Biochimica et Biophysica Acta, 987 (1989) 115–123
Elsevier

BBAMEM 74670

A quantitative assessment of the use of $^{36}Cl^-$ distribution to measure plasma membrane potential in isolated hepatocytes

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(Received 22 June 1989)

Key words: Liver cell; Chloride distribution; Membrane potential

The plasma membrane potential of isolated rat hepatocytes was clamped at different values between 0 and −68 mV by addition of valinomycin in the presence of different extracellular concentrations of K^+, and measured by the distribution of $^{86}Rb^+$ between cells and medium. $^{36}Cl^-$ distribution came to steady state in 10–15 min. This steady-state distribution was compared to the plasma membrane potential over a range of values. $^{36}Cl^-$ distribution provided an accurate measurement of plasma membrane potential between −4 and −40 mV. At higher potentials intracellular chloride concentration is less than 20% of the extracellular concentration and errors due to uncertainties in the measurement of intracellular volume and of the contamination of cell pellets by extracellular medium precluded accurate determination of membrane potential: thus in our experiments $^{36}Cl^-$ underestimated the plasma membrane potential at −68 mV by 8 mV.

Introduction

Various methods have been used to measure the plasma membrane potential of liver cells. These include direct methods where cells are punctured with microelectrodes [1–6] and non-invasive indirect techniques like measurement of the accumulation of the radiolabelled lipophilic cations $TPMP^+$ and TPP^+ [7,8] or the exclusion of permeant anions like thiocyanate [9] and $^{36}Cl^-$ [7,10–13].

Direct measurements with microelectrodes are convenient for work with perfused livers or with cultured hepatocytes attached to a substratum, but are not easy with isolated hepatocytes in suspension. The measurement of plasma membrane potential with microelectrodes in anchored liver cells has yielded widely differing results, from −9.7 to −78 mV (negative inside) although a value around −35 mV is commonly obtained (see Ref. 5). Some workers [5] report that microelectrode readings are biphasic in time. These differences between reports may arise from cell damage during electrode insertion or from differences in the cell sealing process around the impaling electrode.

Indirect ion distribution methods are more suitable for measurement of plasma membrane potential in isolated liver cells. However, measurement of accumulation of cations (such as the lipophilic cations $TPMP^+$ and TPP^+) is generally unsuitable for measurement of plasma membrane potential in liver cells. These ions are accumulated not only across the plasma membrane but also across the mitochondrial membrane. The much greater potential across the mitochondrial membrane results in most of the cation being in the mitochondrial matrix despite the relatively small volume of this compartment. It is hard to overcome this problem. One way is to abolish the mitochondrial membrane potential with uncouplers such as FCCP under conditions in which the plasma membrane potential can be shown not to change; this has been done for lymphocytes [14]. The inhibition of mitochondrial function in liver cells is, however, not always desirable and the rate of glycolysis may be insufficient to maintain cell ATP levels in the presence of inhibitors and ionophores that impair mitochondrial function (see below and Refs. 9 and 12). Attempts to correct for mitochondrial accumulation by subtracting cation accumulation after abolition of the plasma membrane potential [7,15] are theoretically flawed since changes in plasma membrane potential will change the cytoplasmic concentration of the cation and

Abbreviations: $TPMP^+$, triphenylmethylphosphonium; TPP^+, tetraphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; E_{Cl}, E_K, equilibrium potential for Cl^- or K^+; BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate.

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so alter the total amount within the mitochondria even if the mitochondrial potential remains unchanged (see Ref. 14). A second way to avoid the problem of mitochondrial cation accumulation is to assume that the mitochondrial potential does not change under different conditions and to use the total cation uptake as a qualitative measure of plasma membrane potential (see, for example, Ref. 8). This assumption is clearly unsatisfactory when quantitative analysis is required.

Measurement of the exclusion of anions from the cells is in principle a better method of estimating the plasma membrane potential since anions will tend to be excluded rather than accumulated by intracellular organelles. Hoek et al. [9] used the distribution of thiocyanate across the hepatocyte plasma membrane to calculate the plasma membrane potential. In high K^+ medium and in the presence of FCCP or valinomycin these workers expected the plasma membrane to be completely depolarised but found the thiocyanate accumulation to be greater than 1. They introduced a correction term for unspecific binding of thiocyanate to cellular components assuming this binding to be independent of incubation conditions. Such binding corrections introduce considerable uncertainties in the measured values of plasma membrane potential particularly at high values.

