K^+ ACCUMULATION IN THE SPACE BETWEEN GIANT AXON AND SCHWANN CELL IN THE SQUID *ALLOTEUTHIS*

Effects of Changes in Osmolarity

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ABSTRACT In a train of impulses in squid giant axon, accumulation of extracellular potassium causes successive afterhyperpolarizations to be progressively less negative. In *Loligo,* Frankenhaeuser and Hodgkin (9) had satisfactorily accounted for the characteristics of this effect with a model in which the axon is surrounded by a space, width θ, and a barrier of permeability P. In axons isolated from *Alloteuthis,* we found that the model fitted the observations quite well. Superfusing the axon with hypotonic artificial seawater (ASW) caused θ and P to decrease, and, conversely, hypertonic ASW caused them to increase: this would be the case if both the space and the pathway through the barrier were extracellular. In some cases, in normal ASW, the afterhyperpolarizations in a train decreased very little, <0.7 mV. In these extreme cases, θ was estimated to be 190 nm and P to be 7×10^{-4} cm s^{-1}, both several times the values of 30 nm and 6×10^{-5} cm s^{-1} estimated by Frankenhaeuser and Hodgkin (9). We suggest that in vivo the periaxonal space may be considerably wider than that seen in conventionally fixed squid tissue.

INTRODUCTION

During the action potential in unmyelinated nerve fibers, K^+ is released into the extracellular space between the axon membrane and the surrounding Schwann cells. Since changes in $[K^+]$ in the periaxonal space (K_0) can have the physiological consequence of modifying membrane excitability, and the experimental consequence of modifying the currents measured in voltage clamp experiments, clearance of K^+ from periaxonal space has received considerable attention (see references 4, 19, and 20 and references therein). Towards the end of the action potential, the K^+ permeability of the axon membrane is high and the membrane hyperpolarizes to close to the K^+ equilibrium potential. This afterhyperpolarization, or undershoot, is reduced if K_0 rises, and hence can be used as an indicator of changes in K_0. Frankenhaeuser and Hodgkin (9) stimulated giant axons of the squid *Loligo forbesi* with trains of impulses and analyzed the way the successive undershoots in a train decreased (the "FH effect"). They found that the changes in K_0 could be accounted for by a formal model (the "FH model") in which the axon is surrounded by a space of width θ from which K^+ diffuses away through a thin barrier of permeability P. The physical interpretation of the model is uncertain, but the values estimated in *Loligo* for θ and P (30 nm and 6×10^{-5} cm s^{-1}) were compatible with θ corresponding to the extracellular space between the axolemma and the inner membrane of the Schwann cell, and P to extracellular clefts between Schwann cells.

Recently, Abbott et al. (1, 2) have noted that in some, but not all, experiments on axons of the squid *Alloteuthis,* the FH effect was extremely small, which suggests that K_0 rose very little. In some other systems, there is evidence that excess extracellular $[K^+]$ is cleared by passage into or through glial cells (6, 10, 11, 17). We have therefore ana-

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lyzed the FH effect in *Alloteuthis* both when the effect was small and when it was experimentally modified (by changing osmolarity) to see if the FH model and its morphological interpretation remained the simplest explanation.

METHODS

Alloteuthis subulata of mantle length 8–10 cm were kept in tanks for up to 48 h from the time of catching. 2–4 cm of the medial giant axon, diameter 220–260 μm, was dissected out (2) and secured in a chamber perfused by artificial seawater (ASW) of composition (mM): NaCl 470; KCl 10; $MgCl_2$ 55; $CaCl_2$ 11; Tris 10, pH 7.6 at 10°–12°C, unless otherwise stated. The distal end was stimulated by a suction electrode. Recordings were made with a microelectrode, filled with 3 M K citrate, resistance 10–80 MΩ. The experiments described were done on axons that gave action potentials, at the beginning of the experiment, of at least 90 mV from the resting potential.

