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A NEW INTERNAL PERFUSION METHOD FOR TRANSPORT STUDIES IN SQUID GIANT AXONS

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Summary

A new internal perfusion method has been developed which allows control of the internal solute composition in squid axons. The superiority of this technique compared to the old perfusion methods is shown by the experiments performed which have reproduced, both qualitatively and quantitatively, the Na^+ and Ca^{2+} fluxes observed in intact and dialyzed axons. Compared with the internal dialysis, the perfusion method has the advantage that the permeability barrier given by the porous capillary has been eliminated. This allows the introduction into the axon of solutes with very high molecular weight, at the same time that a fast and reliable internal control can be achieved.

Introduction

One of the main technological goals in membrane studies is the ability to control the internal environment of the cells. Axonologists, in particular, have been trying to do this for many years with different degrees of success. In 1962, Baker et al. [1] were able to maintain excitability in squid axons with the axoplasm substantially replaced with isotonic K_2SO_4. This and similar techniques which followed [2,3] (the axoplasm being 'rolled, pushed or sucked out' from the axolemal sheet) are extremely useful in studies on excitability. 'Electrical survival' of the axons was improved when F^- was used as a major

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

intracellular anion [3]. However, F^- has the disadvantage of being a powerful metabolic inhibitor. When similar perfusion techniques were applied to transport studies (basically the Na pump in nerve) the results were not so succesful, with problems ranging from low sensitivity to ouabain (inhibition) and ATP (activation) to very high unspecific and unsteady Na^+ fluxes and short survivals [4—7].

A big step forward for intracellular control in transport studies came with the development by Brinley and Mullins [8] of the internal dialysis technique. In this technique, a capillary tube (glass or plastic), with a high permeability 'porous' region of 10—20 mm is steered through the axon. The passage of fluid through the capillary allows control of the solute composition in the axoplasm surrounding the porous region. The only limitation of internal dialysis is due precisely to the permeability barrier presented by the capillary, which allows the passage of solutes with molecular weight not larger than 1000. This results in: (i) the impossibility of introducing into the axon large molecules of obvious physiological relevance (hormones, enzymes, antibodies, etc.); and (ii) long equilibration times for some solutes like nucleotide polyphosphates.

In the present work we describe a new internal perfusion technique for squid axons. It eliminates the permeability barrier of the porous capillary and has given excellent results when tested in experiments on Na and Ca transport.

Material and Methods

The general design of the perfusion chamber is similar to that described by Brinley and Mullins [8] for internal dialysis, and a detailed description can be found in the original paper. The chamber is a rectangular reservoir made of lucite with internal dimensions of $5 \times 4 \times 3$ cm (width, depth and height, respectively). The base as well as the front and rear walls are held together on the base of a micromanipulator with coarse vertical movement. The side walls can slide on the main body and are attached to micromanipulators which allow vertical and backward or forward displacements. The side walls carry the two end cannulas for mounting the axon, allowing, by their independent movement, the alignment of the axon relative to the tip of the perfusion cannulae, thus avoiding damage of the membrane. A slot, where the nerve will rest when the actual perfusion takes place, is built in the base of chamber (see Fig. 1).

Once the axon is properly mounted the glass cannula A is introduced, with the aid of a micromanipulator, through the left end cannula and is steered throughout the axoplasm until it appears outside the right end cannula. The tip of cannula A is advanced 2—3 mm into the tip of cannula B which is at the right side of the chamber also mounted on a micromanipulator. The overlapped A and B cannulae are then moved together to the left until the tip of cannula B becomes aligned with the right end of the central portion of the slot. At this moment the passage of perfusion fluid begins. Inflow and outflow are carefully matched (at 1—2 μl/min) using a single-channel Sage syringe pump adapted for infusion-withdrawal. The perfusion cannulae are connected to the corre-

