Biochimica et Biophysica Acta 818 (1985) 386–390
Elsevier

BBA 72723

ATP requirements for the electrogenic pump in perfused *Nitella* cells

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(Received February 26th, 1985)
(Revised manuscript received June 4th, 1985)

Key words: Electrogenic process; Perfusion technique; ATP hydrolysis; (*N. translucens*)

The use of the intracellular perfusion technique to determine the dependence on ATP of the electrogenic pump in characean cells was investigated using *Nitella translucens*. Immediately after the cells had regained turgor following perfusion, the ATP concentration was only 0.5 of that in the original perfusion medium and declined further with time. In these cells, the membrane potential did not reach a maximum value below an actual ATP concentration of 0.25 mM. The utility of this type of experiment for determining the K_m for ATP is criticized. The membrane resistance did not vary significantly with internal ATP concentration.

Introduction

The plasmalemma of *Nitella translucens* contains a light-activated electrogenic pump that is capable of hyperpolarizing the membrane potential of the cell to a value more negative than the negative limit set by the equilibrium potential for potassium, E_K [1]. The light activation of the pump is not brought about by a change in the intracellular ATP level [2]. However, the electrogenic pump in characean cells is dependent on ATP [3,4]. To characterize the ATP-dependence of the pump, one must be able to alter the ATP concentration and see what effect it has on the membrane potential. Metabolic inhibitors have been used in *Chara corallina* [5], but the alternative oxidase and internal ATP regulation mechanisms limit the control that can be exercised over the ATP concentration. The most direct approach to determining the ATP requirements for the electrogenic pump in characean cells has been to replace the cell cytoplasm by perfusion with a medium of known ATP concentration and measure the resultant potential [3,6]. As a preliminary to investigating the relationship between the membrane potential, membrane conductance and ATP concentration, we need to know whether the ATP level can in fact be controlled by perfusion and the extent to which the electrical properties of perfused cells reflect those of the intact system. In particular, we felt it was important to determine whether the ATP levels in perfused cells remained at the level present in the perfusion medium.

Materials and Methods

The electrical experiments were conducted with the cells in artificial pond water at pH 6 [1]. Additions to the solutions are indicated for the various experimental treatments. All solutions were CO_2-free and the pH was adjusted as indicated. The methods for measuring the membrane potential and resistance have been described previously [7].

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Abbreviations: E_K, equilibrium potential for K^+; E_m, membrane potential; e.m.f., electromotive force; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

TABLE I

COMPOSITION OF THE PERFUSION MEDIA

The pH was adjusted to 7.0 with KOH.

Compound	Concn. (mM)
Hepes	30
EGTA	5
$MgCl_2$	6
Mes	23.5
Sorbitol	250
ATP	0–2.0

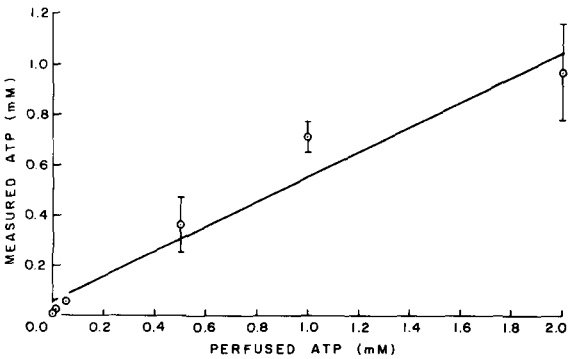

Fig. 1. A comparison of the ATP concentrations of the perfusion media with the ATP concentrations in the perfused cells. The perfusion medium was diluted in the cells as they regained water and turgor. Measurements were made within 15 min after perfusion. Values are mean ± S.E. Error bars for the first three points on the graph fall within the symbols. The straight line is a linear regression given by the equation $y = 0.497x + 0.061$.