RESULTS

Fig. 1, chosen to illustrate the various features of the FH effect, shows (*upper trace*) a train of action potentials at 60/s. The lower trace shows the undershoots with greater amplification, and is similar to the records from *Loligo* shown by FH except that depolarization is shown upwards. The undershoots decrease progressively, the last being 10.5 mV more positive than the first. Other evident features, which were also analyzed by FH, are the build-up and decay of the "negative" (depolarizing) afterpotential and the progressive decrease in spike amplitude. In most axons, especially when first mounted in the chamber, the FH effect was much smaller than that in Fig. 1; Fig. 3 *B* is typical. For each train, we measured the potentials, u, of the undershoots relative to the first ($-\Delta_1 E_+, \ldots, -\Delta_n E_+$ in FH). A plot from one experiment is shown in Fig. 2. To reduce the effects of noise (mainly due to aliasing with 50 Hz interference), at least two trains were recorded for each experimental condition and the measurements averaged. An exponential curve characterized by a time constant τ and a coefficient U was fitted to the points. In the six axons that gave action potentials of 100 mV or more (up to 108 mV), U ranged from 0.49 to 3.7 mV (mean, 1.6 mV; SEM, 0.5 mV) and τ from 19 to 47 ms. There was a small systematic deviation from exponential in that in all of 20 plots the point for the second undershoot lay above the curve, as it does in Fig. 2. As discussed below, this deviation is in the direction to be expected if the barrier round the periaxonal space has a finite thickness.

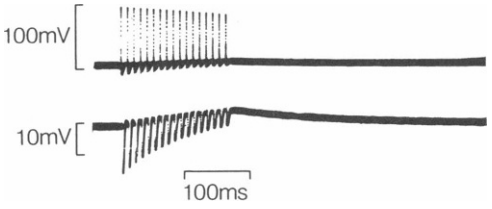

FIGURE 1 Recordings from an *Alloteuthis* axon with an exceptionally pronounced FH effect. The upper trace is at low amplification to show the action potentials. The lower trace shows undershoots at a higher amplification with positive upwards. The progressive increase in K_o not only causes the afterhyperpolarizations to become smaller, but depolarizes the resting potential ("negative" afterpotential). Temperature, 18°C.

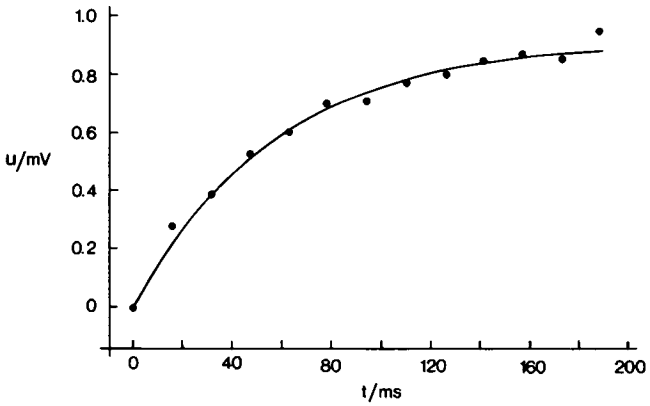

FIGURE 2 Measurements on five consecutive trains of impulses were averaged and the progressive decrease in undershoot, u, was plotted. The continuous line is an exponential.

The Effect of Changing Osmolarity

Fig. 3, *B* and *C* illustrates the effect of switching the superfusate from ASW to ASW diluted to 80%. It is apparent that U increased. The change appeared to be complete ~5 min after the new solution reached the axon. On returning to ASW, U transiently undershot its initial value. Results from six experiments at 10°–12°C are shown in Table I; qualitatively similar results were obtained on nine additional axons at room temperature. Conversely, in ASW made ~17% hypertonic by addition of 0.2 M sucrose, U decreased. The effect was seen in 10 experiments. Results from the five experiments at 10°–12°C are given in Table II. The change, which was complete in ~8 min, was not completely reversed on the return to normal ASW.