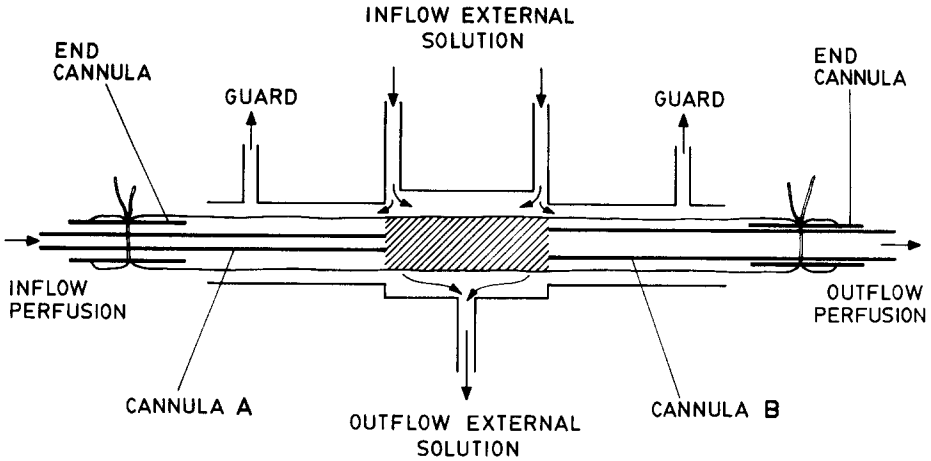

Fig. 1. Diagram (not to scale) representing the basic features of the perfusion chamber and the perfusion technique. The figure is a view from the top of the slot with the axon in place after perfusion has begun. The end cannulae, to which the axon is mounted, are placed across the side walls of the chamber. The perfusion fluid enters the axon through cannula A and leaves it through cannula B. The shaded area represents the region being perfused and is positioned in the central compartment of the slot. Other features are the inflow of external solution (at the ends of the central compartment), the sample collection path (outflow of external solution) and the guard system. See text for details.

sponding syringes (Hamilton gas tied) by lucite holders and polyethylene tubes in the way described for the dialysis technique [8]. The axon is then lowered into the slot and the solution in the upper portion of the chamber is removed by aspiration. Cannula A is slowly removed until its tip reaches the left end of the central compartment: in this way the tips of cannulae A and B will limit the region of the axon being perfused (shaded area in Fig. 1).

The external solution flows into the central compartment of the slot via two paths and comes out through the paths of sample collection (outflow external solution in Fig. 1) and the guards. The function of the guards is to prevent any radioactivity leaving the axon through a non-perfused region of the membrane from reaching the collecting tubes (see Ref. 8 for details); to accomplish this some radioactivity coming from the perfused region must be lost. With the usual flow rates this loss is no more than 15%.

The central compartment is 7 mm long and has a cross-sectional area of 16 mm^2. The inflow rate of external solution is about 1 ml/min.

Excitability can be monitored with the aid of external electrodes.

Solutions

The composition of the standard sea-water was as follows (mM): Na^+, 440; K^+, 10; Mg^{2+}, 50; Ca^{2+}, 10; Tris, 10; Cl^-, 580; EDTA, 0.1. The osmolarity was 1000 mosM and the pH 7.6 (at 20°C). Tris was used as an Na substitute. Ca^{2+}-free sea-water was nominally Ca^{2+}-free and contained in addition 0.5 mM EGTA, with a total Mg^{2+} concentration of 60 mM. K^+ was removed without changing the other constituents. In the experiments on Ca^{2+} efflux, sea-water also contained 1 mM KCN.

The perfusion solutions had the following composition (mM): K^+, 310; Na^+, 65 (40 in Ca^{2+} efflux experiments); Mg^{2+}, 4 (in excess to the ATP con-

centration); Tris, 5; Cl^-, 79; aspartate, 310; EGTA, 1; glycine, 330. The osmolarity was 980 mosM and the pH 7.1 (at 20°C) ATP was added from an ATP/Tris solution with an equal concentration of $MgCl_2$. ATP was obtained from Boehringer and phosphoarginine from Calbiochem. In the experiments on Ca^{2+} efflux, the perfusion solution contained in addition enough $CaCl_2$ to give a Ca^{2+} concentration of 0.05 μm. It also contained 1 mM KCN and 10 μg/ml of oligomycin.

Results

Figs. 2 and 3 illustrate two experiments on Na^+ efflux in perfused squid axons. Because the collection periods for radioactivity measurements began at the moment cannulae A and B were separated, the rising Na^+ efflux during the first few minutes does not represent real flux. The Na^+ efflux is lower because: (i) the isotope has not reached a steady-state distribution; and (ii) the area of the axon surrounding the perfused region of the axoplasm is smaller than the total membrane area bathed by the external solution. The reduction in Na^+ efflux that follows is a consequence of the ATP washout. The very low value of flux obtained after the washout of ATP, in the absence of any metabolic inhibitor, is an indicator of the good state of the axon. The steady efflux of

