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Research Paper

Effect of extracellular pH and Ca^{++} - concentration on the contractility and force generation as verified on *Vorticella* stalk concentration pattern

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Abstract: The prediction of protoosmotic model (as in actin-myosin system) has been applied to pattern of *Vorticella* stalk contraction for its verification. *Vorticella* stalk contraction is effected by the neutralization of negative charges at the end of *spasmin* by Ca^{++}. ATP hydrolysis is only required to sequester the cytosolic Ca^{++}. The effect of pHs in both acidic and alkaline ranges on the stalk contraction pattern was examined in combination with different Ca^{++} concentrations to characterise the contraction mechanism. By increasing H^{+} concentration the frequency of contraction decreases consistently. No change is found in duration of contraction. On returning to normal pH the same frequency of contraction is resumed. The effect of acidic pH thus is reversible. By lowering the H^{+} concentration (pH 8.0 and above) frequency of contraction becomes faster and the duration of contraction also improves compared to the duration at neutral pH. In triton–extracted preparation the contraction is found only at neutral pH for sometimes. No discernible change in contraction is found at changing pH. At both lower and higher pHs the change in Ca^{++} concentrations (from 1 mM to 4 mM) make pronounced effect. The results conformto other biological motile apparatus.

Keywords: Protoosmosis, *Vorticella*, *Spasmin*, frequency, duration.

INTRODUCTION

Vorticella stalk is a contractile device attached from one end with bell or zooid and other end fixed with substratum in extended trophont state. It contracts very rapidly pulling the body towards substratum in less than 10 ms. Stalk consists of a cylindrical sheath containing the contractile thread spasmoneme. The spasmoneme is a rod shaped proteinacious body embedded in the strand, runs helically like ribbon with its outer flat surface towards inside of sheath while its inner surface is covered with a layer of cytoplasm containing many spherical mitochondria. The spasmoneme lies eccentrically within cytoplasmic strand responsible for helical coiling or flexure of the stalk varying as per species of the

Vorticella. Longitudinal fibres lying along the inner surface of the sheath on the opposite side to the spasmoneme are called as *batonnets*. As stiffeners the *batonnets* ensure the helical bending of the sheath, rather than a mere collapse, when the spasmoneme contracts (Faure-Fremiet, 1905). A membranous structure near bundle of filaments encloses vesicles containing Ca^{++} similar to the Ca^{++} in SR of striated muscle. The spasmoneme of *Vorticella* with all organelles are activated rapidly by diffusion of released intratubular Ca^{++}. The tubules appear to form a close system, more similar to the SR than to the T-tubule of a striated muscle fibre. The spasmoneme contraction occurs due entirely to the spasmin molecule that may either change shape or alter its manner of bonding in the filament when Ca^{++} become bound to it (Amos et. al. 1975). There are no close similarities to muscles and large differences in composition are evident. Another clear difference is the positive birefringence with respect to the organelle which substantially reduces during contraction.

The spasmoneme acts as biological spring which stores energy in conformational changes through chemical bonds by converting some form of potential energy to kinetic form of mechanical motion. This acts as latches (Mahadevan and Matsudaira, 2000) on the basis of principles of hydrodymechanics as a result of viscous drag as per in Stoke's formula applied to polyelectric gel (Upadhyaya *et. al*.; 2008). The spasmin proteins are similar to other protein in their biochemical nature of Ca^{++} binding patterns by negatively charged ends neutralisation. Structurally as well as functionally spasmin proteins resemble with *centrin* by 32% and *calmodulin* by 25% in their folding patterns. The force generated for contraction in *spasmoneme* is in the order of a millidyne (103 dynes S^{-1}), whereas the power generated as in few milliergs per second (749 ergs $S^{-1}g^{-1}$).

The protoosmotic model predicts the generation of motive force through electroosmosis by the flow of protons (Singh and Amin, 1989) along the myofilaments responsible for contraction-extension cycle in muscles. Proteins usually carry excess of negative charge in their side groups hence such protein assemblies like F – actin remain surrounded by a layer of cations. The proton flux along the protein chain has to be compensated by the movement of ions in the surrounding as the counter-flux in the direction opposite to the movement of protons. This counter-flux of cations has a hydrodynamic effect. In the context of muscles the possibility of H^+ movement along myofibrils leads to a new way of explaining the energy transduction process. As we conjecture the protoosmosis in case of *Vorticella* stalk contraction is maintained at the level of surrounding medium which facilitates flow of protons along the spasmin proteins.