Perfusion. Nitella internodal cells were cut, trimmed and left in the dark overnight in CO_2-free artificial pond water (pH 6) + 0.4 mM KCl. For perfusion, the cell was blotted and placed on a notched lucite perfusion block. Four knots were loosely tied along the length of the cell and the ends were covered with a drop of perfusion medium (Table I). At the first sign of wilting, the ends of the cell were clipped off and one end of the perfusion block elevated 1 cm to initiate perfusion. Ten to thirty seconds later, the outermost set of knots was tightened, the block lowered, the perfusion medium blotted and the cell covered with 200 mM sorbitol. After partial turgor was regained, the inner set of knots was tightened and the cell was trimmed between the outer and inner set of ligatures. The 'cell' was then allowed to recover in CO_2-free artificial pont water (pH 6) + 0.4 mM KCl, during which time the tonoplast disintegrated. Streaming was visible and vigorous. These new 'cells' were then measured and used for experiments.

Details of the ATP assay protocols have been described previously [5].

Results

There is a linear relationship between the concentration of ATP in the perfusion medium and that observed in the cell following perfusion, measured using the luciferin-luciferase assay. The ATP concentration in the cell was approximately half the original value (Fig. 1). Furthermore, the concentration did not remain constant but declined so that after a recovery period of one hour the value was about one third of the concentration of ATP originally perfused into the cell (Control: 0.77 ± 0.07 (S.E., $n = 27$), recovered for 1 h in light: 0.31 ± 0.15 (S.E., $n = 8$), in dark: 0.40 ± 0.13 (S.E., $n = 8$) mM ATP).

Pump activity, as indicated by the membrane potential, can only be distinguished when the membrane potential exceeds the most negative limit calculated for the passive diffusion potential. In this system, potassium sets the negative limit. The only source of potassium in the perfusion medium was the KOH used to adjust the pH. Perfusion media with higher ATP concentrations required more KOH and there was some variability in the internal K^+ concentration for cells perfused with different concentrations of ATP. This problem was overcome by measuring the membrane potential in artificial pond water (pH 6) + 10 mM KCl at the end of the experiment. Since the cell becomes highly permeable to K^+ under these conditions and the membrane potential becomes equal to E_K [1], this value of the membrane potential can be used to calculate E_K in artificial pond water (pH 6) + 0.4 mM KCl.

There was a correlation between the membrane potential and the ATP concentration in the cell. The membrane potential appeared to saturate at a concentration of 0.25 mM ATP, corresponding to a 0.5 mM ATP perfusion medium (Fig. 2). Thus the pump required 0.25 mM ATP to maintain its

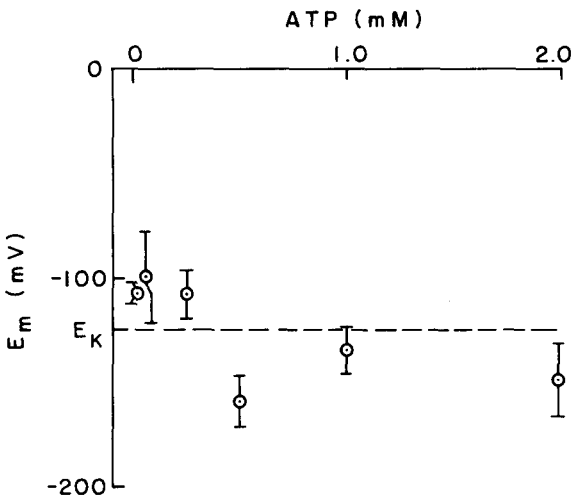

Fig. 2. Membrane potential as a function of ATP concentration in the perfusion medium. The dashed line represents the equilibrium potential for K^+. Points below the dashed line represent membrane potentials that are actively generated by the electrogenic pump.

maximum contribution to the membrane potential.

Unlike the membrane potential, a significant correlation between membrane resistance and the ATP concentration was not observed. The specific membrane resistance did not have a simple dependence on the ATP concentrations in the cell (Fig. 3). The decrease in resistance observed at low ATP concentrations may be related to an increase in K^+ permeability which occurs at lower membrane potentials and may obscure the pattern of increasing resistance evident at intermediate concentrations.