The changes in the quantities U and τ were used to

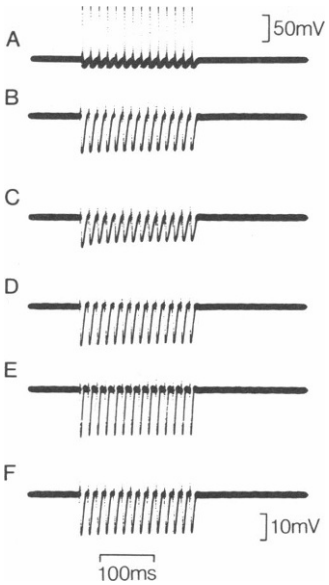

FIGURE 3 Effects of changing osmolarity. *A* shows a train of action potentials and *B* shows the undershoots at higher amplification. (*C*) Undershoots after 6 min in hypotonic ASW; (*D*) 6 min after return to normal ASW; (*E*) after 6 min in hypertonic ASW; and (*F*) 6 min after return to normal ASW. In addition to the changes in FH effect, the changes in the time of recovery of each undershoot that are seen here (slower in hypotonic ASW [*C*] and faster in hypertonic ASW [*E*]) were observed in all experiments.

TABLE I
HYPOTONIC ASW

Exp.	U_1	U_2	U_3	τ_1	τ_2	τ_3	θ_2/θ_1	θ_3/θ_1	P_2/P_1	P_3/P_1
	mV	*mV*	*mV*	*ms*	*ms*	*ms*				
14045	1.32	2.71	1.15	31	27	21	0.40	0.67	0.464	1.005
15008	0.89	2.40	1.09	23	29	39	0.48	1.58	0.396	0.945
18016	3.73	6.01	4.65	35	35	43	0.63	1.04	0.623	0.844
18057	2.08	3.20	1.69	47	42	121	0.57	3.53	0.635	1.475
21002	1.09	2.14	1.07	39	55	51	0.77	1.91	0.565	1.109
21048	1.27	2.39	2.63	27	30	72	0.60	1.52	0.543	0.564
						Mean	0.575	1.71	0.577	0.99
						SEM	0.052	0.40	0.038	0.12
Significance of difference from unity						*P*	<0.001	0.075	<0.001	—

Subscript 1 indicates initial value in ASW; subscript 2, the value 6 min after switching to 80% ASW; and subscript 3, the value 6–8 min after switching back to ASW. *P* was obtained from tables of *t* calculated as $(|\bar{x} - 1|)\,(\sigma/N)^{-1/2}$.

calculate the changes in the two parameters of the FH model, θ, the thickness of the periaxonal space, and P, the permeability of the barrier. If each impulse releases a quantity Q of K^+ ions per unit area of axonal membrane into the periaxonal space, and this is cleared with a time constant τ, then, during a train of impulses, y_r, the excess $[K^+]$ immediately after the rth impulse is

$$y_r = Q/\theta\,[1 - \exp(-rT/\tau)][1 - \exp(-T/\tau)]^{-1}, \quad (1)$$

where T is the interval between impulses. This follows directly from Eq. 7 in FH with $t_1 = T$, $t_2 = 0$. In analyzing the records, the measurements were made from the first afterhyperpolarization so we are concerned with $(y_r - y_1)$, which corresponds to u_{r-1}. When a steady state is reached (i.e., $r \rightarrow \infty$),

$$y_\infty - y_1 = Q/\theta\,(\exp T/\tau - 1)^{-1}. \quad (2)$$

Because u (and therefore y) was small, u would have been approximately proportional to y. One way of seeing this is to consider that during the undershoot, the membrane potential is close to the Nernst potential for K^+,

$$E_K = RT/F\,\{\ln [K^+]_0 - \ln [K^+]_i\}, \quad (3)$$

where $[K^+]_0$, $[K^+]_i$ are the external and internal K^+ concentrations. Differentiating to obtain the effect of a small change in $[K^+]_0$ gives

$$\Delta E_K \simeq RT/F\,(\Delta[K^+]_0)/[K^+]_0. \quad (4)$$

Hence, since $y = \Delta[K^+]_0$ and $u \simeq \Delta E_K$, $u \propto y$. Using this in Eq. 2, we obtain for U, the steady-state value of u during a train of impulses:

$$U = k/\theta\,[\exp(T/\tau) - 1]^{-1}, \quad (5)$$

where k is proportional to Q.