MATERIALS AND METHODS

Vorticellids are the sessile forms normally attached to the aquatic plants submerged in fresh water. The specimens were collected from the shallow stagnant ponds around Chapra (Bihar) city and were cultured in big jars in its natural conditions. The room temperature (20 to 25°C) is suitable for proper growth. It can also be grown in artificial medium. Polished rice with wheat (Frederick Page, 1981) and maize provide good medium for successful culture. The *Vorticella* settles on the maize kernel which slowly gets fully covered with many individuals in their cystic form. To obtain in large numbers it is desirable to make successive subcultures at an interval of few days it started emerging out in their trophont form slowly from their cystic form. Further the maize kernels were transferred from an

old culture (as old as five months) to fresh new medium which yields zooids in one to two days of intervals.

In another method tap water is boiled and filtered and then mixed with 0.1 mM of NaCl, KCl and $CaCl_2$. Boiling egg also provides good nutrition. Its solidified albumin with a little pinch (¼ gm) with yolk is grinded in a bowl contain adequate amount of water. This paste is mixed with filtered water and stirred for a few minutes and the artificial medium APW is now ready. After 2 days of incubation the new batch of *Vorticella* grows out. Glassware with high surface area to volume ratio is desirable to culture *Vorticella* as they require attachment surfaces. Only 50 mL of media is used for culture in 500 mL flask and only 100 mL in 1 L flask. The entire observations were made under Stereo-microscope (Leitz) with 10 X × 18 X magnifications. Other microscopes used were Magnus MS 24/13 and Olympus ch2ibimf. Photo-video-graphic camera [Nikon 12.1 150 3200 P/S/A/M (Coolpix)] was used for photography and video recording. The frequency and duration of stalk contractility of *Vorticella* under known conditions in solutions with different pHs and Ca^{++} concentrations were recorded.

The experimental Procedures

Before experimentation, the specimens of *Vorticella* were kept in standard controlled medium for 24 hours. The culture of *Vorticella* was used to be transferred into its sub-culture after every 2 days. For different Ca^{++} concentrations 1mM, 2mM, 3mM, 4mM and 5mM of pCa were used. The detergent (Triton-X)-extracted specimens were prepared (Naitoh and Kaneko, 1972) under controlled bathing media (pH 7.0) of different Ca^{++} concentrations. The washed specimens of *Vorticella* were kept immersed for approximately 1 minute into the solution containing Triton X-100 in combination with: 20 $mmoll^{-1}$KCl, 10 $mmoll^{-1}$ EDTA and 10 $mmoll^{-1}$Tris-maleate buffer (pH 7.0). The extracted specimens were then washed three times with a washing medium composed of 50 $mmoll^{-1}$KCl, 2 $mmoll^{-1}$ EDTA and 10 $mmoll^{-1}$Tris-maleate buffers (pH 7.0) and it was kept for 15 minutes for the extraction of Triton X-100. The washed extracted specimens were kept immersed into the reactivation media of different Ca^{++} concentration (from 1mM to 5 mM). The reactivation media were consisted of 50 $mmoll^{-1}$ KCl, 10 $mmoll^{-1}$ Tris-maleate buffer (pH 7.0) and Ca^{++} buffer solutions.

The experiments were performed at room temperature (20-25°C) to observe the complete contraction-extension cycles. The lengths of stalks of many *Vorticellids* were measured by linear distances from the base of the stalks i.e. from their attachment point with substratum to the bell/zooids. The length of stalk after contraction under the influence of different Ca^{++} concentrations represents configurationally changes, known as fractional stalk length. The fractional stalk length (L^*) was expressed in terms of Hill's parameter (Ochiai *et. al*., 1979).

