Sensitivity of the Na Efflux in Barnacle Muscle Fibres to the Microinjection of Troponin-C

ASHLEY[1] reported that internal application of EGTA (ethylene glycol bis (β-aminoethyl ether)-N, N'-tetra-acetate) suppressed the contractile response of barnacle muscles fibres to caffeine. This has been confirmed by us but in addition we have not infrequently found a rise or a fall in the Na efflux following microinjection of 100 mM-EGTA. In view of this fact and the possibility that barnacle muscle fibres may lack troponin, it seemed of interest to find out whether or not the behavior of the Na efflux toward this Ca^{2+}-binding protein would be different from that observed with EGTA. The purpose of this paper is to show that the Na efflux is somewhat stimulated and not inhibited by microinjecting troponin-C (TN-C) and that this action does not interfere with the action of external acidification on the Na efflux.

For the measurement of the Na efflux, single muscle fibres from the barnacle, *Balanus nubilus* (and *B. aquila*) were cannulated and then loaded with ^{22}Na by microinjection. The microinjector used was similar to that devised by HODGKIN and KEYNES[2] as modified by CALDWELL and WALSTER[3]. The composition of the artificial sea water (ASW) used was that given by BITTAR and TONG[4]. The methods of measuring ^{22}Na in the washout specimens and the muscle fibres were those described by BITTAR[5] and BITTAR, CALDWELL and LOWE[6]. All experiments were done between 22 and 24 °C. The troponin-C used was obtained from rabbit muscle. This became necessary because preliminary chemical studies carried out on barnacle muscle gave no evidence for the existence of a protein fraction, which by gel electrophoresis could be identified as troponin-C. Rabbit muscle TN-C having a molecular weight of 18,000 daltons was isolated by the method of GREASER and GERGELY[7]. Two TN-C solutions (in water), one 3×10^{-5} M and the other 5×10^{-4} M, the maximal possible using water as solvent, were prepared for microinjection. Myosin also obtained from rabbit muscle, was used in a concentration of 3×10^{-5} M (in 0.4 M – KCl) in control experiments.

In the first group of experiments, 3×10^{-5} M TN-C was microinjected. This resulted in a 18.5 $\pm$ 3% (S.E. of mean) rise in the Na efflux ($n = 10$). A typical experiment is recorded in Figure 1. In the next group of experiments, 3×10^{-5} M myosin was microinjected. The Na efflux showed a prompt rise, the magnitude of which averaged 20.8 $\pm$ 2.3% ($n = 6$). Since this effect could have been due to the KCl used as solvent, more control experiments were done. Microinjection of 0.4 M – KCl was found to cause a transient rise in the Na efflux (see Figure 1). The magnitude of this effect averaged 16.9 $\pm$ 2.7% ($n = 6$).

In order to be certain that the observed effect of TN-C is genuine, a higher concentration of the protein was microinjected. The results obtained with 5×10^{-4} M TN-C indicated a rise in the Na efflux, averaging 37.1 $\pm$ 6.6% ($n = 8$), which is twice the magnitude of that found with a 3×10^{-5} M solution. To avoid or minimize possible intrafiber binding of TN-C, two attempts were made to inject the protein twice, first at the start of the experiment, and then some 40 min following loading of the

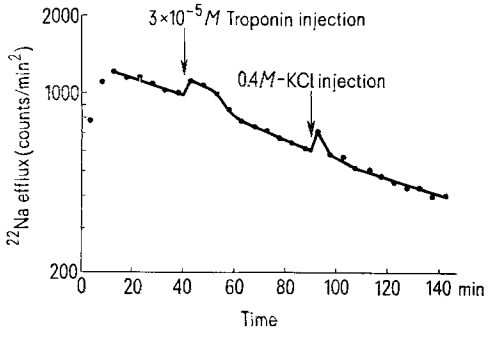

Fig. 1. Effects on the Na efflux of internal application of $3 \times 10^{-5}M$ TN-C, followed by 0.4 M-KCl (semiliog plot).

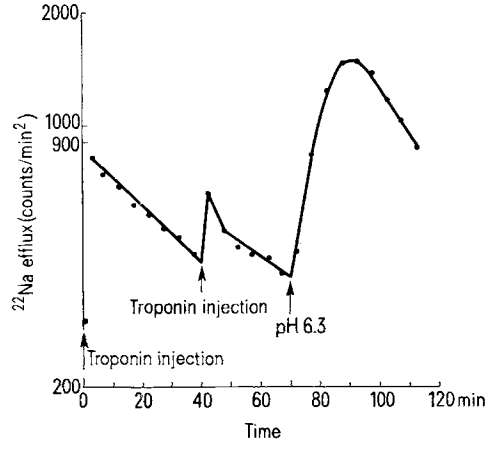

Fig. 2. Effect on the Na efflux of double microinjection of $5 \times 10^{-4}M$ TN-C, followed by external acidification. The first injection was carried out shortly before loading the fiber with Na-22.

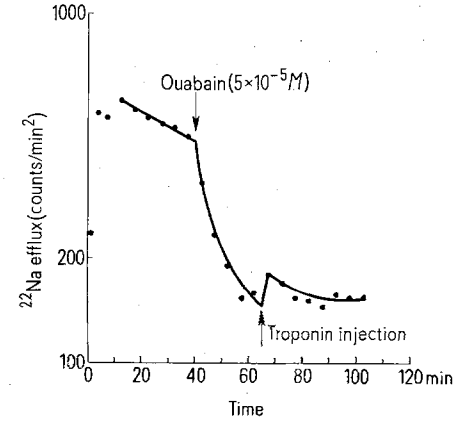

Fig. 3. Effect of internal application of $5 \times 10^{-4}M$ TN-C on the ouabain-insensitive Na efflux.

