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Restoration of Membrane Excitability of Squid Giant Axons by Reagents Activating Tyrosine-Tubulin Ligase

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Summary. Using squid giant axon, an experimental survey was performed on restoration of the membrane excitability which had been partially suppressed. Among reagents examined, a combination of 400 mM KF, 50 μM tyrosine, 1 mM ATP, 1 mM Mg ions and 5 μM cAMP was found to induce the restoration of the excitability to a large extent. Further addition of a small amount of either porcine brain microtubule proteins or the squid axoplasm was found to support complete restoration. The experiments suggest that tubulin-tyrosine ligase contained in the porcine brain microtubule protein fraction or the squid axoplasm maintains the coupling between cytoskeletal structures and the plasma membrane.

In the preceding paper, it was suggested that the cytoskeletal constituents composed of microtubules are associated with the internal surface of the plasma membrane of squid giant axons and that they play a role in the regulation of the membrane excitability. The establishment of experimental conditions restoring the excitablility seems to be requisite for obtaining a clue for investigation of the coupling between microtubules and the plasma membrane.

This paper presents some evidence for restoration of the membrane excitability of squid giant axon by the activation system for tubulin-tyrosine ligase.

Materials and Methods

Intracellular Perfusion of Squid Giant Axons

Giant axons of squid (*Doryteuthis bleekeri*), the method of their preparation for electrophysiological experiments, and the procedures of the electrophysiological experiments

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were the same as those described in the preceding paper (Matsumoto & Sakai, 1979). The standard perfusion medium contained 360 mM KF, 40 mM K phosphate (pH 7.25) and 4 vol% glycerol. All the electrophysiological experiments were carried out under room temperature (15–20 °C).

Preparation of Microtubule Proteins from Porcine Brains and Squid Axons

Porcine and squid microtubule proteins were prepared by one cycle of temperature-dependent polymerization and depolymerization using the same procedures as described in our previous paper (Sakai & Matsumoto, 1978). They were stored at −80 °C in a glutamate-reassembly buffer (pH 6.8) composed of 0.3 M K glutamate, 1 mM EGTA, 1 mM GTP, 5 mM K MES, 0.5 mM $MgSO_4$ at a concentration of 5.6 mg/ml. Squid axoplasm was extruded by the same procedures as also described in our previous paper (Sakai & Matsumoto, 1978), and dissolved in the standard, internal perfusion fluid (*see Materials and Methods* in our previous paper). It was stored on dry ice and used within two days. In some experiments, microtubule assembly was measured viscometrically as reported before (Sakai & Matsumoto, 1978).

Reagents used. Adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP), adenosine 5′-diphosphate (ADP), adenosine 3′,5′-cyclic monophosphate (cAMP), and vinblastine were purchased from Sigma Chemical Co., St. Louis, Mo., and L-tyrosine and L-phenylalanine (analytical grade) were from Takara Kohsan Co., Ltd., Tokyo. L-tyrosine was dissolved in 0.01 N KOH to make a 1 mM solution, followed by addition to the standard perfusion solution to prepare an appropriate concentration of tyrosine.

Results

The ability of a reagent or a group of reagents to restore the membrane excitability was determined by observing the time course of recovery of the action potential, resting potential, and threshold as restoration indicators.

The addition of 1 mM ATP to the standard perfusion solution (Solution I) failed to restore the suppressed excitability as reported by Tasaki, Singer and Takenaka (1965). The combination of 1 mM ATP and 50 μM tyrosine (Solution II) as well as that of ATP and Mg^{2+} also failed to restore the excitability significantly. Addition of 1 mM Mg^{2+} to Solution II (Solution III) was found to induce the recovery of excitability significantly (Fig. 1). In this experiment, excitability was partially deteriorated by internal application of 0.3 mM vinblastine for 43 min. Subsequent application of the standard perfusion solution once improved excitability, but it soon declined. Then the solution was replaced with the standard solution containing 50 μM tyrosine and 1 mM ATP, revealing little restora-