Statistical calculations

Data (i.e. video recording) transferred to the computer was recorded on paper then it was bio-statistically measured by using the formula of mean and standard deviation. The data obtained were represented graphically.

RESULTS

Effect of pH on the contraction pattern
On living specimen of *Vorticella*

Change in pH of the medium is known to affect the biochemical/ physiological performances of the organism by changing the release of Ca^{++} at SR level either in the presence or absence of EDTA. To evaluate the effects of different pH media it was first tried to establish the effect of different pHs alone as a control experiment. Specimens before experimentation were first incubated

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at pH 7.0 and hence as controlled conditions. After administering low pH (6.0) consistently the frequency of contraction was found to decrease but the duration showed no much significant changes. Decreasing pH from 7.4 to 6.2 resulted in three modifications: (1) decrease in the threshold value of free Ca^{++} concentration below 8.9×10^{-8}mol required for contracture of the stalk, (2) a shift to the right of the force-pCa curve with a logarithmic increase in Ca^{++} concentration and it reaches at 100% at about 1.9×10^{-6}mol of Ca^{++} and (3) a decrease in the maximum tension obtained at higher pCa 1.9×10^{-5} mol. The concentration of Ca^{++} of about 1×10^{-7}mol corresponded to 50% contraction of the bell and the stalk.

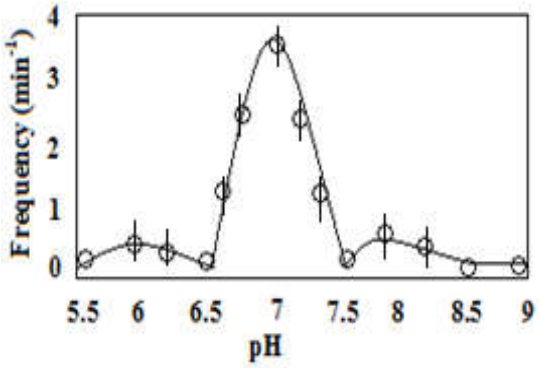

Figure 1: Effect of pH on contraction pattern of living specimen in *Vorticella* representing average frequencies during spontaneous contractility.

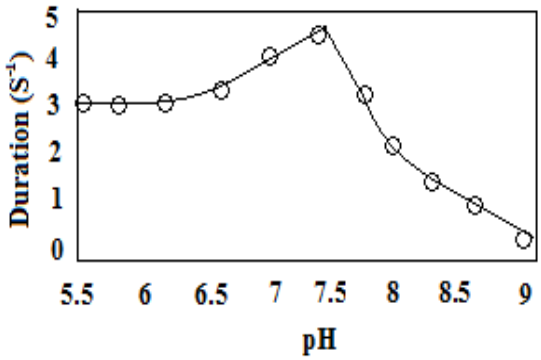

Figure 2: Effect of pH on contraction pattern of living specimen in *Vorticella* representing average duration during spontaneous contractility.

The shrinkage of the cell body is caused by Ca^{++} mediated contraction of the myoneme and the coiling of the stalk is caused by Ca^{++} mediated contraction of the spasmoneme. But when the pH of bathing medium was returned to neutral it again resumed the same frequency and duration as earlier. Thus it is assumed that the effect of acidic pH is reversible. When subjected to higher pH i.e. 8.0 and above the frequency of contraction was faster and the duration also improved compared to the neutral pH even. These effects of different pHs on the same or different specimens were almost identical and reproducible. These experiments were also conducted in the Ca^{++}-free external medium containing EDTA hence no interference of the external Ca^{++} could be presumed with the contractile process.

On Triton-extracted specimen

In fact in Triton-extracted *Vorticella* the contraction could be exhibited at neutral pH for sometimes because the perforated cell membrane allowed the contents of the external solution directly reach the interior of the cell. But the change in pH showed no effect on contraction profile.