From Eq. 5 of FH, the permeability, P, of the barrier is given by

$$P = \theta/\tau. \quad (6)$$

Hence, from Eqs. 5 and 6, the measured values of U and τ can be used to calculate θ/k and P/k.

On the assumption that Q is unaffected by the changes in osmolarity, the changes in θ and P produced by changes in osmolarity are summarized in Tables I and II. With hypotonic ASW, both θ and P were reduced to 58% of their initial values. 6 min after switching the superfusate back to normal ASW, θ had rebounded to 1.7 times its initial value whereas P was back to its initial value. Conversely, hypertonic ASW caused θ to increase remarkably, by a factor of

TABLE II
HYPERTONIC ASW

Exp.	U_1	U_2	U_3	τ_1	τ_2	τ_3	θ_2/θ_1	θ_3/θ_1	P_2/P_1	P_3/P_1
	mV	*mV*	*mV*	*ms*	*ms*	*ms*				
14066	1.15	1.00	1.16	21	51	40	3.55	2.31	1.46	1.20
15023	1.09	0.88	0.92	39	82	167	2.91	5.98	1.39	1.40
18038	4.65	1.91	2.47	43	69	95	4.22	4.59	2.61	2.08
18071	1.69	0.81	0.80	121	90	104	1.22	1.80	1.65	2.10
20043	4.83	1.94	2.82	42	89	104	5.83	4.77	2.54	1.78
						Mean	3.55	3.89	1.93	1.71
						SEM	0.76	0.79	0.27	0.18
Significance of difference from unity						*P*	<0.03	<0.02	<0.03	<0.015

Subscripts analogous to Table I.

3.6. Unlike hypotonic ASW, return to normal ASW, on average, did not reverse the effect and a rebound was never observed.

DISCUSSION

Agreement with the Formal FH Model

In addition to measuring the undershoots during a train of impulses, Frankenhaeuser and Hodgkin (9) analyzed the changes in spike potential and resting potential ("negative afterpotential"), some cumulative effects seen in voltage clamp, and the effects of changing bath $[K^+]$. All these observations could be accounted for by the presence of a periaxonal space from which excess K^+ is cleared by a process obeying the diffusion equation:

$$\theta(dy/dt) = -Py. \quad (7)$$

If we take this as the definition of the FH model, then all our results are compatible with it. In particular, the progressive decrease in the undershoots was close to exponential, as predicted by the model (Eq. 1). (The small deviation consistently observed [the second point lying above the curve] is readily attributed to two causes: [*a*] a finite thickness of the barrier [see Fig. 15 in reference 9], and [*b*] the approximation that $u \propto y$.)

Physical Interpretation of the Model

Frankenhaeuser and Hodgkin (9) estimated θ to be ~30 nm and suggested that it might correspond to the periaxonal extracellular space. Their value of P (6×10^{-5} cm s^{-1}) seemed compatible with K^+ being cleared either through the Schwann cell membrane or through "cracks in the Schwann cell layer." Subsequent work has tended to favor the extracellular route. Stimers et al. (20) accounted for the apparent rising phase of the Na channel gating current by a model involving extracellular clefts. Other evidence comes from experiments in which osmolarity was changed. Electron microscopy of axons fixed after exposure to hypotonic ASW showed narrowing of the extracellular clefts (22); hypertonic ASW produced widening (3). In keeping with this, Adelman et al. (4) found in voltage clamp experiments that periaxonal K^+ accumulation decreased with hypertonic ASW. In the present experiments, the reciprocal effects of hyper- and hypotonic ASW on the FH effect were also compatible with widening and narrowing of extracellular clefts. Another possibility, that Q, the quantity of K^+ released per impulse, changed significantly seems unlikely since changes in osmolarity (4) appear to have little effect on K^+ fluxes under voltage clamp (see also references 13 and 20).

