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ENERGY RELATIONSHIPS BETWEEN ATP SYNTHESIS AND K⁺ GRADIENTS IN CULTURED GLIAL-DERIVED CELL LINE**

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In C6 astrocytoma cells respiring with glucose, 40% of the total production of ATP was provided by glycolysis. Anaerobiosis in the presence of glucose, reduced ATP synthesis by approximately 50%, increased lactate production by 30% and caused a 3-fold decline in [creatine phosphate]/[creatine] and consequently $[ATP]_{free}[ADP]_{free}$. There was no change in $[K^+]_i$ which suggests that glycolytic production of ATP provides sufficient energy to ensure normal operation of the Na^+/K^+ pump. In the absence of glucose, [creatine phosphate]/[creatine] declined to <0.1 in 15 min and there was a loss of K^+ from cells. A comparison of ΔG_{ATP} and $\Delta G_{Na,K}$ under aerobic conditions with and without glucose, showed the former to be larger by 1 - 2 kcal. However, under O_2-limited, glucose-restricted conditions ΔG_{ATP} fell below the level necessary to maintain operation of the Na^+/K^+ pump and led to a collapse in ionic gradients.

It is well known that limitation in oxygen supply to cells and tissues leads to a decrease in cellular energy synthesis which, in turn, restricts the activity of the plasma membrane ion pumps and causes disturbances in ionic balances. One of the key plasma membrane ion pumps, which is responsible for maintaining low $[Na^+]_i$ and high $[K^+]_i$, is sodium/potassium ATPase [1, 2]. This enzyme requires ATP for its operation, hence a decrease in cellular energy level results in influx of Na^+ into and loss of K^+ from the cell. Although this sequence of events has been widely accepted, there is

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surprisingly little information on quantitative relationships between the principal factors involved: the free energy change for ATP hydrolysis and the magnitudes of ionic gradients. The object of this study was to explore these relationships using the C6 astrocytoma cell line. The C6 cells were chosen for the following reasons: 1. they are derived from a rat neural tumor and have been reported to retain several properties which are expressed by glial cells *in vivo* [3 - 5]. Since brain is very sensitive to hypoxia and ischemia [6 - 11], an insight into the behavior of glial cells (one of the two important classes of cells in this tissue) under limitation in oxygen supply is important for our understanding of the mechanism(s) of hypoxic/ischemic damage to the central nervous system; 2. we have studied these cells in some detail and established that they contain a powerful Na^+/K^+ ATPase [12]; 3. they represent a homogenous population of cells which are viable and highly reproducible.

MATERIALS AND METHODS

Cell culture. C6 cells (ATCC NoCCL107) were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland. They were grown in Ham's F-10 medium containing 15% horse serum and 2.5% fetal calf serum and penicillin/streptomycin. The cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C for 3 - 5 days. Cells were removed from the flasks in their exponential phase of growth with trypsin (Difco 1:250)/0.02% EDTA in phosphate buffered saline without calcium and magnesium salts. They were left in this medium at room temperature for approximately 5 min, removed from the flasks and centrifuged at 2000 rpm in a clinical bench top centrifuge. The pellet was washed once in Hanks' medium containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4 and 0.2% bovine serum albumin and finally suspended in the same medium at 7 - 10 mg dry weight/ml. The dry weights of the cell suspension and the medium were determined for each preparation.

Incubations. Cells suspended as described above were placed in a specially designed syringe — cuvette equipped with access ports for potassium and oxygen electrodes and a small magnetic stirring bar driven by an external magnetic stirrer. The concentrations of potassium and oxygen were monitored continuously. At appropriate intervals (see Tables for details) aliquots were withdrawn and either injected into an ice-cold solution of perchloric acid (5% final concentration) or centrifuged rapidly (Beckman microfuge) through a layer of silicone oil to separate cells from the suspending medium. The perchloric acid-treated samples were centrifuged to remove precipitated protein and the clear supernatants were neutralized with 1 M K_2CO_3-0.58 M triethanolamine. Aliquots of the extracts were used for assays of metabolites.

Samples centrifuged through silicone oil were utilized for measurement of intra- and extracellular potassium as described below.

