< 2 \text{ nmol l}^{-1}$ ) and [ $^3\text{H}$ ]inulin ( $2 \times 10^5$  disints  $\text{min}^{-1} \text{ml}^{-1}$ ). Both labelled substances were obtained from Amersham Buchler, Braunschweig, FRG. Details of the analysis of  $^{14}\text{C}$  and  $^3\text{H}$  activity in the muscle and in the medium, of the measurement of medium pH and of the calculation of pHi have been described previously (Pörtner *et al.* 1984).

#### *NMR spectroscopy*

All  $^{31}\text{P}$ -NMR spectra were recorded at 161.5 MHz on a Bruker AM 400 WB spectrometer. Typical acquisition parameters were: sweep width 10 000 Hz, pulse width 7.4  $\mu\text{s}$  (corresponding to a  $45^\circ$  flip angle), 4096 data points, relaxation delay 0.132 s and acquisition time 0.205 s. For each spectrum, 512 scans were accumulated within 2.94 min.

#### *Determination of the intracellular pH by $^{31}\text{P}$ -NMR*

pHi was determined from the chemical shift of the intracellular inorganic phosphate relative to the shift of glycerophosphorylcholine (GPC) as an extracellular chemical shift standard. The chemical shift of GPC (+0.49 p.p.m.) is unaffected by changes in pH. To relate the chemical shift to pHi a muscle extract was titrated. This titration curve (see Zange *et al.* 1990) was used to obtain the relationship between the chemical shift of inorganic phosphate and pHi.

#### *Analysis of cyclic AMP levels*

ABRMs were ground in a small mortar under liquid nitrogen as described by Pette and Reichmann (1982). The concentrations of cyclic AMP were measured in neutralized trichloroacetic acid extracts using a radioimmunoassay test kit obtained from Amersham Buchler, Braunschweig, FRG.

#### *Medium*

The seawater medium used for the DMO experiments had the following composition (in  $\text{mmol l}^{-1}$ ): NaCl, 463; KCl, 10;  $\text{MgCl}_2$ , 24;  $\text{MgSO}_4$ , 28;  $\text{CaCl}_2$ , 10 and Hepes/NaOH, 5 (pH 7.65).  $\text{HCO}_3^-$  ( $0.4 \text{ mmol l}^{-1}$ ) was formed during equilibration with air ( $P_{\text{CO}_2} \approx 0.03 \text{ kPa}$ ). For NMR experiments the concentrations of NaCl and of Hepes were changed to 438 and  $30 \text{ mmol l}^{-1}$ , respectively.

#### *Statistical analysis of the results*

The significance of a difference between two values of pHi (mean  $\pm$  s.d.) was tested using Student's *t*-test ( $P < 0.05$ ).

## Results

### pHi during a contraction–catch–relaxation cycle

#### The estimation of pHi with DMO

In relaxed, maximally stretched ABRMs loaded with 0.1 N, equilibration with DMO and inulin was reached within 15 min. Maximally contracted, unloaded muscles were equilibrated within 45 min, whereas the time for equilibration in tonically contracted ABRMs, which were slowly stretched by a load of 0.1 N during catch, was 30 min.

In resting ABRMs a pHi of  $7.41 \pm 0.06$  ( $N=51$ ) was estimated by the distribution of DMO. In ABRMs in catch (30 min) pHi did not change significantly ( $7.42 \pm 0.05$ ,  $N=10$ ). In muscles in catch for 15 min and then rapidly relaxed by application of serotonin ( $10^{-5} \text{ mol l}^{-1}$ ) for a further 15 min, pHi was significantly increased to  $7.56 \pm 0.05$  ( $N=11$ ; see Fig. 1 and Table 1). Owing to the slow distribution of DMO, pHi measurements could not be performed using this method during the active contraction. Therefore, the  $^{31}\text{P}$ -NMR technique was employed.