[1] C. C. ASHLEY, Am. Zool. 7, 647 (1967).
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[7] M. L. GREASER and J. GERGELY, J. biol. Chem. *246*, 4226 (1971).

fiber with radiosodium. The result of such an experiment, recorded in Figure 2, shows a 59.5% rise in the Na efflux. This value turned out to be almost thrice that obtained with a control fibre involving a single injection of TN-C. In the second experiment, however, the Na efflux showed a 29.6% rise, a value which was not very different from the control value of 20.5%. Figure 2, in addition, provides evidence indicating that double injection of TN-C does not interfere with the sensitivity of the fibre to external acidification. It is, however, worth remembering that since the affinity constant of TN-C for Ca^{2+} is pH-dependent[8], it is not unlikely that some Ca^{2+} bound to the protein had been liberated into the myoplasm following injection. In other experiments 5×10^{-4} TN-C was injected only once, followed by lowering of the external pH from 7.8 to 6.3. The results obtained in 4 experiments showed a $44.2 \pm 13\%$ rise in the Na efflux, and that all fibres were markedly sensitive to external acidification.

The next question coming to mind was whether the effect caused by TN-C involved the ouabain-sensitive or the ouabain-insensitive Na efflux. As shown in Figure 3, external application of 5×10^{-5} *M* ouabain caused a large fall in the Na efflux, while internal application of 5×10^{-4} *M* TN-C caused a small rise in the remaining efflux. The magnitude of the stimulation averaged $23.3 \pm 1.8\%$ ($n = 5$). This result was not wholly in accordance with expectation. This is because the effect of TN-C on the Na efflux in unpoisoned fibres was thought to be due to reduced suppression of the Na^+-K^+ ATPase as the result of reduced $[Ca\ ATP]^{-2}$ formation.

It seems, then, as if TN-C has the ability to stimulate the Na efflux in barnacle fibres. Although the mechanism of this stimulation remains far from clear, there are reasons for supposing that it resembles the mechanism underlying the stimulation obtained by injecting low concentrations of EGTA (BITTAR and SCHULTZ, unpublished data). Since TN-C has a high affinity only for Ca^{2+}, it is tempting to speculate that both TN-C and EGTA, when used in low concentrations, stimulate the Na efflux as the result of removing Ca^{2+} from the myoplasm. Whether EGTA, when applied in high concentrations, inhibits the Na efflux by removing Mg^{2+} from the vicinity of the transport sites, is a question which forms the subject of experiments already in progress.

Zusammenfassung. Nachweis, dass eine Microinjektion von Troponin-C den Na-Ionen-Ausfluss aus Einzelfasern des Entenmuschel-Muskels steigert.

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9 July 1973.

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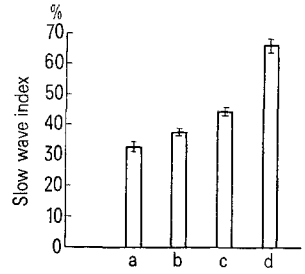
Electroencephalographic Studies in Toad (*Bufo melanostictus*) Following Prolonged Exposure to Heat During Hibernation and Non-Hibernation

Electroencephalographic (EEG) studies have been done exhaustively in hibernating mammals but the studies of the same in hibernating poikilothermic animals is inadequate. EEG studies during hibernation and arousal have been made by CHATFIELD et al.[1] and CHATFIELD and LYMAN[2]. DE, BORAL, DEY and DEB[3] found marked variation in the electrical activities of brain in hibernating toads over the non-hibernating ones. The principal feature observed during hibernation was slowing of the brain potential, which, however, could be replaced by low voltage fast activity (LVFA) following sensory activation.

It has been observed (unpublished) that hibernating toads can withstand an exposure of 56°–58 °C for 8 days, while the non-hibernating animals can tolerate up to 48 °C only for 8 days. This fact aroused the curiosity of the authors to know the electroencephalographic response to this variance in heat tolerance according to season.



Slow wave indices of brain in toads: a, non-hibernation exposed group; b, non-hibernation control group; c, hibernation control group; d, hibernation exposed group.

Materials. Male toads (*Bufo melanostictus*) weighing 45–65 g were taken during hibernation (May to August). These toads were placed in an incubator set at 56 °–58 °C for 8 days with the supply of water being maintained. The rectal temperature of the hibernating toads was 19 °–21 °C, which after the exposure rose to 30 °–33 °C. The rectal temperature of the non-hibernating toads was 29 °–31 °C. After an exposure to 48 °C for a period of 8 days, the rectal temperature was found to vary from 33 °–35 °C.

Methods. After 8 days exposure the animals were taken out of the incubator one by one and made spinal by inserting steel probe downwards through the vertebral column. Steel needle electrodes were fixed on the scalp at 4 points over the right and left cerebral hemispheres and connected to the machine. Electrographic recordings of the brain potential was taken by a Grass Model III-D, 8-channel Electroencephalograph with ink writing pens. The paper was run at a speed of 30 mm/sec.

Brain waves with a frequency of 12 c/sec or less have been designated as slow waves and those ranging above 12 c/sec are designated as fast waves. The slow wave index has been calculated according to the method of

[1] P. O. CHATFIELD, C. P. LYMAN and D. P. PURPURA, Electroenceph. clin. Neurophysiol. *3*, 225 (1951).
[2] P. O. CHATFIELD and C. P. LYMAN, Electroenceph. clin. Neurophysiol. *6*, 403 (1954).
[3] P. K. DEY, M. C. BORAL, C. D. DEY and C. DEB, J. exp. Med. Sci., India *7*, 28 (1963).