Measurements of metabolites. The metabolites were determined by standard enzymatic procedures: ATP and creatine phosphate were assayed in the same sample by the method of Lamprecht & Trautschold [13] and Lamprecht *et al.* [14], respectively. ADP and creatine were measured in the same cuvette by the procedure of Bernt *et al.* [15].

Measurements of intra- and extracellular $[K^+]$. In addition to continuous monitoring of $[K^+]_o$ with the K^+-electrode, intra-and extracellular concentrations of this cation were determined by flame atomic absorption (Varian AA-475 atomic absorption spectrophotometer). The cell pellets were digested in a known volume of concentrated nitric acid for 24 h at room temperature and diluted appropriately in 0.1% cesium chloride in 0.1 M HNO_3. The extracellular medium was diluted directly in the cesium chloride-nitric acid matrix. The concentration of potassium in the samples was calculated from a standard curve (KCl dissolved in CsCl-HNO_3).

Measurement of intracellular water volume. Cells were incubated with tritiated water and [^{14}C]polyethylene glycol ($M_r = 4000$) and centrifuged through silicone oil. Total water volume of the pellet was determined from ^{3}H content and the extracellular water from that of ^{14}C. Intracellular water content was found to be 4.0 ± 0.4 µl/mg dry weight (mean $\pm$ SD, n = 8).

RESULTS

Effect of anaerobiosis on the high-energy phosphate compounds and K^+ gradients in C6 cells incubated with glucose

C6 astrocytoma cells suspended at 7 - 10 mg dry wt/ml with glucose as substrate consumed oxygen dissolved in the medium at a steady and linear rate (2.75 ± 5 nmol/min per mg dry wt. at 25°C) until anaerobiosis was attained (Fig. 1). Simultaneous measurement of the concentration of potassium in the suspension buffer showed that $[K^+]_o$ initially decreased somewhat and then attained a steady-state level which was not altered when oxygen was exhausted and cells remained anaerobic for 15 - 30 min. The high-energy phosphate compounds were measured in fully aerobic samples and at various intervals after the exhaustion of O_2. The results show (Table 1B) that the concentrations of ATP and ADP remained stable for at least 15 min of anaerobic incubation and were equal to those in the aerobic samples. There was, however, a progressive, albeit small, decline in [creatine phosphate] which was accompanied by a rise in [creatine]; consequently the [creatine phosphate]/[creatine] ratio decreased. When the content of potassium inside the cells was measured at the same points in time at which the metabolites

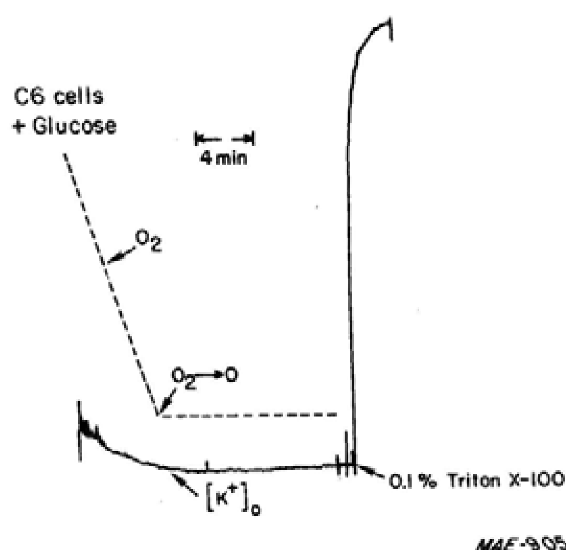

Fig. 1. Effect of anaerobiosis on $[K^+]_o$ in C6 cells incubated with glucose. C6 cells were suspended at 9.4 mg dry wt/ml as described in the Methods and placed in a syringe equipped with potassium and oxygen electrodes. The concentration of sodium in the medium was 145 mM and that of potassium 3 mM; glucose was 10 mM

Table 1

Levels of high energy phosphate compounds and creatine in C6 astrocytoma cells incubated with and without glucose

Concentrations (mM) of high-energy phosphate compounds and creatine were determined by standard enzymatic techniques [13 - 15]. Values are means ±SD for 3 -5 experiments