#### pHi determined by $^{31}\text{P}$ -NMR spectroscopy

pHi was monitored continuously in intervals of 3 min (512 scans) during rest and during a contraction–catch–relaxation cycle. In resting muscles a pHi of

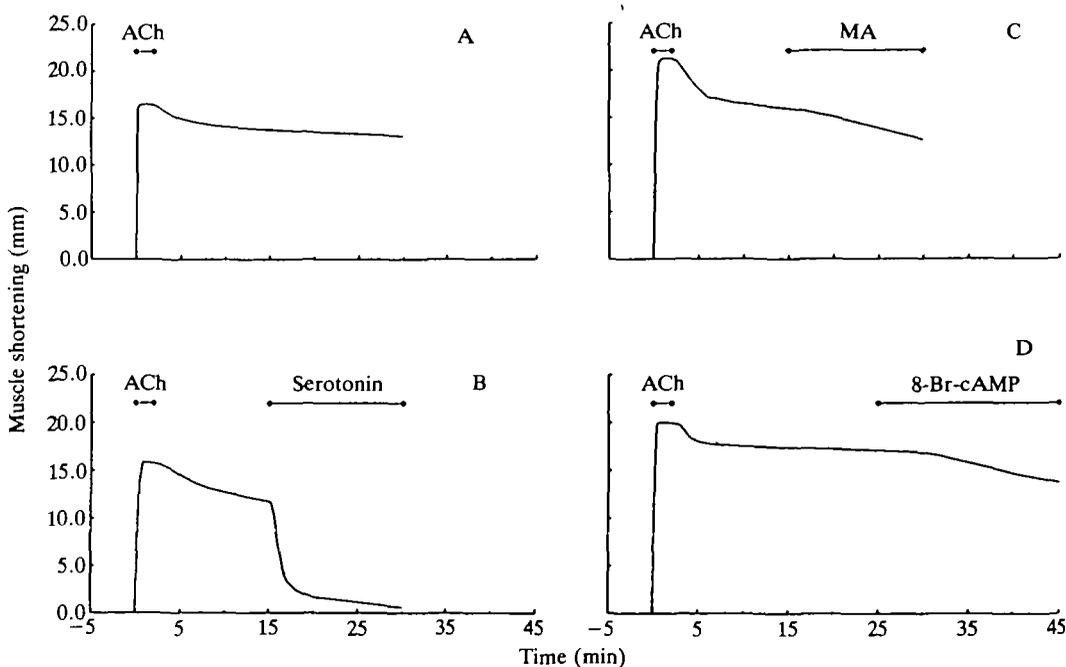


Fig. 1. Typical tonic contractions of ABRMs loaded with 0.1 N elicited with acetylcholine (ACh) (A) and the effects of serotonin (B,  $10^{-5} \text{ mol l}^{-1}$ ), methylamine (MA) (C,  $10 \text{ mmol l}^{-1}$ ) and 8-bromo-cyclic AMP (D,  $5 \text{ mmol l}^{-1}$ ) on catch.

Table 1. *The pHi of resting and contracting ABRMs in the absence and the presence of serotonin ( $10^{-5}$  mol l $^{-1}$ )*

Treatment	pHi	N
Resting control muscles	7.41±0.06	51
Resting muscles in the presence of serotonin (15 min)	7.52±0.04	12
Resting muscles in the presence of serotonin (4.5 h)	7.58±0.03	12
Muscles during catch (30 min; see Fig. 1A)	7.42±0.05	10
Muscles 15 min after the release of catch by serotonin (see Fig. 1B)	7.56±0.05	11

Values are mean±s.d.  
pHi was estimated with DMO.

7.44±0.02 ( $N=5$ ) was estimated. During the active phase of contraction lasting for 3 min, pHi increased significantly to 7.54±0.01 ( $N=5$ ). During the subsequent catch pHi dropped back to resting levels within 3 min. Serotonin ( $10^{-5}$  mol l $^{-1}$ ) elicited a rapid relaxation and a slow increase of pHi. About 1 min after application of serotonin, the ABRMs were stretched at a maximal rate. At this time, pHi had still not increased significantly (7.45±0.03). pHi reached its maximum of 7.57±0.02 after 15–20 min. At this time the muscles had relaxed (see Fig. 2).

#### *The effect of serotonin on resting ABRMs*

Application of serotonin ( $10^{-5}$  mol l $^{-1}$ ) to resting muscles for 15 min also elicited a significant alkalinization (pHi 7.52±0.04,  $N=12$ , DMO method). pHi remained elevated (7.58±0.03,  $N=12$ , DMO method, see Table 1) for 4.5 h.

The effect of serotonin on pHi in ABRMs was dose-dependent. Concentrations of  $10^{-7}$  mol l $^{-1}$  or lower did not change pHi significantly (7.45±0.01,  $N=3$ , NMR method), but serotonin applied at concentrations of  $10^{-6}$  mol l $^{-1}$  or greater increased pHi maximally (7.59±0.02; see Fig. 3).