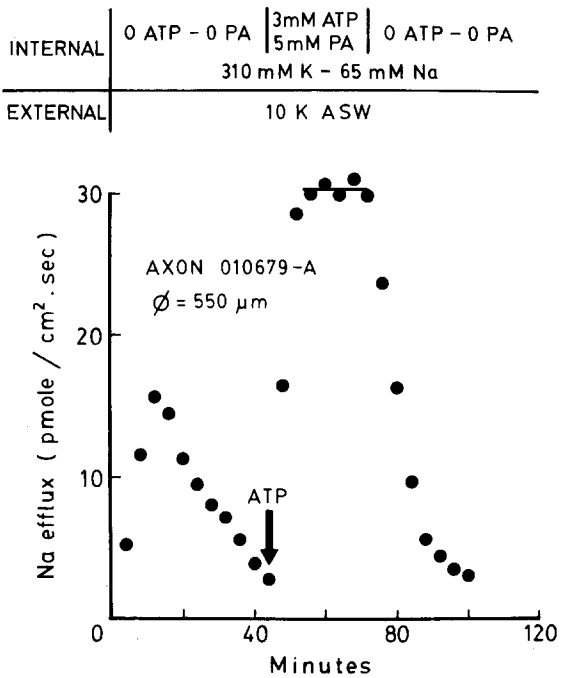

Fig. 2. The ATP dependence of total Na^+ efflux in a perfused squid axon. The collection of samples began at the moment when cannula A was separated from cannula B in the central region of the axon. Therefore, the values of flux during the first minutes do not represent real Na^+ efflux, but are lower, because: (i) the isotope did not reach steady-state distribution; and (ii) the membrane area covering the perfused region of the axon was smaller than the area bathed by the external solution. The axon remained excitable during the course of the experiment. Temperature was 17.5°C. See text for details. PA, phosphoarginine; ASW, artificial sea-water.

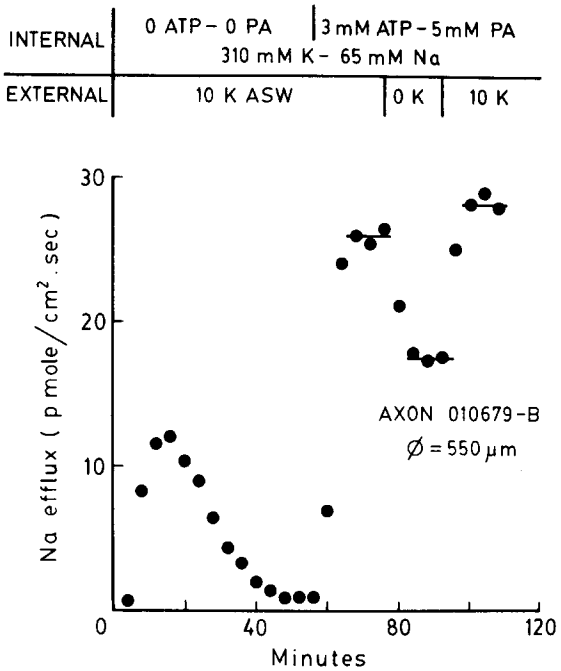

Fig. 3. Effect of ATP depletion and repletion and K_o sensitivity of Na^+ efflux in a perfused squid axon. The general procedure is explained in the text and in the legend to Fig. 2. Temperature was 17.5°C. PA, phosphoarginine; ASW, artificial sea-water.

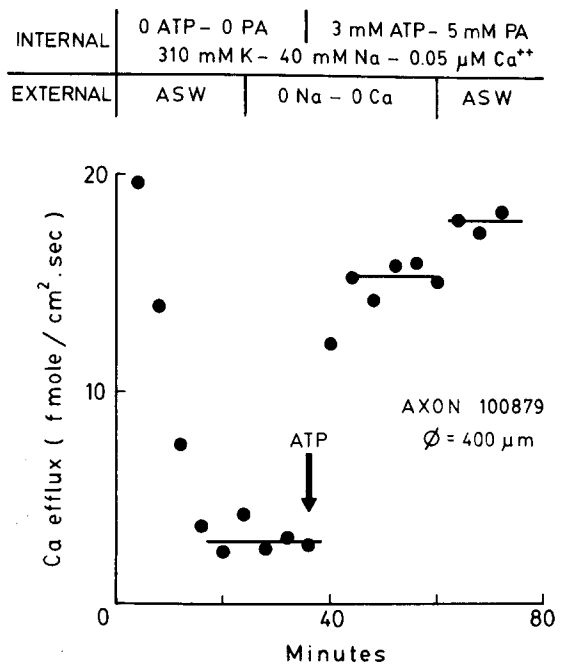

Fig. 4. The ATP dependence and the effects of external Na and Ca on Ca^{2+} efflux in a squid axon perfused with a solution containing physiological Ca^{2+} concentration. Tris was used as Na substitute. For details see text and the legend to Fig. 2. Temperature was 18°C. The axon was excitable at the end of the experiment. PA, phosphoarginine; ASW, artificial sea-water.

