PERIAXONAL K^+ REGULATION IN THE SMALL SQUID *ALLOTEUTHIS*

Studies on Isolated and In Situ Axons

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ABSTRACT A novel giant axon preparation from the squid *Alloteuthis* is described. Properties of in situ and isolated axons are similar. Periaxonal K^+ accumulation is a function of the physiological state of the animal and of the axon and its sheathing layers. Carefully dissected isolated axons, and axons in situ in a healthy mantle, show much less K^+ accumulation than previously reported in squid. It is suggested that the Schwann cells are involved in the observed K^+ regulation.

INTRODUCTION

In classic experiments on the isolated giant axon of the squid *Loligo forbesi*, Frankenhaeuser and Hodgkin (1956) showed that stimulation at high frequency caused accumulation of K^+ in the periaxonal space. The $[K^+]$ was estimated from the undershoot (hyperpolarization) of the action potential during a train of impulses. The results were compatible with K^+ clearance via a permeable barrier that they identified with the Schwann cell layer.

Since that study, it has generally been assumed that an increase in extracellular $[K^+]$ is a necessary consequence of neural activity, and indeed a rise in $[K^+]$ around active neurones has been demonstrated in numerous vertebrate and invertebrate preparations (Orkand et al., 1966; Baylor and Nicholls, 1969; Lux and Neher, 1973; Heinemann and Lux, 1977; Syková and Orkand, 1980; Orkand, 1980). However, it is now known that the nervous system has several mechanisms for minimizing K^+ accumulation, including specializations of glial cell membranes (see, e.g., Coles, 1985). Glial mechanisms have generally been investigated in isolated and cultured glia (Kettenman et al., 1983; Hertz, 1986; Kimelberg et al., 1986), and it has been difficult to extrapolate from these to the in vivo condition.

We chose to reexamine the role of glial cells in extracellular K^+ regulation, using a novel squid axon preparation which has several advantages, and in which normal geometrical relations between axon and glia are preserved. Squid giant axon physiology has been thoroughly investigated, and knowledge of the kinetics of K^+ clearance from the axon surface is important for interpretation of the membrane ion currents (Adelman and Fitzhugh, 1975; Clay, 1986). A great deal is known about the pharmacology of the periaxonal glial (Schwann) cells, and the way they respond to axonal stimulation (Villegas, 1984; Evans et al., 1985, 1986).

The small squid *Alloteuthis subulata*, mantle length 6–10 cm, can be caught with minimal damage and survives well in the laboratory. The thinness and transparency of the mantle makes it a promising preparation for recording from giant axons in situ. The vasculature is accessible for cannulation and perfusion. In this paper we describe the preparation, and compare the properties of isolated and in situ axons. We present evidence that K^+ accumulation is much less marked than previously shown if the axons/Schwann cell sheaths are in good physiological condition. In the subsequent paper, we present a quantitative treatment that explores the mechanisms responsible (Astion et al., 1988).

Preliminary accounts have been published (Abbott et al., 1985; Pichon et al., 1987, 1988).

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METHODS

Animals

Alloteuthis were trawled in shallow water (10–20 m) off the coasts of Plymouth (England) or Roscoff (France) in a net tapering to a plastic bucket with small holes in the walls, designed to minimize trauma. They were immediately transferred to oxygenated seawater in polyethylene bags contained within a larger plastic tank. The soft sides of the bags reduced damage caused by the squid hitting the sides of the tank when swimming at speed. In the laboratory the animals were transferred to large circulating seawater tanks (at least 2-m diam) at 10°C (Roscoff) or 13°–16°C (Plymouth) and generally used within 1–5 d.

Isolated Axons

The viscera and pen were removed, the stellate ganglion ligatured, the sheath around the nerve bundle containing the most medial giant axon was carefully opened by removing the fin nerve, and the giant axon and associated small fibers were removed from the mantle up to the point of the first major bifurcation. The nerve, ~1.5–2-cm long, was mounted in a perspex chamber without further dissection or cleaning. In early experiments the nerve was lightly trapped by fine nylon filaments near each end and passed over a third filament that slightly stretched the giant axon and facilitated impalement. The distal end of the nerve was held by a suction electrode for antidromic stimulation. In later experiments, the nerve rested in a groove in a Sylgard block, with a pin through the stellate ganglion.

In Situ Axons

In early experiments the mantle was cut in the mid-ventral line, the viscera were removed or displaced laterally and the in situ axon preparation was maintained by rapid flow of oxygenated saline through the bath. Later, vascular perfusion was introduced. A ligature was tied around the neck to stop blood supply to the brain, a hole was made in the systemic heart ventricle, and a tapering cannula pulled from 2.5-mm outer diam (OD) polyethene tubing was advanced into the aorta and secured with ligatures. A peristaltic pump was used to perfuse the vasculature with artificial seawater (ASW) lightly dyed with Evans Blue albumin (a few drops of stock 0.5% dye and bovine serum albumin in ASW added to the perfusate). The flow rate was designed to mimic the normal cardiac output, ~20 μl/s. The mantle veins were cut to allow perfusate drainage. Microelectrodes were inserted into the axon through the intact sheath unless otherwise indicated.