#### *The relationship between cyclic AMP levels and pHi*

In resting control muscles, cyclic AMP levels were estimated to be 3.2±0.2 nmol g $^{-1}$  wet mass ( $N=6$ ). Serotonin ( $10^{-5}$  mol l $^{-1}$ , 15 min) induced a significant increase in cyclic AMP levels to 38.9±8.4 nmol g $^{-1}$  ( $N=6$ ). Therefore, we were interested in the question of whether cyclic AMP, as the second messenger of serotonin, causes an increase in pHi.

To simulate the effect of serotonin on cyclic AMP levels we used forskolin, which is known to stimulate adenylate cyclase (Seamon and Daly, 1981) and IBMX (isobutyl-1-methylxanthine), an inhibitor of phosphodiesterase (Pannabecker and Orchard, 1986). A significant increase in cyclic AMP levels (8.4±1.5 nmol g $^{-1}$ ) could be induced only if forskolin (50  $\mu$ mol l $^{-1}$ ) and IBMX (1 mmol l $^{-1}$ ) were applied together.

In resting ABRMs, forskolin and IBMX induced a significant alkalinization

( $7.59 \pm 0.03$ ,  $N=12$ , DMO method; Table 2). The application of 8-bromo-cyclic AMP (8-Br-cAMP) ( $5 \text{ mmol l}^{-1}$ ) to isotonically contracted ABRMs during catch also elicited a significant increase in pHi ( $7.53 \pm 0.05$ ,  $N=9$ , DMO method; Table

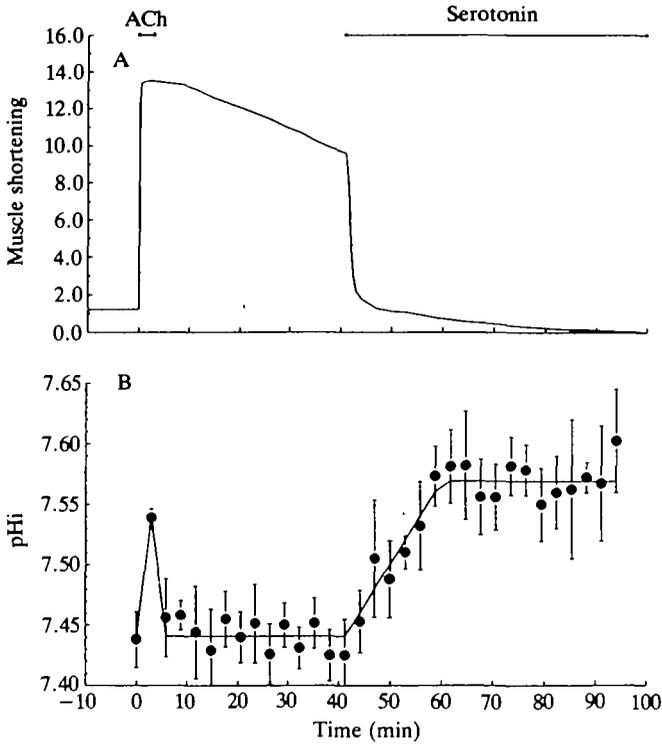


Fig. 2. Tracing from a typical contraction-catch-relaxation cycle (A) and pHi values (B) during such a cycle measured by  $^{31}\text{P}$ -NMR (means  $\pm$  s.d.,  $N=5$ ).

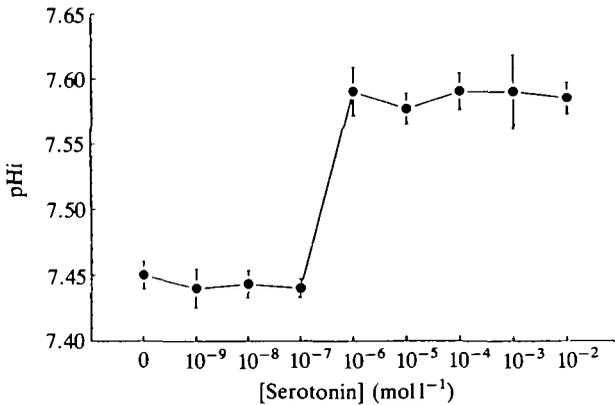


Fig. 3. The dose-response curve for the effect of serotonin on pHi in ABRMs measured by  $^{31}\text{P}$ -NMR spectroscopy (means  $\pm$  s.d.,  $N=3$ ).

