Arsenazo III Ca^{2+} -Transients of *Xenopus* Skeletal Muscle during Repetitive Stimulation in Hypertonic Solution

Kazunori OCHI and Moto MATSUMURA

Department of Physiology, Kawasaki Medical School,
Kurashiki, 701-01 Japan

Summary The arsenazo III Ca^{2+} -transients (AZ signals) were measured in hypertonic solution. The muscle fiber of *Xenopus* was stimulated at the frequency of 0.1/s. The AZ signal responding to the first stimulus in hypertonic Ringer solution was nearly similar to that in normal Ringer solution. After 8 repetitive stimuli, however, time to peak was increased to 3 times and decay time constant was up to 4 times as long as the control. The apparent inhibition of Ca^{2+} uptake into sarcoplasmic reticulum was supposed to be the origin of the prolongation in time to peak and decay time course.

Key words: E-C coupling, arsenazo III, hypertonic solution.

The intracellular Ca^{2+} concentration of the muscle fiber following an electrical stimulation is now measured with the Ca^{2+} -sensitive dyes, one of which is arsenazo III (AZ). It has been well known that the muscle fibers exposed to hypertonic Ringer solution are inhibited in regard to initiating twitch, although the action potential takes place as it does in normal Ringer solution (HODGKIN and HOROWICZ, 1957). TAYLOR *et al.* (1975) reported that the aequorin Ca^{2+} -transients were prolonged in hypertonic solution without noticeable change in peak amplitude. The present work was performed to explain how the Ca^{2+} -transients were influenced by exposure to hypertonic solution, especially during repetitive stimulation at the low rate.

A single layer of muscle fibers dissected out from the thoracic muscle (m. sternoradialis) of *Xenopus* was placed horizontally in the muscle chamber containing normal or hypertonic Ringer solution. Both ends were connected to micrometers by stainless steel hooks. The muscle was so stretched that the movement might be completely abolished, where the sarcomere length was 3.8–4.0 μm . The AZ (Sigma) was dissolved in a solution of 10 mM KHCO_3 . Hypertonic Ringer solution (NaCl 118, KCl 2.5, CaCl_2 1.8, sucrose 240, HEPES 2.5 mM, and pH 7.2)

was prepared by adding sucrose to normal Ringer solution. Osmolality of this hypertonic solution was about 475 mOsm/kg H₂O and about 2.1 times that of normal Ringer solution, if calculated by the equation of DYDYŃSKA and WILKIE (1963). After 20 min, AZ was injected iontophoretically into the muscle fiber through a glass microelectrode so that the final intracellular AZ concentration might be about 1 mM. This electrode was also used for the intracellular stimulation. The AZ signal was detected by a pair of photomultipliers (Hamamatsu Photonics, R 1463) as the difference of transmitted light intensity at 658 and 723 nm. The AZ signal was held by PCM data recording system (NF Electronic Inst., RP-880) and then displayed on the chart recorder (NEC San-ei Inst., 8K-31) through the transient memory (Kawasaki Electronica, TM-1410). If necessary, the averager (Kawasaki Electronica, TMC-300) was used. The AZ signals were expressed in terms of absorbance change ΔA (OCHI, 1984).

In normal Ringer solution, the AZ signal rose rapidly after 1.5 ms latency, attained the peak and then fell in an approximately exponential manner (Fig. 1). Upon repetitive stimulation at frequency of 0.1/s, the peak of the AZ signal gradually decreased. The cause of this phenomenon may be the effect of preceding stimulation on Ca²⁺ release from the sarcoplasmic reticulum, SR (BLINKS *et al.*, 1978). Time to peak of the AZ signal to the 33rd-34th stimulation was 5 ms, while that to the 1st-2nd stimulation was 4.5 ms. Decay time constant for the 33rd-34th AZ signal was 40 ms compared with 25 ms for the 1st-2nd signal. Namely, the time course was only slightly prolonged after 34 stimulations. As to the AZ signal responding to the first stimulating pulse in hypertonic Ringer solution, these time parameters were slightly prolonged. The time to peak was 9 ms and the decay time constant was 28 ms. The most striking feature of the AZ signal in hypertonic Ringer solution was the marked prolongation of both rising and falling phases during repetitive stimulation (Fig. 2). Although the maximum amplitude of the 8th AZ signal decreased to only 84% of the first one, the time to peak of the 8th AZ signal was prolonged from 9 to 27 ms and the decay time constant was increased from 28

Fig. 1. AZ signals evoked by stimulation at 0.1/s in normal Ringer solution. a, b, and c are the averaged responses of the 1st-2nd, 16th-17th, and 33rd-34th signals. Calibration of the signal is indicated by ΔA measured in trace a. 23.6°C.