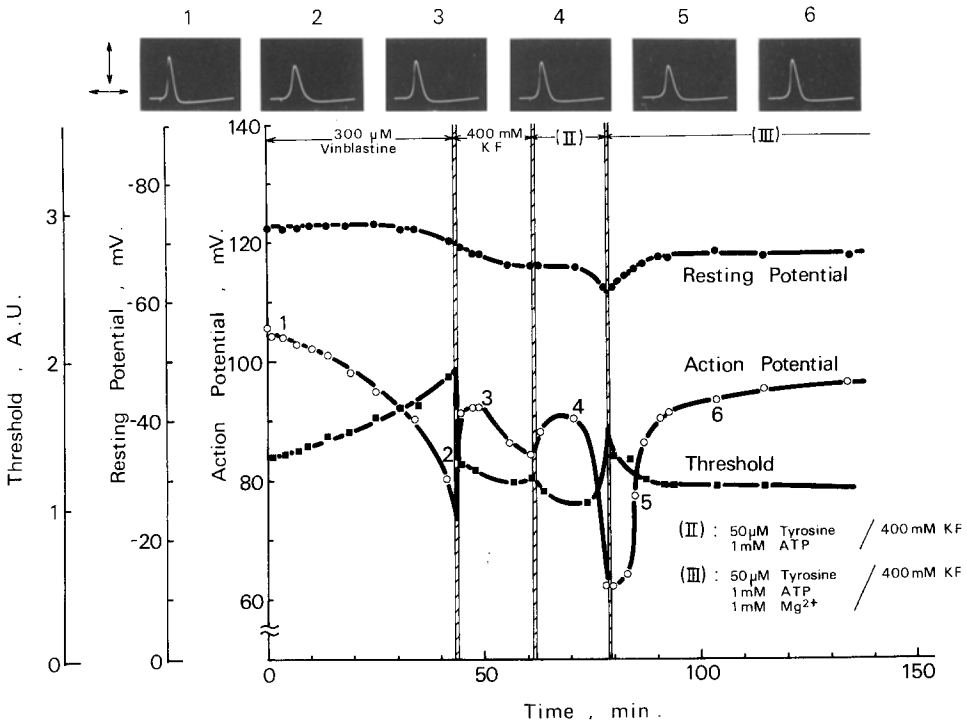

Fig. 1. Effect of combination of tyrosine, ATP, and Mg^{2+} upon the restoration of membrane excitability. The figure shows the time course of changes in action potential, resting potential, and threshold during subsequent internal perfusion with 400 mM KF containing 300 μM vinblastine, 400 mM KF, Solution II and III. The vertical breaks represent the time necessary to switch the solutions. Six representative oscilloscope records are shown on the top. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

tion. Finally, Solution III was perfused, resulting in excitability being steadily recovered, though the restoration was incomplete.

As a poor substrate for tubulin-tyrosine ligase, phenylalanine (50 μM) was tested in the presence of 1 mM ATP and 1 mM $MgCl_2$. The ability of phenylalanine to recover excitability was far weaker than that of tyrosine, slightly restored excitability soon declining during the application of the amino acid.

Other nucleotides, CTP, GTP, UTP and ADP, were then substituted for ATP for the restoration experiments. These nucleotides were capable of inducing restoration to some extent but were less effective than ATP.