Table 2. *The relationship between cyclic AMP levels and pHi (DMO method) in the ABRMs*

Treatment	Cyclic AMP (nmol g <sup>-1</sup> wet mass)	N	pHi	N
Resting controls	3.2±0.2	6	7.41±0.06	51
Forskolin (50 mmol l <sup>-1</sup> ) and IBMX (1 mmol l <sup>-1</sup> ) applied to resting muscles (60 min)	8.4±1.5	6	7.59±0.03	12
8-Br-cAMP (5 mmol l <sup>-1</sup> ) applied during catch (15 min, see Fig. 1)	—	—	7.53±0.05	12
Serotonin (10 <sup>-5</sup> mol l <sup>-1</sup> ) applied to resting muscles (15 min)	38.9±8.4	6	7.54±0.04	9
Dopamine (10 <sup>-5</sup> mol l <sup>-1</sup> ) applied to resting muscles (15 min)	3.4±0.5	6	7.45±0.03	12
Electric pulses (10 V, 100 ms, 2 Hz, 20s) applied during catch	3.6±0.6	6	—	—

Values are mean±s.d.

2). Therefore, it seems likely that serotonin induces an increase in pHi *via* cyclic AMP as a second messenger.

In those experiments in which the effect of serotonin (10<sup>-5</sup> mol l<sup>-1</sup>) on catch and pHi was investigated, the isotonicity contracted ABRMs loaded with 0.1 N were extended at a rate of 0.05±0.02 mm min<sup>-1</sup> (N=9) during catch. Serotonin induced a maximum relaxation rate of 5.0±1.8 mm min<sup>-1</sup> after 1 min.

Before 8-bromo-cyclic AMP was added to the isotonicity contracted ABRMs, the muscles stretched at a rate of 0.05±0.02 mm min<sup>-1</sup> (N=9). This rate was significantly increased to 0.18±0.08 mm min<sup>-1</sup> within 15 min of 8-bromo-cyclic AMP application. The maximal rate of relaxation induced by serotonin was 28 times faster than that induced by 8-bromo-cyclic AMP.

The catch-releasing transmitter dopamine (10<sup>-5</sup> mol l<sup>-1</sup>) did not elicit significant changes in pHi (7.45±0.03, N=12, DMO method) or in cyclic AMP levels (3.4±0.5 nmol g<sup>-1</sup>, N=6; Table 2).

#### *The relationship between pHi and the rate of relaxation during catch*

The rate at which an ABRM was stretched by a load of 0.1 N ( $V_s$ , mm min<sup>-1</sup>) was used as an indicator of the holding capacity of the muscle during catch.  $V_s$  was not significantly correlated with the wet mass or with the muscle tension (N cm<sup>-2</sup>). Therefore, experiments were conducted without any standardization of  $V_s$ .

Our results show that alkalinization elicited by the weak base methylamine (10 or 20 mmol l<sup>-1</sup>) induced a significant acceleration of  $V_s$ , whereas acidification elicited by unlabelled DMO (10 mmol l<sup>-1</sup>) induced a significant decrease in  $V_s$  (Table 3).

The effect of alkalinization elicited by 10 mmol l<sup>-1</sup> methylamine<sup>■</sup>

Table 3. The effects of changes in pHi on the relaxation rate ( $V_s$  in  $\text{mm min}^{-1}$ ) of isotonicly (0.1 N) contracted ABRMs during catch

Treatment	pHi	$\Delta\text{pHi}$	$V_{s1}$	$V_{s2}$	$\Delta V_s$	$N$
Control	7.42±0.05	+0.01±0.05	0.05±0.02	—	—	10
Methylamine (10 mmol l <sup>-1</sup> )	7.54±0.05	+0.13±0.05	0.05±0.03	0.16±0.04	+0.11±0.04	8
Methylamine (20 mmol l <sup>-1</sup> )	7.61±0.07	+0.20±0.07	0.06±0.03	1.04±0.71	+0.98±0.70	7
DMO (10 mmol l <sup>-1</sup> )	7.28±0.04	-0.13±0.04	0.09±0.03	0.05±0.02	-0.04±0.02	7
8-Br-cAMP (5 mmol l <sup>-1</sup> )	7.53±0.05	+0.12±0.05	0.05±0.02	0.18±0.09	+0.13±0.07	9

$V_s$  was measured before ( $V_{s1}$ ) and after ( $V_{s2}$ ) treatment.  
pHi was estimated using [<sup>14</sup>C]DMO.  
 $\Delta\text{pHi}$  was calculated relative to the pHi of resting control muscles (7.41, see Table 1).  
Values are mean±s.d.