Fig. 2. A: AZ signals evoked by stimulation at 0.1/s in hypertonic Ringer solution. a, b, c, d, e, f, g, and h indicate the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th signals. Calibration of the signal is indicated by ΔA measured in trace a. 22.4°C. B: falling phase of AZ signals in hypertonic solution. The peak amplitude of every signal is taken as 100%, and the time is taken from the peak of the signal.

Fig. 3. Changes of 3 parameters in AZ signals responding to the stimulation at the rate of 0.1/s. Squares, triangles, and circles indicate the peak response size, decay time constant, and time to peak. Measurements in normal Ringer solution are shown by closed symbols and those in hypertonic solution by open symbols.

to 113 ms (Fig. 3). Latency of the AZ signal in hypertonic Ringer solution was 5 ms, which was about 3 times as long as the one in normal Ringer solution.

According to BLINKS *et al.* (1978), the aequorin Ca^{2+} -transients signal depended on the stimulus frequency between 0.003 and 3/s (15°C), where each twitch tension was not summed but separated. They suggested that the descending staircase of aequorin signal reflected the progressive decrease in Ca^{2+} release. Our result obtained in normal Ringer solution indicated about 15% decrease after 33–34 stimulation and was qualitatively consistent with their result.

Several possibilities explaining the dissociation of contraction from excitation in hypertonic Ringer solution have been pointed out. They are structural changes in T-tubule and SR (DYDYŃSKA and WILKIE, 1963), existence of the activation heat and deterioration of contractile proteins (HOWARTH, 1958), water loss and resulting volume decrease (CAPUTO, 1968) and the inhibition of myofibrillar ATPase activity (PODOLSKY and SUGI, 1967). All of these explanations indicate that the contraction failure is caused not by the process leading to Ca^{2+} release but by the contractile proteins themselves. Therefore, it is expected that Ca^{2+} transients signal will not be much affected by exposure of the muscle fiber to hypertonic solution. This is valid in regard to the first AZ signal. The decrease in peak by 10–20% was observed after 20 min immersion in hypertonic solution, but if the diffusion of the injected dye was considered (BAYLOR *et al.*, 1982) the AZ signal would be increased in hypertonic solution. PALADE and VERGARA (1982) also demonstrated that the AZ signal of cut fiber under voltage control was not significantly affected by exposure to hypertonic solution, although TAYLOR *et al.* (1975) showed clearer prolongation of aequorin signal than our result wherein the time to peak and decay time constant of the 1st–2nd AZ signal are increased from 4.5 to 9 ms and from 25 to 28 ms. It is uncertain but probable that the increase in ionic strength or in free Mg ions concentration is related to the amplitude and time course. Even if the changes in ionic environment would affect the AZ signal, they would not explain further changes during repetitive stimulation.

The most remarkable changes in hypertonic Ringer solution are observed after repetitive stimulations. They are delayed onset and progressive slowing of the time courses both in rising and in falling phases. The underlying mechanism is uncertain but several explanations are possible. 1) The charge movement or the Ca^{2+} release mechanism is easily inactivated in hypertonic Ringer solution and is not fully recovered within the interval of 10 s. 2) ENDO (1977) suggested that swelling of SR induced Ca^{2+} release accompanied by SR membrane depolarization in intact fiber exposed to hypertonic solution. As the result of the maintained Ca^{2+} release from SR at the resting state in hypertonic Ringer solution, the amount of Ca within SR is partly consumed or SR membrane is more or less inactivated. This state becomes progressively dominant during repetitive stimulation, resulting in a slow rising phase of the 8th AZ signal. 3) Ca ions released in hypertonic solution are in the forms of Ca-troponin and Ca-parvalbumin. These proteins, in addition to rested-state Ca bind further Ca^{2+} released following stimulation. During relaxation, Ca^{2+}

is dissociated from troponin and parvalbumin to form AZ-Ca complex. This corresponds to the falling phase of the AZ signal. If rested-state Ca bound to parvalbumin is increased in hypertonic solution and activated-state Ca is added to it, Ca dissociation from parvalbumin during relaxation takes a longer time and the falling phase of the AZ signal is prolonged. 4) The falling phase is mainly determined by Ca²⁺ uptake into SR (MILEDI *et al.*, 1977). During exposure time to hypertonic Ringer solution, the number of Ca²⁺-binding sites of SR will have been decreased and they become progressively saturated by each stimulating pulse. In fact, the delay of the falling phase is more remarkable at the stimulation rate of 1/s, although not investigated precisely. 5) The amplitude of the recorded AZ signal in hypertonic solution is a little smaller than the control. One possibility is the Ca²⁺ release from SR is reduced because of the decrease in concentration gradient between the inside of the SR and the myoplasm. These explanations are speculative. But the apparent inhibition of Ca²⁺ uptake of SR due to swelling will play a role in prolongation of the falling phase during repetitive stimulation.

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