	ATP	ADP	CrP	Cr	ATP + ADP
A. no glucose					
aerobic	5.76 ± 0.52	0.82 ± 0.21	3.58 ± 0.34	4.74 ± 0.31	6.58
anaerobic					
0.5 min	5.46 ± 0.42	0.65 ± 0.10	3.61 ± 0.75	5.32 ± 1.10	6.11
7 min	3.68 ± 0.18	1.40 ± 0.30	0.81 ± 0.24	7.53 ± 1.20	5.08
15 min	2.13 ± 0.45	1.45 ± 0.29	0.63 ± 0.15	8.50 ± 1.92	3.58
B. 10 mM glucose					
aerobic	6.46 ± 0.60	0.37 ± 0.03	4.78 ± 1.20	3.23 ± 0.73	6.80
anaerobic					
0.5 min	6.25 ± 0.15	0.32 ± 0.01	4.39 ± 1.10	3.79 ± 0.88	6.57
7 min	5.98 ± 0.63	0.35 ± 0.05	3.80 ± 0.93	4.42 ± 0.73	6.33
15 min	6.08 ± 0.81	0.38 ± 0.02	2.97 ± 0.33	5.16 ± 0.65	6.46

were determined, it was found to remain constant at a value of 143 - 148 mM (Table 2B).

Glycolytic activity of C6 cells was evaluated by measuring the levels of lactate and pyruvate in the medium. Under aerobic conditions the cells produced lactate at a linear rate of 10.5 ± 0.5 nmol/min per mg dry wt. In the absence of oxygen the rate increased by about 30% to 13.8 ± 1.1 nmol/min per mg dry wt. (Both values are means $\pm$SD for 5 experiments).

Table 2

Relationships between the energy level and $[K^+]_i$ in C6 cells

[CrP] and [Cr] are taken from Table 1. $[K^+]_i$ was measured by atomic absorption. (Values are means $\pm$SD for 5 - 6 experiments.) $[ATP]_{free}/[ADP]_{free}$ was calculated assuming near equilibrium in creatine phosphokinase reaction (CPK). K_{CPK} at pH 7.4 and 1 mM free $[Mg^{2+}]$ was taken to be 66 [17]

	[CrP]/[Cr]	$[ATP]_{free}/[ADP]_{free}$	$[K^+]_i$ (mM)
A. no glucose			
aerobic	0.81	53	146 ± 5
anaerobic			
0.5 min	0.68	45	143 ± 8
7 min	0.11	7.3	127 ± 11
15 min	0.07	4.6	115 ± 15
B. 10 mM glucose			
aerobic	1.48	98	148 ± 7
anaerobic			
0.5 min	1.16	77	146 ± 5
7 min	0.86	57	148 ± 11
15 min	0.58	38	143 ± 7

At the concentration of cells used in this study millimolar levels of lactate were synthesized within minutes. By contrast, the amounts of pyruvate in the medium remained low (<0.1 mM) and did not change with time.

Effect of glucose withdrawal on the energy and K^+ levels in C6 cells

C6 cells incubated in the absence of glucose also rapidly consumed dissolved oxygen (Fig. 2) which suggests that endogenous substrates other than glucose (amino acids?) can serve as fuel for the respiratory chain. However, in these latter cells the levels of creatine phosphate and ATP were lower, whereas those of creatine and ADP were higher than the corresponding values in cells incubated with glucose. Moreover, upon exhaustion of oxygen, glucose-deprived cells leaked potassium into the external medium (Fig. 2, Table 2). This loss of K^+ from cells was accompanied by a rapid fall in

[creatine phosphate] and [ATP] and an increase in [creatine] and [ADP] so that the [creatine phosphate]/[creatine] ratio decreased by more than 10-fold in 15 min. It is important to note that the increase in the amount of ADP was smaller than the corresponding decline in the level of ATP.