(pHi = 7.54±0.05,  $N=8$ , DMO method) on  $V_s$  (0.16±0.04  $\text{mm min}^{-1}$ ) was similar to the effect of 8-bromo-cyclic AMP on pHi and  $V_s$ .

### Discussion

The pHi of resting ABRMs was found to be 7.41±0.06 ( $N=51$ , DMO method) and 7.44±0.02 ( $N=5$ , NMR spectroscopy) when the muscles were incubated in aerated seawater medium at pH 7.65 in the absence of serotonin. The value of pHi estimated with DMO was significantly lower than that estimated by NMR. Nevertheless, results from both methods can be considered to show good agreement because a difference of 0.03 pH units is low.

During the active phase of a tonic contraction under isotonic conditions, pHi rapidly and significantly increased from 7.44±0.02 to 7.54±0.01 ( $N=5$ , NMR). This alkalosis can probably be correlated with a breakdown of the phosphagen phospho-L-arginine, by which ABRMs support most of their energy demand during the working phase of contraction (Nauss and Davies, 1966; Zange *et al.* 1989). The splitting of a phosphagen generally causes consumption of protons because the pK value of the phosphate group in the phosphagen is higher than that of inorganic phosphate (Pörtner, 1987).

When catch was elicited by the removal of acetylcholine, pHi returned to resting levels within 3 min. This fast reacidification could be correlated with the resynthesis of phospho-L-arginine (Nauss and Davies, 1966; Zange *et al.* 1989).

Contractile filaments of the living ABRMs maintain tension and shortening during catch at a pHi of 7.44. When skinned fibres of the ABRM were incubated at this physiological pH, however, they failed to enter the catch-like state which can be elicited by the removal of  $\text{Ca}^{2+}$  after an active contraction. Therefore, studies on the catch-like state of skinned fibres have been performed using acidic media

between pH 6.2 and 6.8 (Johnson, 1959; Rüegg, 1971; Ishii, 1987). Pfitzer and Rüegg (1982) found striking similarities between the mechanical behaviour of living ABRMs during catch and skinned fibres of the ABRM during the catch-like state at pH 6.5. For the time being we cannot resolve this discrepancy of the functional involvement of pHi in catch in living ABRMs compared with skinned fibres.

In the skinned fibre experiments mentioned above, the incubation medium contained about  $150 \text{ mmol l}^{-1} \text{ Cl}^{-}$ . In their study on skinned axonemes from sea urchin sperm, Gibbons *et al.* (1985) have shown that the activity of the dynein ATPase depends on the concentration of  $\text{Cl}^{-}$  in the medium, the enzyme being 1.5 times more active at a concentration of  $150 \text{ mmol l}^{-1}$  than in the presence of  $30 \text{ mmol l}^{-1} \text{ KCl}$ . Using organic anions to make up the same ionic strength had no effect. Gibbons *et al.* (1985) showed that the relative concentration of  $\text{Cl}^{-}$  and organic anions in the incubation medium could affect some contractile systems and therefore must be taken into account. Future research must also ensure that catch in the living ABRMs and catch in skinned fibres are based on the same molecular mechanism.

In intact ABRMs, both alkalization elicited by methylamine and acidification caused by unlabelled DMO induced changes in stretch resistance of the ABRM during catch. Methylamine was used because the membrane permeability of methylamine is known to be slightly lower than that of DMO (Boron and Roos, 1976). Therefore, changes in pHi induced by methylamine could be monitored by the distribution of DMO. Catch was examined by the rate at which an ABRM loaded with 0.1 N was stretched ( $V_s$ ). When pHi was increased to  $7.54 \pm 0.05$  ( $N=8$ , DMO method), the relaxation rate was increased from  $0.05 \pm 0.03$  to  $0.16 \pm 0.04 \text{ mm min}^{-1}$  (3.2 times). A further rise in pHi to  $7.61 \pm 0.07$  increased the rate of relaxation 18-fold. Acidification (pHi  $7.28 \pm 0.04$ ) diminished the relaxation rate by 56%. Previous assumptions, drawn from skinned fibre experiments, that the maintenance of catch by a living ABRM depends on pHi (Johnson, 1959; Rüegg, 1971), have therefore been confirmed in this study. It should, however, be kept in mind that the pH-sensitive range of catch in living ABRMs is about 0.6–1.2 pH units above that of skinned fibres.