The ability to restore excitability was examined for cAMP at the concentration ranging from 5 μM to 1.5 mM using the standard perfusion medium containing 50 μM tyrosine, 1 mM ATP and 1 mM $MgCl_2$ (Solu-

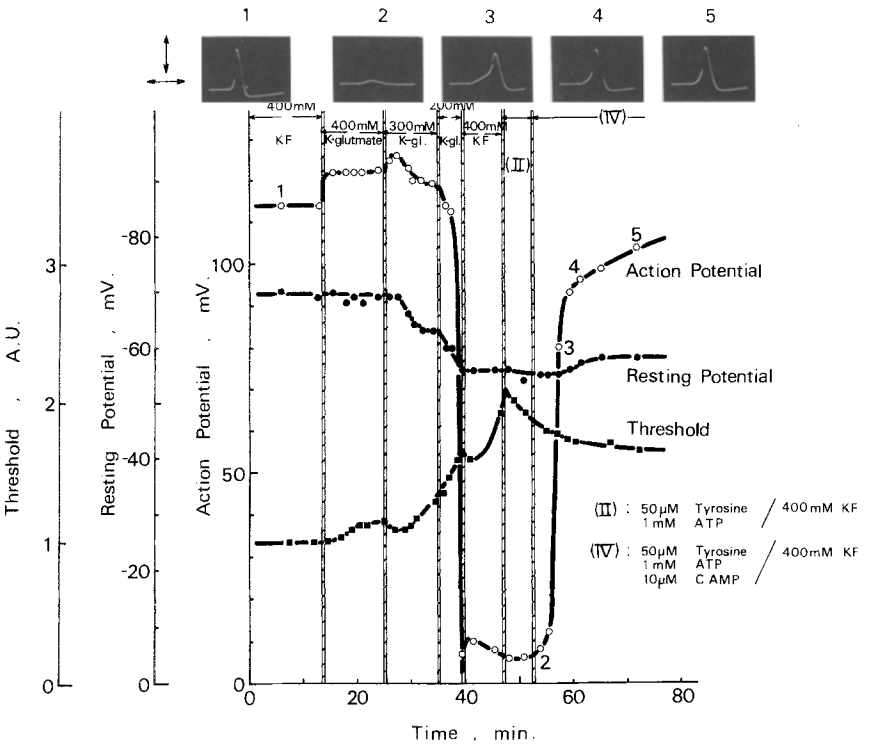

Fig. 2. Effect of cAMP upon the restoration of membrane excitability. The figure shows the time course of changes in action potential, resting potential, and threshold during subsequent intracellular perfusion with 400 mM KF, 400 mM K glutamate, 300 mM K glutamate, 200 mM K glutamate, 400 mM KF, Solution II and IV. Five representative oscillograph records are shown on the top. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

tion III). Higher concentration (0.5 ~ 1.5 mM) of cAMP always failed to restore excitability. Since cAMP-dependent protein kinase is known to be activated by the micromolar level of the cyclic nucleotide (Guthrow, Allen & Rasmussen, 1972; Miyamoto *et al.*, 1973; Murofushi, 1974), the concentration was fixed to 5 to 10 μM in all the experiments described below.

Figure 2 shows the record of the restoration of excitability by the combination of 50 μM tyrosine, 1 mM ATP and 10 μM cAMP (Solution IV). After the failure of restoration by the standard perfusion medium (Fig. 2, from 40 to 47 min) and by Solution II in succession (Fig. 2, from 47 to 52 min), the excitability was partially recovered by further addition of cAMP.