Solutions

The ASW contained (mM): NaCl 470, KCl 10, $CaCl_2$ 11, $MgCl_2$ 55, Tris buffer 10 (pH 7.6). Variations in $[K^+]$ were made by substituting KCl for NaCl. In low $[Na^+]$ solutions, Tris Cl replaced NaCl. Temperature was 18°–20°C unless otherwise stated.

Microelectrodes were pulled from 1.5-mm OD filament glass (Clark Electromedical, Reading, UK). When back-filled with 3 M KCl immediately before use they had a resistance of 5–30 MΩ. Electrodes were mounted directly on the headstage of a WPI KS 700 amplifier, and capacity compensated.

Statistics

Results are expressed as mean ± SE (standard error of mean).

RESULTS

State of Animals

Animals classed as "in good physiological state" were alert, moved fast, showed rapid and dramatic color changes, were able to catch live mysids as food, and to shoal, copulate, and fight. Their nervous systems and giant fiber jetting reflexes were clearly healthy.

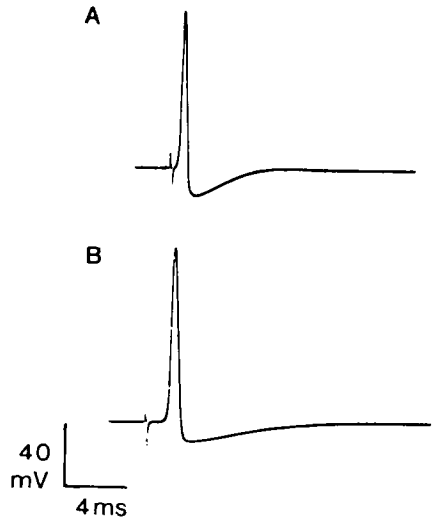

FIGURE 1. Representative action potentials from (*A*) isolated and (*B*) in situ axons. Baseline (resting) potential: (*A*) −59; (*B*) −63 mV.

Resting and Action Potential in Isolated Axons

The resting potential (RP) in isolated axons in ASW at 20°C was −62 ± 0.7 mV (n = 30) with a 47 mV change in RP per decade change in $[K^+]$ above 40 mM $[K^+]$. At 10°C, RP was −59 ± 3 mV (n = 16) with a 49 mV change for a 10-fold change in $[K^+]$. The mean action potential (AP) overshoot in ASW was 32 ± 2.5 mV (20°C) with a near-Nernstian decline on changing to 75 or 50% $[Na^+]$ ASW. In all preparations in 25% $[Na^+]$ ASW, and in some in 50% $[Na^+]$ ASW, the decline in overshoot was steeper than 58 mV per decade change in $[Na^+]$.

Resting and Action Potential in In Situ Axons

In axons in situ in the superfused mantle at 18°–20°C, the mean RP was −62 ± 0.8 mV (n = 11), not significantly

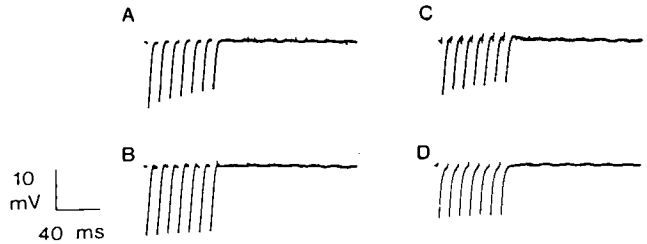

FIGURE 2 Isolated (*A, B*) and in situ axons (*C, D*) stimulated for 70 ms at 100 Hz. Action potential undershoots are shown at high gain (spikes off scale upwards). Axons showing undershoot decline during the burst can be seen in both isolated and in situ preparations (*A, C*), but axons in better condition frequently show less decline (*B, D*). Baseline: (*A*) −66; (*B*) −59; (*C*) −57; (*D*) −65 mV.

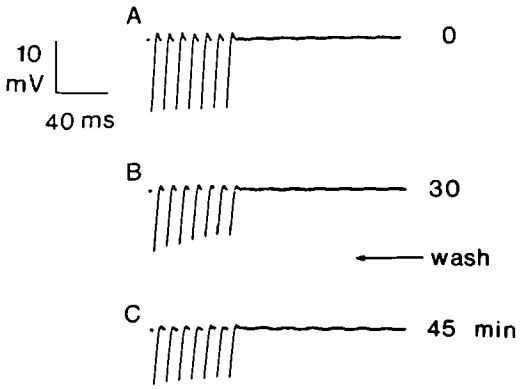

FIGURE 3 An isolated axon initially showing little undershoot decline (*A*, at time zero) showed increased decline after 30 min in stagnant medium (*B*), and partial recovery on re-starting flow (*C*). 100-Hz stimulation for 70 ms. Baseline: −59 mV.

different from isolated axons. The mean AP overshoot was 31 ± 2.2 mV ($n = 11$), again not significantly different from isolated axons. Fig. 1 shows representative APs in isolated and in situ axons.