Effect of different Ca^{++} concentrations in combination with different EDTA concentrations and pHs

On the living specimen

At neutral pH the different concentrations of Ca^{++} shows no effect upon the contraction frequency but slight change could be found at 5 mM Ca^{++} concentration. The effect of varying pHs on the relation with free Ca^{++} concentrations were studied in vitro experimentation in the presence of 4 mM of total EDTA. The level of plateau was not significantly different at any given pCa and pHs either at 4 mM EDTA or 10 mM of it. The tension developed was slower in the presence of 4 mM of EDTA than the 10 mM

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of it at pH 6.2 and free Ca^{++} concentration lower than 6. But, when the pH was changed to lower side (acidic side) the change in Ca^{++} in the medium showed apparent change. At pH 5.5 the effect was more pronounced and almost similar change was observed at Ca^{++} concentrations ranging from 1 mM to 4 mM.

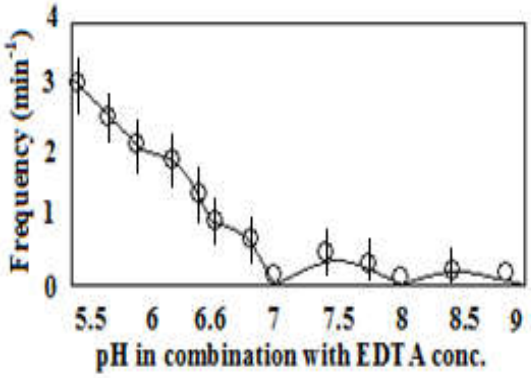

Figure 3: Effect of Ca^{++} concentration on contraction pattern of living specimen in *Vorticella* in combination with different EDTA concentrations at different pHs representing average frequencies during spontaneous contractility.

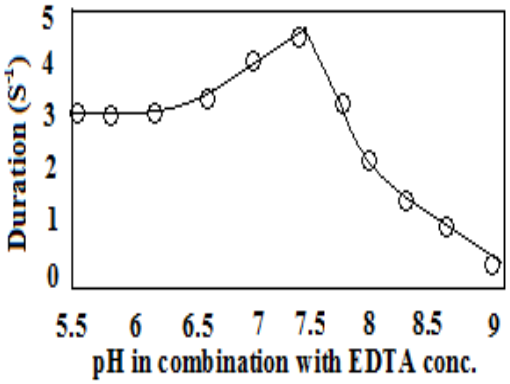

Figure 4: Effect of Ca^{++} concentration on contraction pattern of living specimen in *Vorticella* in combination with different EDTA concentrations at different pHs representing average duration during spontaneous contractility.

This shows the external Ca^{++} concentration matters little in terms of evoking change in frequency and duration of contraction of both stalk and the bell. But, the early and apparent response at lower pH reminds of the higher H^{+} concentration causing change in membrane potential of ER membrane affecting its permeability and consequently release of intracellular Ca^{++} into the cytosol which promotes contractility.

Table 1: Ca^{++}-ion induced contractility of *Vorticella* stalk in µm/s represented by mean with standard deviation where N=5:

pH	1 mM	2 mM	3 mM	4 mM	5 mM
5.5	06.00±0.60	05.00±0.22	04.00±0.05	03.00±0.00	02.00±.0.00
5.6	05.80±0.08	05.00±0.22	04.00±0.05	03.00±0.00	02.00±.0.00
5.7	05.60±0.08	04.90±0.22	03.90±0.05	02.90±0.04	01.90±.0.00
5.8	05.40±0.08	04.70±0.67	03.80±0.05	02.70±0.00	01.80±.0.00
5.9	05.20±0.09	04.50±0.19	03.70±0.05	02.50±0.00	01.70±.0.00
6.0	05.00±0.08	04.30±0.18	03.60±0.05	02.30±.0.00	01.60±.0.00
6.1	10.00±0.08	05.00±0.22	04.00±0.05	03.00±.0.00	02.00±.0.00
6.2	15.00±0.08	10.00±1.48	05.00±0.05	06.00±.0.00	04.00±.0.00
6.3	20.00±0.08	15.00±1.50	10.00±0.05	09.00±.0.00	08.00±.0.00
6.4	25.00±0.08	20.00±1.50	15.00±0.05	12.00±.0.00	09.00±.0.00
6.5	30.00±0.08	25.00±1.49	20.00±0.05	15.00±.0.00	10.00±.0.00
6.6	25.00±1.25	20.00±1.48	15.00±0.05	12.00±.0.00	08.00±.0.00
6.7	15.00±0.08	15.00±1.48	10.00±0.05	09.00±.0.00	06.00±.0.00

