Biochimica et Biophysica Acta, 775 (1984) 182–188
Elsevier

BBA 72233

THE INFLUENCE OF pH ON THE CELL MEMBRANE POTENTIAL OF PRIMARY CULTURED RAT HEPATOCYTES AS MEASURED WITH TETRAPHENYLPHOSPHONIUM AND DIMETHYLOXAZOLIDINE-2,4-DIONE

VOLKER EHRHARDT

1. Zoologisches Institut, Berliner Strasse 28, D-3400 Göttingen (F.R.G.)

(Received March 13th, 1984)

Key words: Membrane potential; pH gradient; Tetraphenylphosphonium distribution; Proton permeability; Intracellular pH; (Rat hepatocyte)

The accumulation of tetraphenylphosphonium in cultured rat hepatocytes is increased upon alkalization of the extracellular pH. External acidification causes a decrease in the ratio of the intracellular to the extracellular concentration of the cation. The addition of bicarbonate to the incubation medium induces an increase in the tetraphenylphosphonium distribution ratio whereas the effect of NH_4^+ is to decrease it. Concomitant measurements of the distribution of dimethyloxazolidine-2,4-dione show that the intracellular accumulation of tetraphenylphosphonium is a function of the pH difference across the plasma membrane, i.e. it depends on the magnitude and direction of the (normally outwardly directed) transmembrane proton concentration gradient. Since the distribution of the lipophilic cation qualitatively monitors changes of the electrical plasma membrane potential of the liver cells, it is concluded that the changes of the tetraphenylphosphonium distribution occurring with changes of the transmembrane pH difference reflect modulations of the cellular membrane potential. Taking into consideration the very low permeability of the liver cell membrane to passive proton movements, it is suggested that the plasma membrane of the liver cells contains an electrogenic proton-translocating mechanism which is accelerated by increasing and is slowed down by decreasing the transmembrane pH difference ($pH_i < pH_e$).

Introduction

In hepatocytes, as in other animal cells endowed with transport system A, the uptake of amino acids catalyzed by that system depends in a characteristic way on the extracellular pH. Maximal transport velocities are observed at slightly alkaline pH values, whereas a lowering of the incubation medium pH below pH 7.0 leads to a marked reduction of amino acid transport [1,2]. McGivan's investigation of the mechanism of the stimulating effect of bicarbonate on the uptake of serine and alanine in freshly isolated hepatocytes showed that the uptake of these system A-reactive amino acids is a linear function of the pH difference between intra- and extracellular space ($pH_i < pH_e$) [3]. Correspondingly, studies in our laboratory revealed that the uptake of the system A-specific amino acid 2-(methylamino)isobutyric acid is not a simple function of external or internal pH but rather parallels the proton concentration gradient across the plasma membrane [4].

In the discussion of his observations, McGivan proposed that the uptake of amino acids together with sodium ions occurs in exchange with protons. Measurements in our laboratory, however, did not reveal any change in the intracellular pH during

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium cation.

amino acid transport [4]. Furthermore, it has been shown that the system A-catalyzed transport into the liver cells is an electrogenic process [5].

An alternative explanation for the effect of pH changes on amino acid transport is suggested from the influence of a proton conductor on the accumulation of a lipophilic cation and the uptake of 2-aminoisobutyric acid in Ehrlich ascites tumor cells [6]. In these experiments it has been shown that the electrical potential difference across the plasma membrane (which is a driving force for the Na^+-coupled transport) is modulated by the magnitude and the direction of an imposed pH gradient. As an interpretation of their observation the authors proposed that the uncoupler shifts the plasma membrane potential towards an H^+-diffusion potential.

In view of these results it was of interest whether the proton distribution across the cell membrane also affects the membrane potential when the membrane permeability to passive proton movements is not artificially increased. In an attempt to detect a correlation between the transmembrane pH gradient and the electrical potential difference across the plasma membrane, the intracellular accumulation of the lipophilic cation TPP^+ as well as the distribution of 5,5'-dimethyloxazolidine-2,4-dione were determined in rat hepatocyte monolayer cultures under selected pH conditions. In addition, attempts were made to assess the passive proton permeability of the liver cell membrane. The results of these experiments support the existence of an electrogenic H^+-efflux mechanism whose contribution to the electrical cellular membrane potential depends on the magnitude of the transmembrane proton concentration gradient ($pH_i < pH_e$).