Estimated values of P are compatible with extracellular clefts being the pathway for clearance of K^+. To estimate P from our data, we use the result of Landowne and Scruggs (14) that Q is ~7.5 pmol cm^{-2} at 11°C. We consider the case of the smallest U we observed, 0.5 mV. τ was 28 ms, so, from Eqs. 5 and 6, θ/k was 2.48 and P/k was 0.090. Frankenhaeuser and Hodgkin ([9], Table II) found that $u/\text{mV} \simeq y/\text{mM}$. This provides a link between Eqs. 2 and 5 and gives $\theta = 187$ nm (we come back to this), and, from Eq. 6, $P = 6.7 \times 10^{-4}$ cm s^{-1}. For extracellular diffusion through a layer of tissue of small thickness L, $P = DA/L$, where D is the diffusion coefficient in free solution and A is a factor representing the amount by which diffusion is restricted. $D = 1.5 \times 10^{-5}$ cm s^{-1}, so $L/A = 220$ μm. This result predicts that if the barrier were, say, 2 μm thick and penetrated by straight channels, the channels would need to occupy $<1/100$ of the area, a value that may reasonably be assigned to extracellular clefts (3). In summary, the model in which K^+ accumulates in a periaxonal space and diffuses away through extracellular clefts has many attractive features.

Occurrence of Large Values of θ

We most often saw small FH effects in fresh preparations giving relatively large action potentials. This observation tends to support the conclusion of Abbott et al. (2), based on more extensive investigations, that in vivo the FH effect is small and there is little periaxonal K^+ accumulation. The value of θ that interests us is therefore for the experiments in which the FH effect was smallest. In the extreme case of the axon with the smallest U, for which P was calculated above, we estimated θ to be 187 nm. This is about six times the mean value previously estimated for *Loligo* by Frankenhaeuser and Hodgkin (9), and larger than other published values for squid axon. However, we see no discrepancies or evidence of species differences. Nearly all previous work on periaxonal K^+ accumulation has been concerned with axon preparations used to study axon membrane properties; these preparations were more elaborate than ours and usually involved internal perfusion, or at least introduction of an axial electrode. Even in our relatively simple preparation, the magnitude of the FH effect was extremely variable. For the six axons that gave action potentials of over 100 mV, the estimated θ ranged from 187 to 33 nm (mean 77 nm). Adelman et al. (4; Tables II and III) report a largest value of ~60 nm for *Loligo*, which is within the range of our values for *Alloteuthis*. Pichon et al. (15, 16) found little K^+ accumulation in freshly dissected axons from *Loligo*, as well as *Alloteuthis*. Stimers et al. (20, p. 531) report a value of 26 nm for *Loligo*, but this was from a preparation whose Na gating current had an apparent rising phase. Their fresh preparations often had no rising phases and according to their interpretation this was because they had wider spaces. For the giant axon of *Myxicola*, a species from a different Class, a width as large as 224 nm has been reported (5).

Electron microscopy of squid axons has shown periaxonal clefts ~10-nm wide (3, 22), much less than the larger of the physiological estimates of θ. However, it is notoriously difficult to preserve the volume of extracellular space during fixation, and only by the use of special measures has it been possible to reconcile the physiological

and anatomical estimates of extracellular space in, for example, vertebrate nervous tissue (7, 21).

CONCLUSIONS

(*a*) It has been suggested in other preparations that an increase in K_0 might be a signal to coordinate glial and neuronal activity (12, 18), but the results of the present work confirm those of Abbott et al. (2) and Pichon et al. (15) in showing that clearance of extracellular K^+ round squid axon can be rapid enough to prevent K_0 from increasing by more than a few percent during neuronal activity. This result suggests that other signals, such as neurotransmitters, may be necessary (8).

(*b*) Physiological experiments are unanimous in indicating the presence of a periaxonal space. The present results suggest that in some preparations the space may be wider than hitherto accepted, e.g., more than 100 nm.

(*c*) The qualitative effects of varying osmolarity suggest that clearance of periaxonal K_0 depends primarily on extracellular routes. However, the same model implies the existence of a surprisingly wide periaxonal space. Hence, until the morphology is better known, it would be unwise to rule out radically different interpretations, perhaps involving movement of K^+ across Schwann cell membranes.

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