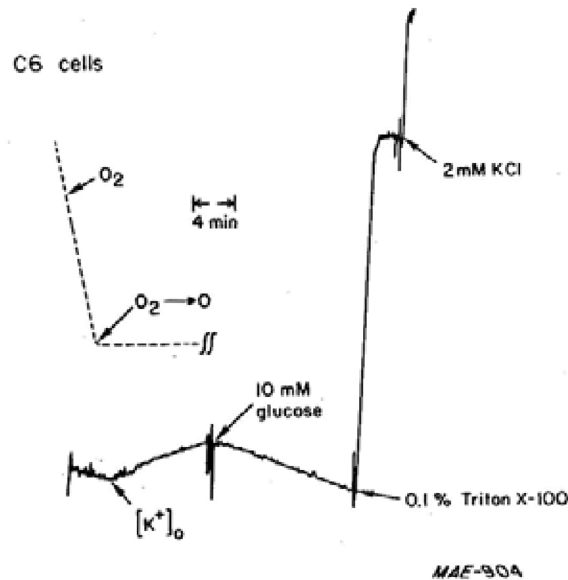

Fig. 2. Effect of anaerobiosis on $[K^+]_o$ in glucose-depleted cells. Conditions are the same as in Fig. 1 except that glucose was omitted from the initial incubation. Cell concentration was 9.6 mg dry wt/ml. At the end of the experiment 2mM KCl was added to calibrate the electrode

Hence, after 15 min of anaerobic incubation the sum of the two decreased to about one half of that present in the aerobic sample. This suggests that ADP was converted to AMP and perhaps even further to dephosphorylated products.

Figure 2 also shows that, when glucose was introduced to an anaerobic suspension of cells, the concentration of potassium in the medium began to decrease. This behaviour indicates that synthesis of ATP through glycolysis can support the activity of the Na^+/K^+ pump and lead to restoration of the K^+ gradient.

DISCUSSION

The results of this study show, in agreement with our previous work [12], that C6 astrocytoma cells incubated with glucose as substrate maintain large values of energy parameters ([creatine phosphate]/[creatine] and [ATP]/[ADP]) and high levels of intracellular potassium. The presence of creatine phosphate and creatine allows calculations of the free cytosolic [ATP]/[ADP]

ratios which are found to be substantially higher (Table 2) than those determined from measurements of the total ATP and ADP content of cells (Table 1). This difference is caused, to a large extent by binding of ADP and by compartmentation of the adenine nucleotides into intracellular organelles [16 - 18].

C6 cells, like all other cells in culture, are capable of powerful glycolytic activity. It can be calculated from the rates of O_2 uptake and lactate production that mitochondrial oxidative phosphorylation provides 16.5 nmol ATP/min per mg dry wt., while glycolysis supplies 10.5 nmol ATP/min per mg dry wt, i.e., almost 40% of the total amount of energy produced. (The stoichiometric factors used to calculate ATP synthesis are 6 for O_2 and 1 for lactate). It is interesting that under anaerobic conditions, the ATP produced by glycolysis is able to maintain normal levels of intracellular potassium, i.e., the operation of the Na^+/K^+ pump, even in the presence of a somewhat lowered $[ATP]_{free}/[ADP]_{free}$ (by a factor of 3, Table 2) and a decrease of about 50% in total ATP synthesized. This suggests that the amount of energy available from ATP hydrolysis is under these conditions sufficient to fuel the Na^+ pump (see below for the calculations). It is also possible that ATP produced by glycolysis may be utilized preferentially by the plasma membrane-bound ATPase.

Withdrawal of glucose together with oxygen from cell suspensions, eliminates both sources of energy production. Under such conditions the cellular ratio of $[ATP]_{free}/[ADP]_{free}$ falls and potassium leaks from cells, most likely because of the lack of energy for operation of the Na^+ pump. The free energy required to fuel the plasma membrane Na/K ATPase can be estimated assuming a stoichiometry of $3\ Na^+_{out}:2\ K^+_{in}:1$ ATP for this enzyme:

$$\Delta G_{Na,K} = RT \ln \{([Na^+]_o/[Na^+]_i)^3 ([K^+]_i/[K^+]_o)^2\} - FE \qquad \text{eq. 1}$$