Effect of Repetitive Stimulation

In early experiments, stimulation for 70 ms at 100 Hz produced clear decline of the undershoots in both in situ and isolated axons (Fig. 2, *A* and *C*), evidence of significant K^+ accumulation.

However, when care was taken to avoid stretching, drying, or touching the nerve during the dissection of isolated axons and the preparation of in situ axons, less decline of the undershoots was frequently observed (Fig. 2, *B* and *D*), consistent with less K^+ accumulation. Fig. 3 shows an isolated axon with initially little undershoot decline, showing greater decline after 30 min without flow through the chamber, and partial reversal of the decline on re-starting flow. In situ axons appeared more stable, and in

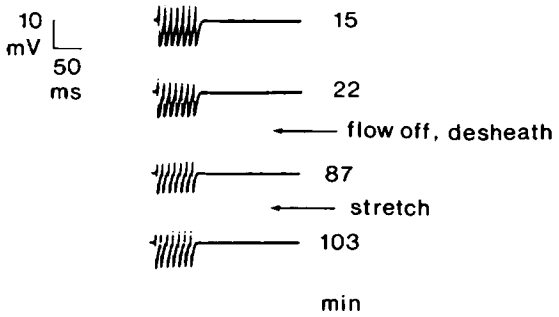

FIGURE 4 Undershoots during 70-ms stimulation at 100 Hz monitored in an in situ axon. Blood perfusion was stopped at time zero, superfusion stopped at 37 min, the electrode was withdrawn, and the outer nerve sheath opened at 67 min, and the electrode was re-inserted. Gentle stretching was applied at 100 min with the electrode in place. There was gradual decline of the first undershoot of the burst during the experiment, but little change in the rate of undershoot decline during the burst. Baseline −58 to −60 mV throughout.

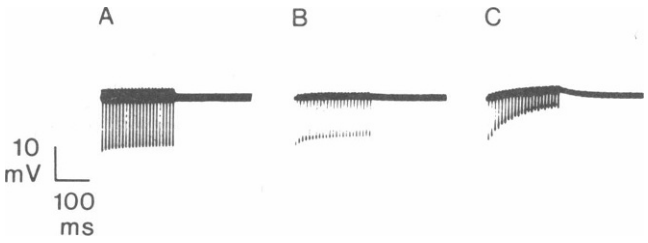

FIGURE 5 Undershoots during 200-ms stimulation at 100 Hz in three different isolated axons to show typical appearance of type A (*A*), type B, (*B*) and type C (*C*) response. Baseline: (*A*) −53; (*B*) −54; (*C*) −52 mV.

axons showing little K^+ accumulation initially, stopping perfusion and superfusion had little effect over 1–3 h (Fig. 4).

In an attempt to identify procedures causing increased K^+ accumulation in isolated axons, a 200-ms period of stimulation at 100 Hz was introduced, to show the changes in undershoot more clearly. Axon responses could be classified into three broad classes: Types A, B, and C (Fig. 5); (*A*) negligible undershoot decline; (*B*) some undershoot decline without change in DC level; (*C*) pronounced undershoot decline with change in DC level. When first set up in the chamber, an axon could show any one of the three responses. Once dissection procedures had been standardized, the greatest cause of variability in isolated axons was the physiological state of the animal, freshly caught and fast-moving squid generally showing less K^+ accumulation than those from damaged or sluggish animals, particularly those with opaque mantles. There was generally a change in the response over minutes to hours, always in the order A to B to C (Fig. 6). Fig. 7 shows responses from an axon impaled first near the stellate ganglion (*A*), then more distally (*B*). The response in the first position changed from type A to type C over ~2 h, but re-impalement in the second position at ~4 h showed that the response here was still type A/B. Of several experimental manipulations tested, including stretching, drying, and high $[K^+]$ pulses,

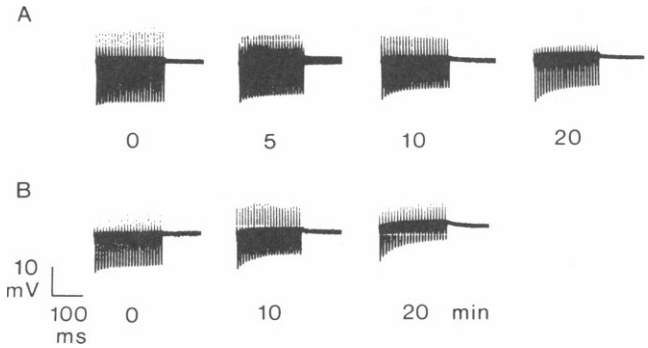

FIGURE 6 Progressive change with time of undershoot decline during stimulation for 200 ms at 100 Hz, of two different isolated axons (*A* and *B*). The time zero trace was taken within a few minutes of first impalement of each axon. In both cases, change from type A to B to C response (Fig. 5) is seen. Baseline: (*A*) −53; (*B*) −52 mV.