Methods

Adult rat hepatocytes were isolated by means of a modification of the collagenase perfusion technique described by Bonney [7]. The cells were suspended in Leibovitz L-15 medium containing 15% fetal calf serum and then plated into plastic petri dishes. After 18–20 h of culturing the medium was exchanged for L-15 without serum but additionally buffered with 10 mM Hepes at pH 7.4. The monolayers were used for the experiments on the third day of culturing.

Uptake experiments were started by incubating the cells at 37°C in a buffer solution containing the desired radiolabelled compound. The uptake was stopped by aspirating the radioactive medium and rinsing the monolayers with ice-cold buffer.

The buffer solution used for the uptake studies was a modified Hanks' salt solution (137.2 mM Na^+/5.8 mM K^+/1.3 mM Ca^{2+}/0.8 mM Mg^{2+}/143.6 mM Cl^-/0.8 mM SO_4^{2-}/0.8 mM PO_4^{3-}/20.0 mM Hepes/8.0 mM glucose). In some experiments the K^+ concentration in the buffer was varied while the concentrations of the other constituents except Na^+ (37.2 mM) remained unchanged. The osmolarity of the solutions was kept constant by adding appropriate amounts of choline$^+$.

The apparent internal pH of the liver cells was calculated from the distribution of 5,5'-dimethyloxazolidine-2,4-dione in equilibrium. As previously shown, the distribution of 5,5'-dimethyloxazolidine-2,4-dione across the liver cell membrane attains a steady state within 5 min [4]. The intracellular water volumes were determined as the intracellular space available to the non-metabolizable hexose 3-*O*-methyl-D-glucose [8]. ATP levels were measured in neutralized perchloric acid extracts with the luciferase/luciferin method. Electrophysiological control measurements were performed with the patch clamp system described by Hamill et al. [9].

Results and Discussion

The accumulation of TPP^+ with time is shown in Fig. 1. The distribution of the cation approaches an equilibrium within about 15 min. Assuming a partitioning of TPP^+ into a single compartment, its equilibrium distribution yields a value of about −100 mV for the cellular membrane potential. This seems to be a gross overestimation as the highest reported values, found in perfused rat livers with electrophysiological methods, are approx. −50 mV [10]. As can be inferred from measurements of the distribution of lipophilic cations with other respiring cells (see, for example, Ref. 11), the excessive accumulation of TPP^+ in the liver cells is due to its intramitochondrial accumulation. This opinion is

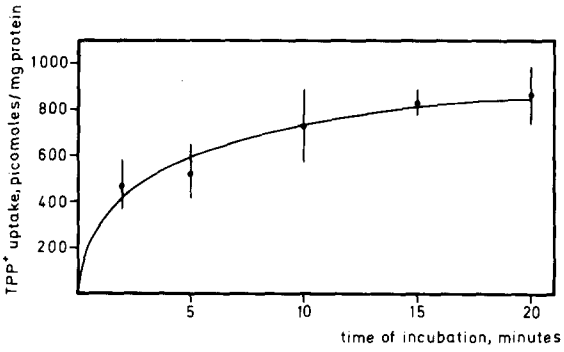

Fig. 1. The distribution of TPP^+ across the plasma membrane with time. The monolayers were incubated for the indicated time periods in a buffer solution (pH 7.4) containing [^{14}C]TPP^+ (10^{-5} M). The points represent the mean of at least four determinations ± S.D.

supported by the differential effect of inhibitors of mitochondrial metabolism on the TPP^+ distribution: In the presence of inhibitors known to reduce the electrical potential difference across the inner mitochondrial membrane the amount of TPP^+ taken up by the liver cells is decreased. The addition of oligomycin, however, leads to a marked increase of the intracellular TPP^+ accumulation (unpublished data). As oligomycin is known to prevent mitochondrial ATP synthesis but not the energization by substrate in isolated mitochondria, it is reasonable to conclude that the increased intracellular TPP^+ accumulation in the presence of this inhibitor mirrors a hyperpolarization of the mitochondrial membrane potential.