Taking the intracellular $[Na^+]$ to be 5.8 mM with an external $[Na^+]$ of 145 mM, E of -90 mV [12] and utilizing the values of internal $[K^+]$ from Table 2 at the external concentration of 3 mM, one obtains an estimate for $\Delta G_{Na,K}$ of 12.33 kcal. The amount of free energy available in ATP hydrolysis can be calculated from the equation:

$$-\Delta G_{ATP} = \Delta G^{o'}_{ATP} + RT\ln \frac{[ATP]_{free}}{[ADP]_{free}[Pi]}$$

where the standard free energy for ATP hydrolysis, ΔG_{ATP} is 7.6 kcal/mol [19], $[Pi]_i$ is 3.61 mM [12] and $[ATP]_{free}/[ADP]_{free}$ ratios are listed in Table 2. Substituting the appropriate numbers into eq. 2, one obtains a figure of 13.62 kcal/mol in the presence of glucose and 13.27 kcal/mol in its absence. These values are 1 - 2 kcal higher than $\Delta G_{Na,K}$. On the other hand, under anaerobic conditions in the absence of glucose a decline in $[ATP]_{free}/[ADP]_{free}$ and a rise in $[Pi]_i$ substantially decrease ΔG_{ATP} and hence limit

energetically the operation of the pump. This, in turn, will cause an increase in $[Na^+]_i$ and a decline in $[K^+]_i$, consistent with the results shown in this investigation.

Another interesting observation described in the present study deserves comment. In anaerobic cells in the absence of glucose, the increase in [ADP] was much smaller than the decrease in [ATP]. A similar phenomenon has been noted in intact brain and shown to be accompanied by a rise in [AMP] [20, 21]. This suggests involvement of the adenylate kinase reaction which converts 2 molecules of ADP to AMP and ATP. It is possible that the latter reaction helps to maintain ATP levels and prevents the decline in [ATP]/[ADP].

Finally, it may be timely to discuss briefly the relevance of our *in vitro* studies on a glial cell line to the *in vivo* situation in the brain. It is well known that the concentration of intracellular potassium and the membrane electrical potential are much larger in cerebral glia than in neurons [22 - 26]. It would seem, therefore, that the concentrations of internal sodium are appropriately lower in the former cells. Hence $\Delta G_{Na,K}$ in the glial cells may be rather high and the presence of creatine phosphate and creatine might be necessary to maintain ATP at a level which is high enough to ensure proper operation of the Na^+/K^+ pump. Although no information is currently available on te glycolyti capacity of glial cells *in vivo*, it is possible that it is large enough to provide a substantial proportion of the total ATP produced. Moreover, if ΔG_{ATP} exceeds $\Delta G_{Na,K}$ by a relatively small amount of 1 - 2 kcal, a situation observed in C6 astrocytoma, this may mean that during cerebral ischemia. i.e.. in the absence of both oxygen and glucose, glial cells will easily lose potassium and gain sodium.

REFERENCES

1. Skou, J. C. (1965) Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.*, **45**, 596 - 617.
2. Glynn, I. M. & Karlish, S. J. D. (1975) The sodium pump. *Ann. Rev. Physiol.*, **37**, 13 - 55.
3. Pfeiffer, S. E., Herschman, H. R., Lightbody, J. & Sato, G. (1970) Synthesis by a clonal line of rat glial cells of a protein unique to the nervous system. *J. Cell. Physiol.*, **75**, 329 - 339.
4. Browning, E. T. & Nicklas, W. J. (1982) Induction of glutamine synthetase by dibutyryl cyclicAMP in C-6 glioma cells. *J. Neurochem.*, **39**, 336 - 341.
5. Higashida, H., Miki, N., Tanaka, T., Kato, K., Nakano, T., Nagatsu, T. & Kano-Tanaka, K. (1982) Receptor-associated changes of the catecholamine-sensitive adenylate cyclase in glioma cells doubly transformed with Moloney sarcoma virus. *J. Cell. Physiol.*, **110**, 107 - 113.
6. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J. Biol. Chem.*, **239**, 18 - 30.