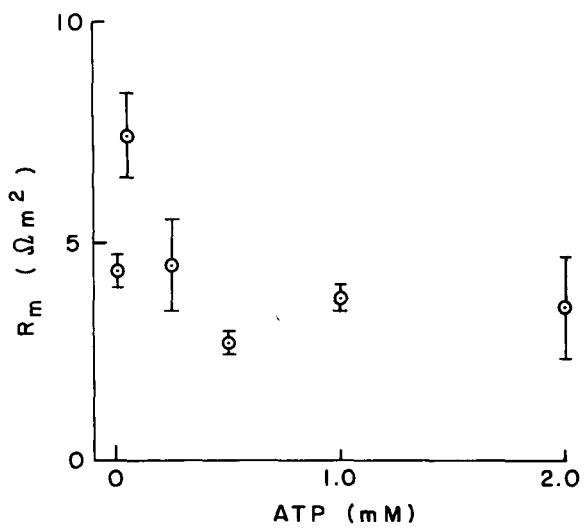

Fig. 3. Specific membrane resistance as a function of ATP concentration in the perfusion medium. Values are given as the mean ± S.E.

Discussion

Using perfused cells of *Nitella translucens* and varying the internal concentration of ATP, it was found that, in order to generate a potential that was more negative than the potassium equilibrium potential, a concentration of 250 μM ATP was required. This contrasts with the work of Mimura et al. [6] where the effect of ATP appeared to saturate at 50 μM with an apparent K_m of 10 to 30 μM. This difference probably reflects the species difference and also differences in protocol and analysis.

Our results indicate the existence of problems with the perfusion technique which do not appear to have been considered adequately in the past. Upon recovery of turgor, the internal concentration of ATP in perfused and ligated cells is reduced to 50% of the value in the original perfusion medium (Fig. 1), and declines further with time (see Results). Mimura et al. [16] claim that use of an ATP regenerating system in the perfusion medium results in an internal ATP concentration 80 to 100% of that in the perfusion medium, but supply no further information. Clearly, the dilution and hydrolysis of the ATP in the perfusion medium is a problem that must be confronted if this method is to be used to make quantitative determinations of the relationship of the electrogenic pump to ATP concentration. For example, it could affect the interpretation of the experiments of Kawamura et al. [8] on the effect of internal ATP on the response of the membrane potential and resistance to external pH in the light and dark. Measurements in the light were made immediately following perfusion while those in the dark were made 2 to 3 h after perfusion. During this period, the loss of internal ATP might account for some of the difference in the response of the membrane potential to external pH, observed in the dark, in perfused cells. This response was intermediate between cells in the light (measured

soon after perfusion) and those perfused with medium containing no ATP.

A second problem involves the occurrence of abnormally low membrane potentials. For example, Mimura et al. [6], in their Table II, show that rather more than half the cells prepared by the ligation method had abnormally low membrane potentials. They claimed that these cells are in the excited state and excluded them from their calculation of the K_m for ATP. However, the average membrane potential for all their sets of cells falls within the range of E_K. While this procedure may be justified for *Nitellopsis*, in the case of *Nitella translucens* we did not observe a clear bimodal distribution of the membrane potentials and have not culled data for cells with lower membrane potentials.

Mimura et al. [6] also used a Pb^{2+} pretreatment with the open vacuole method and with one of the sets of ligated cells to suppress excitation. Although ATP produced a hyperpolarization beyond the very low value of the membrane potential in the 'zero ATP' perfusion (about -50 mV), it is not evident that the maximum value of the potential exceeded E_K in either case. We have shown [9] that one effect of lead is to reduce the permeability of the membrane to K^+. The long-term effects include inhibition of the electrogenic pump and will be dealt with elsewhere.

In addition to the technical problems, there are theoretical problems associated with interpretation of the ATP-dependent hyperpolarization in terms of the activity of the electrogenic pump. The simplest case is one in which a constant-current electrogenic pump is acting in parallel with passive channels having a conductance independent of the membrane potential (i.e. a linear current-voltage relationship). In this case, and this case only, the hyperpolarization produced by the pump will be proportional to the current through the pump, assuming a constant stoichiometry. It would then be valid to compare the K_m for ATP derived from the concentration-dependence of the membrane potential with the K_m for ATP derived from the concentration-dependence of ATP hydrolysis by the membrane ATPase. Although this situation exists to a good approximation in *Neurospora* [10], it does not appear to hold true for the Characeae because the electrogenic pump contributes a large fraction of the membrane conductance, and the pump conductance varies with the ATP level [4,5].