It follows from these observations that the absolute size of the cellular membrane potential of the hepatocytes cannot be determined by simply measuring the steady-state distribution of TPP^+ without any knowledge of the contribution of the mitochondria to the over-all accumulation of the lipophilic cation. However, as shown below, the TPP^+ distribution can serve as a qualitative indicator for changes of the electrical potential difference across the liver cell membrane.

In order to prove the sensitivity of the TPP^+ distribution to variations of the plasma membrane potential, the intracellular accumulation of the cation was measured at 0, 50 and 100 mM extracellular K^+ in the presence of 10^{-10} M valinomycin. This low valinomycin concentration was chosen because in concentrations regularly used for the experimental modulation of membrane potentials (about 10^{-6} M) the ionophore leads to a severe depolarization of the liver cell membrane potential [10,12]. The reason for this is not known. The concentration used in the present study seems sufficient to shift the plasma membrane potential of the hepatocytes towards a K^+ diffusion potential (Ref. 10 and own unpublished results). As can be inferred from the fact that 10^{-10} M valinomycin does not depress the ATP content of the liver cells (unpublished results), the mitochondrial potential of the liver cells is obviously not considerably affected by 10^{-10} M valinomycin.

The data shown in Table I demonstrate that the equilibrium distribution of TPP^+ decreases with increased extracellular K^+ concentration, i.e. under conditions known to lead to a depolarization of the plasma membrane potential. As revealed by electrophysiological control measurements, the cellular membrane potential of the hepatocytes depolarizes from about −15 mV in the K^+-free buffer solution to about −3 mV when the extracellular K^+ concentration is increased to 100 mM (unpublished data). As a consequence, one has to assume that the TPP^+ distribution qualitatively monitors changes in the electrical potential difference across the plasma membrane of the hepatocytes.

By using TPP^+ attempts were now made to investigate whether the cellular membrane potential is influenced by the pH. For this purpose, the intracellular steady state accumulation of TPP^+ was measured at different external pH values. The

TABLE I

EFFECT OF THE EXTRACELLULAR K^+ CONCENTRATION ON THE TPP^+ DISTRIBUTION

The TPP^+ distribution was measured after incubating the monolayers for 15 min in valinomycin (10^{-10} M)-supplemented solutions (pH 7.4) with the indicated K^+ content. The data are expressed as the average of 16–20 measurements ± S.D. (c_i, c_e: intracellular, extracellular concentration).

Extracellular K^+ concentration (mM)	TPP^+ distribution ratio (c_i/c_e)
0	60.5 ± 6.2
50	32.3 ± 3.4
100	21.0 ± 2.0

TABLE II

RELATIONSHIP BETWEEN THE TPP^+ DISTRIBUTION AND THE pH DIFFERENCE ACROSS THE PLASMA MEMBRANE

For the measurements of the TPP^+ and the 5,5'-dimethyloxazolidine-2,4-dione (DMO) distributions the hepatocytes were incubated for 15 min with [^{14}C]TPP^+ (10^{-5} M) or [^{14}C]-DMO ($5 \cdot 10^{-4}$ M) at the indicated external pH values. The TPP^+ distribution ratios and the 5,5'-dimethyloxazolidine-2,4-dione data (from which the apparent pH differences were calculated) are expressed as the mean of at least 12 measurements ± S.D. (c_i, c_e: intracellular, extracellular concentration).

pH_e	Addition	DMO distribution ratio (c_i/c_e)	Apparent ΔpH ($pH_e - pH_i$)	TPP^+ distribution ratio (c_i/c_e)
7.0	–	1.07 ± 0.10	−0.03	29.4 ± 2.8
7.4	–	0.76 ± 0.10	+0.13	35.2 ± 2.7
7.7	–	0.44 ± 0.06	+0.27	44.8 ± 2.3
8.0	–	0.35 ± 0.06	+0.47	49.8 ± 9.0
7.7	20 mM HCO_3^-	0.28 ± 0.05	+0.58	65.7 ± 5.6
8.0	20 mM NH_4^+	0.66 ± 0.11	+0.17	37.0 ± 3.2
8.0	1 mM ouabain	0.34 ± 0.03	+0.48	53.2 ± 6.1

results of these experiments show that the intracellular TPP^+ accumulation increases when the extracellular pH is raised from pH 7.4 to pH 8.0, whereas it decreases when the external pH is lowered from pH 7.4 to pH 7.0 (Table II). As the TPP^+ distribution can obviously serve as a qualitative indicator of changes in the electrical plasma membrane potential of the hepatocytes, these findings indicate that the pH has an influence on the electrical potential difference across the liver cell membrane. Electrophysiological control measurements revealing a depolarization of about 3 mV at pH 7.0 and a hyperpolarization of about 5 mV at pH 8.0 compared with the membrane potential at pH 7.4 confirm the interpretation of the TPP^+ data (unpublished results).