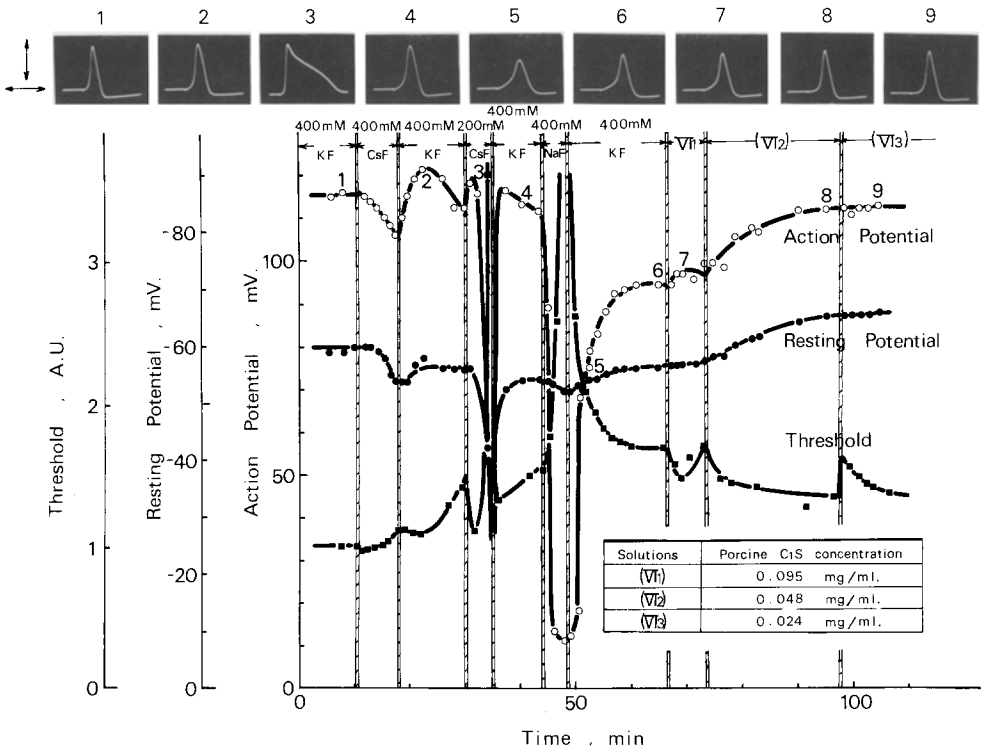

Fig. 3. Effect of porcine microtubule proteins C_1S upon the restoration of the membrane excitability after it was partially suppressed. The figure shows the time course of changes in action potential, resting potential, and threshold during subsequent internal perfusion with 400 mM KF, 400 mM C_8F, 400 mM KF, 200 mM C_8F, 400 mM KF, 400 mM NaF, 400 mM KF, Solution VI with three different concentrations of C_1S. Nine representative oscillograph records are shown on the top. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

The combination of 50 μM tyrosine, 1 mM ATP, 1 mM $MgCl_2$ and 5 μM cAMP (Solution V) was then found to be the best among the solutions described above causing the restoration to occur quite consistently, when the membrane excitability had been suppressed by perfusing the axon with the standard 400 mM NaF medium or with 200 mM K glutamate medium.

The effects of porcine brain microtubule proteins (C_1S) supplemented into Solution V was then examined. The solution supplemented with C_1S (Solution VI) was confirmed to be more capable of inducing the restoration than Solution V when the concentration of C_1S was appropriately chosen, though full recovery still was not attained (Fig. 3).

Finally, the squid axoplasm was dissolved in Solution V (Solution VII)