The analysis Mimura et al. [6] used to derive the K_m of the electrogenic pump for ATP involves both practical and theoretical difficulties. In the first place, they assume that the pump activity is proportional to E_a, where

$$E_a = E_m - E_d \tag{1}$$

and E_m is the membrane potential measured in the presence of ATP and E_d is the diffusion potential which they equate with the membrane potential after a double perfusion of the cell with a medium containing no ATP. In fact, the values of E_d estimated in this way, which are in the range of -50 to -80 mV, are much more positive than the known diffusion potentials measured in intact cells under conditions in which the pump is inhibited [1]. In addition, the values of E_d obtained in this way are in the region of the membrane potential in which the membrane becomes excitable. That this is having an effect is evident from the low membrane resistance of cells perfused with zero ATP (Fig. 4 in Ref. 6). Thus the value of E_d estimated in this way is probably more positive than the value appropriate for cells containing higher ATP concentrations. The effect of this is to exaggerate the apparent value of E_a in the low range of ATP concentrations and hence decrease the apparent value of K_m.

The theoretical problem results from the conductance of the electrogenic pump in the Characeae. Using the simple equivalent circuit for the electrogenic pump having an e.m.f., E_P, in parallel with the passive diffusion channels [1], it can be shown that

$$E_a = g_P(E_P - E_D)/(g_D + g_P) \tag{2}$$

where E_D is the diffusion potential of the passive channels, g_P is the conductance of the electrogenic pump and g_D is the conductance of the passive channels. The dependence of E_a on ATP concentration clearly does not bear a simple relationship to the K_m for ATP hydrolysis or the flux through the pump since both E_P and g_P are dependent on the ATP concentration. In addition, the presence of g_D in this equation emphasizes the

importance that changes in this parameter could have on E_a as the membrane potential becomes more positive and the passive membrane conductance increases at low ATP concentrations.

Since the K_m for ATP is related to the rate of ATP hydrolysis and of H^+ transport, it would be more relevant to consider the effect of ATP on the current, i_P, through the pump, which is given by

$$i_P = g_P(E_P - E_m) \quad (3)$$

Note that this equation gives a value for i_P that is opposite in sign to the equations used by Tazawa and Shimmen [11] and Kishimoto et al. [12]. However, it is consistent with the convention [13] that a net flux of ions into the cell is positive and hence a net flux of positive ions into the cell will also give a positive current. Thus, according to the convention used here, a positive current is directed from outside to in.

An expression for E_P for an electrogenic H^+ pump was derived previously, assuming that the pump derived energy from ATP hydrolysis characterized by a chemical potential difference $\Delta\bar{\mu}_P$ [1]

$$E_P = \Delta\bar{\mu}_P/F\nu_H - (RT/F)\ln(H_i^+/H_o^+) \quad (4)$$

Expanding the term $\Delta\bar{\mu}_P$ and substituting Eqn. 4 in Eqn. 3, the dependence of i_P on the ATP concentration is given by:

$$i_P = -g_P\{(2.3RT/F\nu_H)(\log K_{ATP} + \log[ATP]/[ADP][P_i]) + (2.3RT/F)\log(H_i^+/H_o^+) + E_m\} \quad (5)$$

where ν_H is the number of H^+ transported per ATP molecule hydrolysed and K_{ATP} is the equilibrium constant for the hydrolysis of ATP. Although this expression does not relate i_P to the K_m for ATP for the pump, it is interesting to note that, given constant values of g_P, E_H and E_m, the equation predicts a linear relationship between i_P and log[ATP]. To a first approximation, Smith and Walker [14] did in fact observe such a relationship between the clamp current and the ATP concentration in cells of *Chara corallina* clamped at −180 mV and perfused using the open vacuole method.

The apparent independence of the membrane resistance and the internal concentrations of ATP (Fig. 3) may be a function of the variability of the system although, as noted above, the decrease in resistance at the lowest ATP concentration may be the result of an increase in the conductance of the passive diffusion channels associated with the reduction of the membrane potential. This may obscure a continuation of the increase in the membrane resistance that appears at intermediate ATP concentrations as the ATP concentration is lowered. Further investigation of this point will require use of the voltage clamping technique.

In conclusion, we suggest that caution should be exercised in both the design and interpretation of experiments with perfused cells.

Acknowledgement

This study was supported by grant number PCM 81-11007 from the National Science Foundation.

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