1 pmol/cm^2 per s in Fig. 3 is in excellent agreement with similar residual Na^+ efflux obtained in dialyzed axons under otherwise identical experimental conditions (Refs. 9 and 10, see also Ref. 8). It is worth noticing the fast depletion and repletion times for ATP. The magnitude of the total ATP-dependent efflux of Na^+ and its sensitivity to external K are also coincident with recently reported values for dialyzed axons [9,10]. The axons were excitable during the whole experiment.

Fig. 4 is an experiment on Ca^{2+} efflux in an axon perfused with a Ca^{2+} concentration within the physiological range [11]. Following the washout of ATP, a very low steady Ca^{2+} efflux was obtained (about 3 fmol/cm^2 per s) which was insensitive to the removal of external Na and Ca. Readdition of ATP, in the absence of Na_o and Ca_o, produced a marked stimulation of Ca^{2+} efflux (uncoupled efflux). This ATP-dependent efflux of Ca^{2+} was only slightly increased when external Na and Ca were restored. Altogether, the experiment of Fig. 4 exactly reproduces recent observations reported by DiPolo and Beaugé [12] in dialyzed squid axons.

Discussion

The micro-injection technique [13], was until now, the only way of introducing large molecules into axons for the purpose of transport studies. If several substances were to be introduced several injections would be required, and no internal control was achieved. Some improvement could be accomplished with the combination of a single injection followed by internal dialysis, but once dialysis begins further injections are no longer possible. The new technique proposed here has the tremendous advantage of combining the possibility of repetitive introduction of substances with high molecular weight (enzymes, antibodies, hormones, etc.) with the simultaneous control of the intracellular environment.

The superiority of this perfusion technique compared to the old ones [4—7] can be asserted on the basis of the experiments performed which have reproduced both qualitatively and quantitatively, the Na^+ and Ca^{2+} fluxes observed in intact and dialyzed axons. Worth stressing are the dependence on ATP (97% for total Na^+ efflux and 80% for uncoupled Ca^{2+} efflux) and the very low values for 'leak' fluxes. The low Ca^{2+} leak is particularly important for it has been shown that this leak is an excellent indicator of the membrane resistance [14] and, consequently, the health of the membrane.

When tested, the survival of the axons (based on excitability and low 'leak' fluxes) averaged a little more than 2 h. The axons we used were rather small, with diameters ranging from 400 to 550 μm. Perfect match between inflow and withdrawal must be critical in order to avoid development of differences in hydrostatic pressures. It was possible that, even with the care we have taken, some differences in hydrostatic pressure did develop, and this might have been more damaging in small axons. At any rate, an overall survival time of about 2 h does not represent a real handicap considering the speed at which changes made reach their new steady state with the consequent reduction in the duration of an experiment.

So far the new perfusion method has only been used for efflux experiments.

However, it can be easily adapted for influx by introducing a four-way valve and two syringes for withdrawal.

Acknowledgements

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References

1 Baker, P.F., Hodgkin, A.L. and Shaw, T.I. (1962) J. Physiol. 164, 330—354
2 Tasaki, I., Watanabe, A. and Takenata, T. (1962) Proc. Natl. Acad. Sci. U.S.A. 48, 1177—1184
3 Alwater, I., Benzanilla, F. and Rojas, E. (1969) J. Physiol. 201, 657—672
4 Shaw, T.I. (1966) J. Physiol. 182, 209—216
5 Baker, P.F., Foster, R.F., Gilbert, D.S. and Shaw, T.I. (1971) J. Physiol. 219, 487—506
6 Canessa-Fisher, M., Zambrano, F. and Rojas, E. (1968) J. Gen. Physiol. 51, 162s—171s
7 Rojas, E. and Canessa-Fisher, M. (1968) J. Gen. Physiol. 52, 240—257
8 Brinley, F.J. and Mullins, L.J. (1967) J. Gen. Physiol. 50, 2303—2331
9 Beaugé, L.A. and DiPolo, R. (1978) Nature 271, 777—778
10 Beaugé, L.A. and DiPolo, R. (1979) Biochim. Biophys. Acta 553, 495—500
11 DiPolo, R., Requena, J., Brinley, F.J., Mullins, L.J., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433—467
12 DiPolo, R. and Beaugé, L.A. (1979) Nature 278, 271—272
13 Hodgkin, A.L. and Keynes, R.D. (1956) J. Physiol. 131, 592—616
14 Mullins, L.J. and Brinley, F.J. (1975) J. Gen. Physiol. 65, 135—152