In order to see whether the observed modulations of the TPP^+ distribution are related in some way to changes in the intracellular H^+ concentration, it was necessary to obtain an estimate of the pH_i of the liver cells. The pertinent determinations were performed by using the equilibrium distribution of 5,5'-dimethyloxazolidine-2,4-dione. The results of these measurements show that, although the intracellular pH of the hepatocytes changes with the variations in the external pH, the proton concentration difference across the plasma membrane ($pH_i < pH_e$) increases with increasing extracellular pH values. On the basis of this finding it is considered that modulations of the liver cell membrane potential observed with changes of the extracellular pH are not a simple function of external or internal pH, but correlate with variations in the steepness of an outwardly directed transmembrane H^+ concentration gradient. In order to verify this assumption, the TPP^+ and 5,5'-dimethyloxazolidine-2,4-dione distributions were measured in the presence of bicarbonate or ammonium ions. Whereas bicarbonate is known to produce an increase in the transmembrane pH difference because of passive diffusion of CO_2 or H_2CO_3 into the cells leading to a net influx of protons, the incubation with ammonium ions produces a decrease in the transmembrane pH difference due to entry of NH_3 which then combines with protons (see, for example, Ref. 13).

As shown by the apparent pH_i values derived from 5,5'-dimethyloxazolidine-2,4-dione distributions, a steep outwardly-directed H^+ concentration gradient develops during the incubation of the liver cell cultures in buffer solutions supplemented with 20 mM bicarbonate. At external pH values between 7.0 and 8.0 the apparent pH difference across the cellular membrane is in the range of or even exceeds the ΔpH found with the bicarbonate-free medium at pH 8.0. Beyond its effect on the transmembrane proton concentration gradient, bicarbonate also influences the distribution of TPP^+. At comparable values of external pH the intracellular accumulation of the lipophilic cation was found to be higher when bicarbonate was present. As a reproducible control of the pH

of the bicarbonate medium during the 15 min-period of incubation proved to be difficult in the petri dishes, the 5,5′-dimethyloxazolidine-2,4-dione and TPP^+ distributions obtained in a representative series of experiments are shown here. In the course of these experiments the pH of the bicarbonate-supplemented medium shifted to the same final value of pH 7.7 (Table II).

In agreement with the findings concerning the effect of bicarbonate on the TPP^+ distribution in the liver cells, a depolarization of the plasma membrane potential of several types of cultured mammalian cells has been observed by Hülser when he reduced the intracellular HCO_3^- concentration from 44 mM to 6 mM [14]. Although measurements of the intracellular pH were not performed in this study, it is reasonable to assume that the omission of bicarbonate led to an increase in the internal pH of these cells and that the observed depolarizations correlated with the decrease in the transmembrane pH difference.

Since it can be expected that the effect of ammonium ions on the intracellular pH is more pronounced at higher extracellular pH values, the effect of ammonia on the TPP^+ and 5,5′-dimethyloxazolidine-2,4-dione distributions across the liver cell membrane was studied at a value of external pH of 8.0. Table II shows that the addition of 20 mM NH_4^+ to the incubation medium causes the apparent internal pH to become more alkaline. Furthermore, the addition of ammonia leads, probably due to a depolarization of the electrical potential difference across the liver cell membrane, to a decrease in the TPP^+ distribution ratio.

Considering the effects of bicarbonate and ammonium ions on the 5,5′-dimethyloxazolidine-2,4-dione and TPP^+ distributions, the assumption that the electrical cellular membrane potential of the liver cells is not simply a function of extra- or intracellular pH but is correlated with the steepness of an outwardly directed H^+ concentration gradient seems to be justified. The plot of the data given in Table II with the TPP^+ distributions plotted against the calculated differences in pH ($pH_e - pH_i$) supports this view, since all values of the TPP^+ distributions obtained under the different experimental conditions lie on the same line (not shown).