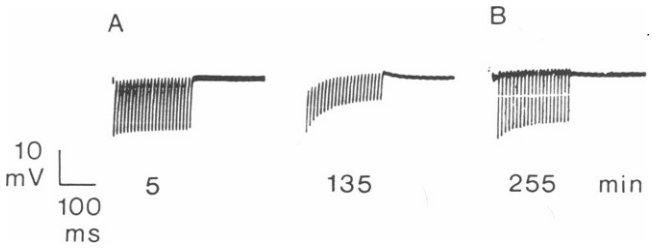

FIGURE 7 Undershoots during stimulation for 200 ms at 100 Hz in an axon impaled at time zero near the stellate ganglion (*A*) and at 250 min more distally (*B*). The response at the first position changed from type A to C over 135 min, but when recorded at 255 min in the second position, was still type A/B. Baseline: (*A*) −63; (*B*) −62 mV.

some of which appeared to hasten the change from type A to type C response, only changes in osmolarity of the bathing medium caused reversible changes in undershoot decline (see Astion et al., 1988).

DISCUSSION

Alloteuthis as Experimental Preparation

The RP and AP of dissected *Alloteuthis* axons show very similar properties to those described for *Loligo forbesi,* in dependence on $[K^+]$ and $[Na^+]$, respectively (Hodgkin and Katz, 1949).

Carefully isolated axons had RPs and APs similar to those of in situ axons. In earlier studies, Hodgkin and Keynes found a slightly lower RP in isolated than in in situ *Loligo forbesi* axons (see Hodgkin, 1958), possibly reflecting the more extensive cleaning procedure used to isolate *Loligo* axons. Resting potentials recorded in situ in *Loligo pealeii* were initially around −70 mV, declining to near −60 mV after 1 h (Moore and Cole, 1960). The agreement between in situ and isolated *Alloteuthis* axons suggests that the isolated axons were in good physiological condition. It also indicates that it is not unreasonable to extrapolate from the results in vitro to the in vivo situation. The vascularly perfused preparation promises to be useful for studies where it is essential to preserve the normal geometry of the system, which comprises the giant axon and its ensheathing layers, the blood supply, and the mantle muscle fibers. The similarity to *Loligo* in basic axonal properties permits useful comparisons between the species, while *Alloteuthis* may have significant advantages in studies aimed at elucidating normal physiological mechanisms in situ. Unlike previous in situ squid preparations (Hodgkin, 1958; Moore and Cole, 1960), the *Alloteuthis* preparation permits control of the perfusing medium, and is stable over several hours.

K^+ Accumulation during Repetitive Stimulation

If the decline of the undershoot is an indication of K^+ accumulation in the periaxonal space, then accumulation is much less marked around carefully handled *Alloteuthis* axons than previously observed in other squid preparations. The sensitivity of accumulation to the state of the animal, the carefulness of dissection, and in some cases the rate of perfusion, suggests that it is necessary to avoid mechanical and metabolic trauma to the cellular components to preserve the normal mechanisms of K^+ regulation. Since axons with similar RPs and APs may have markedly different rates of undershoot decline, axonal differences alone are unlikely to be responsible for the differences in K^+ accumulation. A more likely possibility is that the Schwann cells are healthier in preparations showing less K^+ accumulation. Healthy Schwann cells could contribute to K^+ removal either by transcellular transport mechanisms, or by preserving a large extracellular diffusion pathway. Further experiments designed to investigate the mechanism of K^+ regulation are presented in the accompanying paper (Astion et al., 1988).

Possible Species Differences

The relative lack of K^+ accumulation in fresh *Alloteuthis* axons, yet pronounced accumulation in most published studies using *Loligo* axons, could be taken to indicate marked species differences. However, there is considerable variability even among *Loligo* axons, with clear signs of less accumulation in fresh axons (Frankenhaeuser and Hodgkin, 1956; Pichon et al., 1987; Stimers et al., 1987; Pichon et al., 1988; Lieberman, E. M., and S. Hassan, unpublished results). The results point rather to little periaxonal K^+ accumulation being the normal physiological situation in squid, and increased accumulation being the result of procedures that disturb the normal state of axon–Schwann cell relations.

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