6.8	10.00±0.08	10.00±1.40	05.00±0.05	06.00±.0.00	04.00±.0.00
6.9	05.00±0.08	05.00±1.49	01.00±0.04	03.00±.0.00	02.00±.0.00
7.0	00.00±0.00	00.00±0.00	00.00±0.00	00.00±.0.00	00.00±.0.00
7.1	02.00±0.08	02.00±0.22	01.00±0.04	01.00±.0.00	01.40±.0.00
7.2	04.00±0.08	04.00±0.80	02.00±0.05	02.00±.0.00	01.80±.0.00
7.3	06.00±0.06	06.00±0.87	03.00±0.05	02.50±.0.00	02.20±.0.00
7.4	08.00±0.08	08.00±0.86	04.00±0.05	03.00±.0.00	02.40±.0.00
7.5	10.00±0.08	08.60±0.61	06.60±0.05	04.60±.0.00	02.60±.0.00
7.6	09.00±0.08	07.50±0.61	06.00±0.05	04.40±.0.00	02.60±.0.00
7.7	08.00±0.08	07.00±0.22	05.50±0.23	04.20±.0.00	02.40±.0.00
7.8	07.00±0.08	06.50±0.22	05.00±0.05	03.90±.0.00	02.40±.0.00
7.9	06.60±0.08	06.00±0.22	04.60±0.05	03.60±.0.00	02.30±.0.00
8.0	06.30±1.21	05.30±0.22	04.30±0.05	03.30±.0.00	02.30±.0.00
8.1	06.40±0.04	05.40±0.22	04.30±0.05	03.30±.0.00	02.30±.0.00
8.2	06.50±0.08	05.50±0.22	04.20±0.05	03.10±.0.00	02.20±.0.00
8.3	06.60±0.08	05.60±0.26	04.20±0.05	02.80±.0.00	02.10±.0.00
8.4	06.80±0.08	05.80±0.23	04.10±0.05	02.40±.0.00	01.80±.0.00
8.5	07.00±0.08	06.00±0.23	04.00±0.05	02.00±.0.00	01.40±.0.00
8.6	06.00±0.60	05.00±0.28	03.50±0.05	01.80±.0.00	00.00±.0.00
8.7	05.00±0.60	04.00±0.26	03.00±0.05	01.60±.0.00	00.00±.0.00
8.8	04.00±0.08	03.00±0.00	02.60±0.05	01.40±.0.00	00.00±.0.00
8.9	02.00±0.08	02.00±0.00	02.00±0.05	01.20±.0.02	00.00±.0.00
9.0	00.00±0.00	00.00±0.00	00.00±0.00	00.00±.0.00	00.00±.0.00

DISCUSSIONS

In our experiments the variation of frequency and durations of contraction were minimized due to the variables other than pH, like Ca^{++} concentrations. pH induced variation of Ca^{++} sensitivity was of particular concern at acidic pH where the effect was most likely to occur. Proton concentration was expected to vary throughout the stalk length as it is involved in ATPase activity and the proton is generated in hydrolysis of ATP. Protons are also transported from the fibres (*spasmoneme*) at steady state by diffusion from the bathing medium. Almost all studies on other systems show that the frequency of contraction declines with acidification except as in barnacle muscle fibres (Ashley and Moisescu, 1974). Decrease in pH reduces the opening probability of ER-Ca^{++} release channels but other findings suggest that reduction in pH causes an increase in cytosolic free Ca^{++}. Changes in pH and Ca^{++} concentrations are capable of loading the ER. Thus we can say that *Vorticella* stalk contraction is energy compensation process as needs no ATP hydrolysis at the level of spasmoneme which is rather energised by proton flow along protic cable of spasmin protein. The pattern of current flow along the contractile proteins is similar to the flow of electrons in conductor wire and it alters the diffusivity of Ca^{++} as resistance in wire does in artificial system and thus affects the rate of contraction-extension cycle. The kinetics of protein folding (spasmoneme of the stalk) governed by the second law of thermodynamics (Lin, G. N. *et. al*., 2010) is greatly affected by the flow of protons

which regulate Ca^{++}-flux along the *spamin* protein.