Serotonin ( $10^{-5} \text{ mol l}^{-1}$ ) induced a rapid relaxation of the ABRM, an increase in cyclic AMP levels from 3.2 to  $38.9 \text{ nmol g}^{-1}$  and a slow increase of pHi which reached its maximum of  $7.57 \pm 0.02$  ( $N=5$ , NMR method;  $7.56 \pm 0.05$ , DMO method) after 15 min. Serotonin also induced an alkalization in resting muscles. pHi remained at the higher level as long as serotonin was present. Alkalization, which was also elicited by the combined effect of forskolin and IBMX or by 8-bromo-cyclic AMP, proves that cyclic AMP acts as a second messenger of serotonin in terms of a modulation of pHi. The dose-response curves of serotonin show that a minimum concentration of  $10^{-6} \text{ mol l}^{-1}$  is necessary to induce significant effects on both cyclic AMP levels (Köhler and Lindl, 1980) and pHi (see Fig. 3). Much lower concentrations of serotonin ( $10^{-9} \text{ mol l}^{-1}$ ) induce significant effects on the maintenance of catch in living ABRMs (Twarog, 1954).

Application of cyclic AMP abolishes the catch-like state of skinned fibres (Cornelius, 1982; Pfitzer and Rüegg, 1982). If the release of catch *in vivo* depends on cyclic AMP, applications of either serotonin or 8-bromo-cyclic AMP to living muscles should have similar effects on catch. 8-bromo-cyclic AMP ( $5 \text{ mmol l}^{-1}$ ) induced an acceleration of  $V_s$  from  $0.05 \pm 0.02$  to  $0.18 \pm 0.09 \text{ mm min}^{-1}$  ( $N=7$ ). Serotonin, however, induced a significantly faster rate of relaxation than 8-bromo-cyclic AMP ( $5.0 \pm 1.8 \text{ mm min}^{-1}$ , 28 times). Striking similarities between the effects of 8-bromo-cyclic AMP and alkalization elicited by  $10 \text{ mmol l}^{-1}$  methylamine (see Fig. 1 and Table 3) suggest that the effect of 8-bromo-cyclic AMP on catch was elicited indirectly by an increase in pHi.

Application of dopamine ( $10^{-5} \text{ mol l}^{-1}$ ), another catch-releasing transmitter (Twarog and Cole, 1972), did not result in a significant increase of cyclic AMP levels (see Table 2; Ishikawa *et al.* 1981) or pHi (see Table 2). Electrical stimulation of an ABRM with rectangular pulses (Winton, 1937) during a tonic contraction abolished catch but did not increase cyclic AMP levels (see Table 2).

The application of serotonin may, therefore, activate two mechanisms that are triggered separately *in vivo*. A more serotonin-sensitive ( $10^{-9} \text{ mol l}^{-1}$ ; Twarog, 1954) mechanism causes the rapid relaxation of an ABRM during catch. Another, less serotonin-sensitive ( $10^{-6} \text{ mol l}^{-1}$ ) cyclic-AMP-dependent mechanism causes a rise in pHi, increasing the relaxation velocity of the ABRM during catch.

The dual effect of serotonin, triggered by different receptors, includes the involvement of further intracellular messengers. Serotonin ( $>10^{-9} \text{ mol l}^{-1}$ ) induces the release of  $\text{Ca}^{2+}$  from intracellular stores (Bloomquist and Curtis, 1975*a,b*). As the concentration of free  $\text{Ca}^{2+}$  remains constant after application of serotonin (Ishii *et al.* 1989), extrusion of  $\text{Ca}^{2+}$  from the intracellular space might be coupled with an equimolar release of  $\text{Ca}^{2+}$  from the contractile filaments, thus releasing catch. As serotonin does not affect the membrane potential (Hidaka *et al.* 1967), these  $\text{Ca}^{2+}$  movements might be triggered by an inositol lipid response (Berridge, 1989). The validity of these assumptions should be examined in the future.

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