7. Duffy, T. E., Nelson, S. R. & Lowry, O. H. (1972) Cerebral carbohydrate metabolism during acute hypoxia and recovery. *J. Neurochem.*, **19**, 959 - 977.
8. Brown, A. W. & Brierley, J. B. (1973) The earliest alterations in rat neurons and astrocytes after anoxia-ischaemia. *Acta Neuropathol.*, **23**, 9 - 22.
9. Kogure, K., Scheinberg, P., Utsunomiya, Y., Kishikawa, H. & Busto, R. (1977) Sequential cerebral biochemical and physiological events in controlled hypoxemia. *Ann. Neurol.*, **2**, 304 - 310.
10. Fahn, S., Davis, J. N. & Rowland, L. P. (Eds.) (1979) Cerebral Hypoxia and its Consequences. *Adv. Neurol.*, **26**, Raven Press, New York.
11. Siesjö, B. K. (1981) Cell damage in the brain: a speculative synthesis. *J. Cereb. Blood Flow Metab.*, **1**, 155 - 185.
12. Erecińska, M. & Silver, I. A. (1986) The role of gial cells in regulation of neurotransmitter amino acids in the external environment. I. Transmembrane electrical and ion gradients and energy parameters in cultured glial-derived cell lines. *Br. Res.*, **369**, 193 - 202.
13. Lamprecht, W. & Trautschold, I. (1974) Adenosine-5′-triphosphate: determination with hexokinase and glucose-6-phosphate dehydrogenase; in *Methods of Enzymatic Analysis*. Vol. 4, (H. U., Bergmeyer, ed) pp. 2101 - 2110 Academic Press, New York.
14. Lamprecht, W., Stein, P., Heinz, F. & Weisser, H. (1974) Creatine phosphate: determination with creatine kinase, hexokinase and glucose-6-phosphate dehydrogenase; in *Methods of Enzymatic Analysis*. Vol. 4, (Bergmeyer, H. U., ed.) pp. 1777 - 1781 Academic Press, New York.
15. Bernt, E., Bergmeyer, H. U. & Möllering, H. (1974) Creatine; in *Methods of Enzymatic Analysis*. Vol. 4, (H. U., Bergmeyer, ed.) pp. 1772 - 1776 Academic Press, New York.
16. Veech, R. L., Lawson, J. W. R., Cornell, N. W. & Krebs, H. A. (1979) Cytosolic phosphorylation potential. *J. Biol. Chem.*, **254**, 6538 - 6547.
17. Lawson, J. W. R. & Veech, R. L. (1979) Effects of pH and free Mg^{++} on the K_{eq} of the creatine kinase reaction and other hydrolases and phosphate transfer reactions. *J. Biol. Chem.*, **254**, 6528 - 6537.
18. Wilson, D. F., Nelson, D. & Erecińska, M. (1982) Binding of the intramitochondrial ADP and its relationship to adenine nucleotide translocation. *FEBS Lett.*, **143**, 228 - 232.
19. Guynn, R. W. & Veech, R. L. (1973) The equilibrium constants of the adenosine triphosphate hydrolysis and the adenosine triphosphate-citrate lyase reactions. *J. Biol. Chem.*, **248**, 6966 - 6972.
20. Kleihues, P., Kobayashi, K. & Hossman, K-A. (1974) Purine nucleotide metabolism in the cat after one hour of complete ischemia. *J. Neurochem.*, **23**, 417 - 425.
21. Kobayashi, M., Lust, W. D. & Passonneau, J. V. (1977) Concentrations of energy metabolites and cyclic nucleotides during and after bilateral ischemia in the gerbil cerebral cortex. *J. Neurochem.*, **29**, 53 - 59.
22. Dennis, M. J. & Gerschenfeld, H. M. (1969) Some physiological properties of identified mammalian neuroglial cells. *J. Physiol. (London)*, **203**, 211 - 222.
23. Futamachi, K. J. & Pedley, T. A. (1976) Glial cells and extracellular potassium: their relationship in mammalian cortex. *Br. Res.*, **109**, 311 - 322.
24. Kuffler, S. W. (1967) Neuroglial cells: physiological properties of glial cells in the central nervous system of amphibia. *Proc. Roy. Soc. London, Ser. B.*, **168**, 1 - 21.
25. Kuffler, S. W., Nicholls, J. G. & Orkand, R. K. (1966) Physiological properties of glial cells in the central nervous system of amphibia. *J. Neurophysiol.*, **29**, 768 - 787.
26. Somjen, G. G. (1975) Electrophysiology of neuroglia. *Ann. Rev. Physiol.*, **37**, 163 - 190.