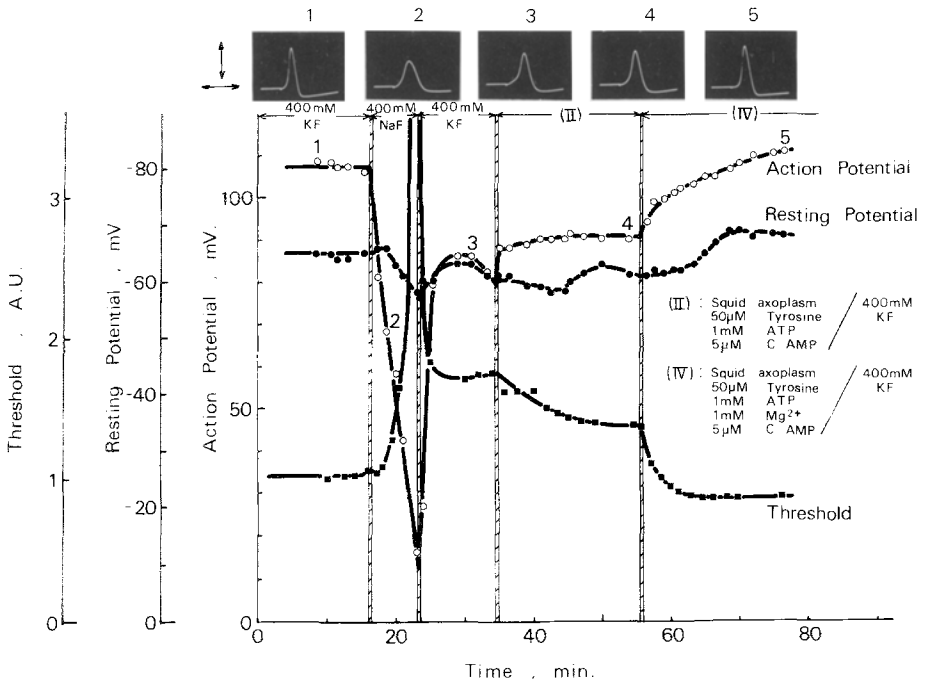

Fig. 4. Effect of squid axoplasm upon the restoration of the membrane excitability. The figure shows the time course of changes in action potential, resting potential, and threshold during subsequent internal perfusion with 400 mM KF, 400 mM NaF, 400 mM KF, Solution VII deprived of Mg^{2+} and Solution VII. Five representative oscillograph records are shown on the top. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

to test the restoration-inducing ability. The restoration was satisfactory, judging from the shape and amplitude of action potential, resting potential, and threshold except for the conduction latency (Fig. 4). The degree of the restoration also depended on the concentration of the axoplasm dissolved as observed for porcine brain C_1S. Figure 4 also shows that the axoplasm dissolved in Solution V exhibited restoration larger than that dissolved in Solution V deprived of Mg ions.

Discussion

The present results clearly indicated that the combination of 50 μM tyrosine, 1 mM ATP and 1 mM Mg^{2+} was inevitable to maintain the excitability. This strongly suggests that tubulin-tyrosine ligase is involved in the maintenance of the membrane excitability. A preliminary experi-

ment perfusing with purified tublin-typosine ligase further supported this idea (G. Matsumoto, T. Kobayashi & H. Sakai, *unpublished data*). The enzyme catalyzing tyrosylation of α-tubulin (Barra *et al.*, 1974; Raybin & Flavin, 1975; Deanin & Gordon, 1976) requires ATP, Mg^{2+}, and K^+ (Raybin & Flavin, 1975; Deanin & Gordon, 1976). Poor ability of phenylalanine to restore the excitability was supported by the fact that incorporation of phenylalanine into α-tubulin was 30 times weaker than that of tyrosine (Deanin & Gordon, 1976). These accordingly would lead to an assumption that only microtubules containing tyrosylated tubulin interact with the plasma membrane. The results that ADP, CTP, GTP or UTP could support the restoration in a little less degree as a substitute of ATP can be attributed to the presence of nucleoside diphosphate kinase in the brain (Kobayashi & Shimizu, 1977) for ADP and to the ability of GTP (Raybin & Flavin, 1975) and possibly CTP and UTP as well to activate the enzyme to some extent.

The effect of cAMP upon restoration could not be attributed to the stimulatory effect of cAMP on microtubule assembly as demonstrated with platelet tubulin (Steiner, 1978) because no stimulation of microtubule assembly was induced by cAMP using squid tubulin. The effect might be related to photophorylation of some species of proteins in the axon as measured by Pant *et al.* (1978) with two major components of axoplasmic proteins having chain weights of 2×10^5 and of more than 4×10^5 daltons. The analysis for the role of cAMP awaits further investigation.

It should be noted here that in the restoration of the excitability due to application of porcine brain microtubule proteins or squid axoplasm, the resting potential was also restored to or above the original level. This suggests that not only the amplitude of action potential and threshold but also resting potential were governed by the cytoskeletal constituents. From the present experiments we propose the idea that tubulin-tyrosine ligase functions in restoring membrane excitability through the maintenance of coupling between the plasma membrane and cytoskeletal frameworks.

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