The experiments on triton X-100 treated specimen activated by external Ca^{++} imply that the primary site where protons act and reduce the contraction frequency and duration in the spasmin-Ca^{++} binding process rather than on the release of Ca^{++} i.e. from the ER. This is similar to (Chase and Kushmerick, 1988) intact muscle fibre that proton induced shift of force–pCa relation provides a mechanism which also affect the twitch.

Ca^{++} evokes (Cheng *et. al*., 1993) elementary calcium-release units (Horne and Mayer, 1997) or ryanodine receptor of the stalk to become more pronouncedly charged by acidic media than the alkaline by regulating the ionic flux in both directions of the stalk length.

The putative release of Ca^{++} during Ca^{++} overload in ER serves as a site for initiation and propagation of Ca^{++} waves along the spasmin proteins by affecting terminal domains and motifs. The Ca^{++} addition neutralizes negatively charged amino acid (tyrosine) moiety of spasmin proteins causing them to collapse in sweeping pattern. In this case in the absence of Ca^{++} in extended state, force of attraction and the force of repulsion between the spasmoneme and *batonnets* are maintained. When Ca^{++} comes in between them the state of force of attraction and repulsion is upset. With the increase of force of attraction the force of repulsion also increases to maintain their conformational equilibrium as per the law of energy and force. The increased centripetal forces on batonnets cause spasmoneme to bend and swell in helical form by the action of Ca^{++}. These forces around spasmoneme and battonets cause the randomly distributed spasmin proteins to contract in criss-cross bending pattern around the centralized Ca^{++}.

Protons accelerate reactions by reducing the free energy of relaxation (ΔG). This may be interpreted in terms of transition state theory which states that reactants proton (P) and spasmin (S) form the proton-spasmin (P-S) complex and passes through activated transition state (P-S). Here certain amount of activation energy (ΔG^{o}) is transduced. When proper H^{+} concentration is present less energy is required for activation ($\Delta G^{o'}$). In other words we can say that the P-S complex creates a new reaction path with lower energy.

On the basis of energy transduction principle we can assume an alternate equation for protoosmosis in terms of Hill's parametric equation where $[Ca^{++}]$ can be replaced by $[H^{+}]$ as:

$$P^{*} = \frac{[S]}{[S] + [S.H^{+}]} = \frac{1}{1 + ([H^{+}]/K_{m})^{n}}$$

Here P* indicates the protoosmotic parameter for the fractional stalk length dependent on energy expenditure in overall contraction-extension cycles. This is independent of Ca^{++} sequestration throughout the process which requires ATP hydrolysis for its sequestration at the level of ER. Here K_m indicates association constant instead of dissociation constant and $[H^{+}]$ & [S] indicate concentrations of H^{+}& S respectively.

In the surrounding buffer system, protons act actively below the isoelectric point (pI) value of 7. To change the pI value by permeability detergent affects the membrane by altering colligative properties of the lipid molecules in the medium and thus generates motive force continuously to facilitate the flux of protons within it in a cable form connecting up on the membrane and affecting its membrane potential. The concentrations of these protons directly affect the zwitterion characteristic of amino acid residues of spasmin proteins and energize their active terminal domains responsible of contraction-extension cycles.

Conclusion: Thus we can say that the change in pI of the surrounding medium directly affects the contraction-extension cycles of *Vorticella* stalk more positively in favour of protoosmotic model than other model systems. This results change in the colligative properties of the surrounding medium of spasmin protein. By further investigation it could be established whether the H^+ movement process could be capitalized as the basic machinery to energize the biological contractile systems as an alternative. This property of H^+ movement affecting the dynamics of Ca^{++} binding to the Ca^{++} binding proteins along actin (as with spasmin in *Vorticella* stalk) may help in regulating the muscle contraction processes in the muscular dystrophy or atrophy diseases. The spasmin like artificial system may be generated in the affected muscles to take advantages of H^+-Ca^{++} conducting cables.

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