The assumption that the electrical membrane potential of the hepatocytes is a function of the transmembrane pH difference is further supported by measurements of the transport of the system A-specific amino acid 2-(methylamino)isobutyric acid. When the uptake of this amino acid was studied under the same experimental conditions as the TPP^+ distribution, the velocity of uptake was found to increase with an increase in the transmembrane pH difference (unpublished results). As it is known that the electrical plasma membrane potential is a driving force of the system A-catalyzed transport, the variations in the velocity of amino acid transport occurring with the changes in the transmembrane proton concentration gradient probably mirror modulations of the electrical plasma membrane potential.

The picture emerging from the results presented here is similar to the findings of Burckhardt and Pietrzyk [6] with Ehrlich ascites tumor cells. The authors observed an increase in the intracellular TPP^+ accumulation and 2-aminoisobutyric acid transport after a sudden extracellular alkalization, indicating a hyperpolarization of the electrical cellular membrane potential. As these studies were carried out with ATP-depleted cells in the presence of an uncoupler, the hyperpolarization of the Ehrlich cell membrane potential could be readily explained by the contribution of an H^+ diffusion potential. In the experiments with the liver cells, however, the modulations of the cell membrane potential which can be seen when the transmembrane proton concentration gradient is varied occur without artificially increasing the passive proton permeability of the membrane. Therefore, it was of interest to assess the permeability of the liver cell membrane to passive proton movements. The pertinent experimental test was performed by measuring the 5,5′-dimethyloxazolidine-2,4-dione distribution in liver cells incubated in valinomycin-supplemented buffer solutions containing different K^+ concentrations. As the 5,5′-dimethyloxazolidine-2,4-dione distribution demonstrates, the pH_i of the liver cells is not affected by the experimental modulation of the plasma membrane potential. Only after the addition of the uncoupler FCCP does the pH_i readjust to the membrane potential (Table III). Consequently, one has to assume that the cellular membrane of the hepatocytes is impermeable to passive proton move-

TABLE III

EFFECT OF VARYING THE CELLULAR MEMBRANE POTENTIAL ON THE 5,5'-DIMETHYLOXAZOLIDINE-2,4-DIONE DISTRIBUTION

The distribution of 5,5'-dimethyloxazolidine-2,4-dione (DMO) was measured after having incubated the monolayers for 15 min in valinomycin (10^{-10} M)-supplemented buffer solutions (pH 7.4) with the indicated K^+ content. In one series of experiments FCCP (10^{-5} M) was added to the incubation media. The results are expressed as the average of 6–14 measurements ± S.D. (c_i, c_e: intracellular, extracellular concentration).

Extracellular K^+ concn. (mM)	Addition of FCCP	DMO distribution ratio (c_i/c_e)	Apparent pH_i
0	–	0.30 ± 0.02	6.82
50	–	0.26 ± 0.02	6.73
100	–	0.29 ± 0.01	6.81
0	+	0.10 ± 0.02	6.33
50	+	0.15 ± 0.04	6.55
100	+	0.19 ± 0.04	6.70

TABLE IV

EFFECT OF INHIBITORS OF MITOCHONDRIAL METABOLISM ON THE APPARENT INTERNAL pH AND THE ATP CONTENT OF THE LIVER CELLS

The 5,5'-dimethyloxazolidine-2,4-dione (DMO) distribution and the ATP content of the cells were measured after a 15-min incubation of the monolayers in buffer solutions (pH 7.4) containing either antimycin A (2.5 μg/ml) or oligomycin (1 μg/ml) or none of the inhibitors. The apparent internal pH values were calculated from the 5,5'-dimethyloxazolidine-2,4-dione distribution. The 5,5'-dimethyloxazolidine-2,4-dione data and the intracellular ATP concentrations were expressed as the mean of at least ten determinations ± S.D. (c_i, c_e: intracellular, extracellular concentration).

Additions	DMO distribution ratio (c_i/c_e)	Apparent pH_i	ATP content (mM)
Control	0.76 ± 0.10	7.27	6.93 ± 0.93
Antimycin A	0.19 ± 0.10	6.57	0.39 ± 0.08
Oligomycin	0.24 ± 0.03	6.70	0.34 ± 0.11

ments. Hence, it is unlikely that proton diffusion is of importance for the relation between the magnitude of the transmembrane H^+ concentration gradient and the liver cell membrane potential.

There are several other possible explanations for the mechanism of modulation of the electrical plasma membrane potential due to changes in the transmembrane proton concentration gradient. A modulation of the electrogenic Na^+/K^+-pump activity as one possible explanation might be considered. However, as shown by the insensitivity of the TPP^+ distribution to ouabain, the Na^+/K^+-pump is obviously not involved in the observed variations of the electrical plasma membrane potential (Table II).

An alternative explanation for the correlation between the steepness of an outwardly directed proton concentration gradient and the electrical potential difference across the liver cell membrane is offered by studies with tight urinary epithelia. These experiments indicate that the apical membranes of a certain population of the epithelial cells contain an electrogenic proton-translocating ATPase in addition to an electroneutral Na^+/H^+ exchanger [15]. It is conceivable that the liver cells also possess an electrogenic proton extruding mechanism. Its acceleration on increasing and its slowing down on decreasing the transmembrane pH difference ($pH_i < pH_e$) would reasonably explain the modulations of the liver cell membrane potential occurring with changes in the pH difference across the cellular membrane. Possibly, plasma membranal electrogenic proton-translocating mechanisms are common to many types of animal cells. The repeatedly reported characteristic increase in the system A-catalyzed amino acid transport with an increased extracellular pH may be a hint to the contribution of such systems.

As far as the liver cell is concerned, an indication for the existence of an H^+ secreting mechanism which uses ATP hydrolysis as the driving force is given by the severe acidification of the pH_i of the hepatocytes observed in ATP-depleted cells (Table IV). Since more direct information about the H^+-translocating mechanism(s) involved in the regulation of the pH_i of the liver cells is lacking up to now, however, further conclusions are more than speculative. Therefore, the possibility that the hepatocytes are endowed, in addition to the postulated electrogenic mechanism, with an electroneutral system participating in the translocation of protons delivered by metabolic processes cannot be disregarded. This electroneutral mechanism could be a passive one such as an Na^+/H^+

antiporter, or an exchange pump such as the ($H^+ + K^+$)-ATPase proposed for the gastric mucosa [16].

Acknowledgement

The author is greatly indebted to Dr. J. Bormann (Max-Planck-Institut für Biophysikalische Chemie, Göttingen) for the electrophysiological control measurements. The studies described in this paper were supported by the Deutsche Forschungsgemeinschaft.

References

1 Oxender, D.L. and Christensen, H.N. (1963) J. Biol. Chem. 238, 3686–399
2 Le Cam, A. and Freychet, P. (1977) J. Biol. Chem. 252, 148–156
3 McGivan, J.D. (1979) Biochem. J. 182, 697–705
4 Ehrhardt, V. (1982) Arch. Int. Physiol. Biochim. 90, 103–109
5 Kristensen, L.Ø. (1980) J. Biol. Chem. 255, 5236–5243
6 Burckhardt, G. and Pietrzyk, C. (1980) Biochim. Biophys. Acta 601, 403–414
7 Bonney, R.J. (1974) In Vitro 10, 130–142
8 Kletzien, R.F., Pariza, M.W., Becker, J.E. and Potter, V.R. (1975) Anal. Biochem. 68, 537–544
9 Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100
10 Friedman, N. and Dambach, G. (1980) Biochim. Biophys. Acta 536, 180–185
11 Deutsch, C., Erecinska, M., Werrlein, R. and Silver, I.A. (1979) Proc. Natl. Acad. Sci. USA 76, 2175–2179
12 Hoek, J.B., Nicholls, D.G. and Williamson, J.R. (1980) J. Biol. Chem. 255, 1458–1464
13 Roos, A. and Boron, W.F. (1982) in Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions (Nuccitelli, R. and Deamer, D.W., eds.), Kroc Foundation Series, Vol. 15, pp. 205–219, Alan Riss, New York
14 Hülser, D.F. (1971) Pflügers Arch. 325, 174–187
15 Steinmetz, P.R. and Andersen, O.S. (1982) J. Membrane Biol. 65, 155–174
16 Forte, J.G., Machen, T.E. and Öbrink, K.J. (1980) Annu. Rev. Physiol. 42, 11–126