For estimates of plasma membrane potential from the $^{36}Cl^-$ distribution across the hepatocyte plasma membrane it is necessary that $^{36}Cl^-$ is passively distributed according to the Nernst equilibrium and that its binding to intracellular components is negligible. Direct measurements of membrane potential using microelectrodes in perfused livers have been compared with the activity of intracellular chloride measured by tracer studies [1] and Cl^- sensitive electrodes [3,4] over a range of membrane potentials. The good correlation between membrane potential and Cl^- distribution suggested that Cl^- is passively distributed according to membrane potential in liver cells in situ. The pathway and the role of electrophoretic Cl^- transport in liver are becoming clearer. There is recent evidence for the existence of a DIDS-sensitive Cl^- channel in liver [7]. Chloride efflux through this channel may be activated by raised cAMP levels [16] in response to increased plasma membrane potential [12]; this may be important in the formation of bile [16]. Other pathways for Cl^- transport may also exist. For example it is reported that bilirubin stimulates a Cl^-/organic anion exchange mechanism in perfused livers [17]. In epithelial tissues Na^+-coupled Cl^- transport occurs [18]. However, under unstimulated conditions ion replacement studies have found no consistent evidence for Na^+ and Cl^- flux coupling in liver cells [4,6]. Although Cl^-/HCO_3^- exchange occurs in liver cells, its physiological role remains unclear [19].

The distribution of $^{36}Cl^-$ has also been used to measure the plasma membrane potential in isolated hepatocytes [10–13]. A good qualitative demonstration that $^{36}Cl^-$ distribution follows the plasma membrane potential has been provided by Bradford et al. [12]. These workers observed a hyperpolarisation of the plasma membrane potential in the presence of cAMP and a depolarisation of the plasma membrane potential of cells incubated in high K^+ medium as measured by the distribution of $^{36}Cl^-$. They compared these values of plasma membrane potential with those measured directly with microelectrodes in perfused liver by other workers under similar conditions and found good agreement. Bear et al. [7] proposed that [^{3}H]TPP$^+$ accumulation and $^{36}Cl^-$ distribution measured the same value of plasma membrane potential in hepatocytes at high values (−40 mV) in the presence of valinomycin and at low values (−29 mV) in the absence of valinomycin, but the correction they used for TPP$^+$ binding was inappropriate (see above). Despite these studies, a quantitative evaluation of the use of $^{36}Cl^-$ distribution to measure plasma membrane potential over a range of values has not been reported. Experiments in which the mitochondrial membrane potential is measured in situ with lipophilic cation probes [9,11] and safranine dyes [10] require simultaneous and accurate measurement of the plasma membrane potential. This is particularly important for those conditions in which the plasma membrane potential may change: hormonal treatments [2,20], the nutritional status [2,3], alterations in intracellular pH [21] and temperature [22].

To test the possibility that non-electrogenic Cl^- transport may affect the transmembrane distribution of Cl^- we have compared the distribution of $^{36}Cl^-$ with the equilibrium potential for K^+ (E_K) over a range of values. Valinomycin was present to increase the K^+ conductance of the membrane to high values and so clamp the plasma membrane potential at E_K. Plasma membrane potential was set at different values by varying extracellular [K^+], and measured using $^{86}Rb^+$ accumulation. The results show that the distribution of $^{36}Cl^-$ provides accurate measurement of the plasma membrane potential over a range of −4 to −40 mV. At high potential (−68 mV) the $^{36}Cl^-$ distribution underestimates the actual potential. The use of $^{36}Cl^-$ for measurement of high plasma membrane potentials may be limited by the inaccuracy in determinations of cell volume and extracellular contamination of cell pellets.

Experimental

Materials. ^{86}RbCl, [^{3}H]TPMP iodide, Na^{36}Cl, hydroxy[^{14}C]methylinulin, and ^3H_2O were from Amersham International plc, Amersham, U.K. Valinomycin, oligomycin, BSA, inulin and Trypan blue were from Sigma Chemical Company Ltd., Poole, U.K. Myxothiazol was from Boehringer Mannheim, Mannheim, F.R.G. Dinonyl phthalate and silicone fluid D.C.

550 were from BDH Chemials Ltd., Poole, U.K. TPMP bromide was from Aldrich Chemical Co. Ltd., Gillingham, U.K. Rhodamine 6G was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Firefly Lantern extract was from Sigma Chemical Co., P.O. Box 14508, St. Louis, U.S.A.

Preparation of hepatocytes. Hepatocytes were prepared from fed female Wistar rats (230–280 g) by a modification [23] of the method of Berry and Friend [24]. The viability of freshly isolated cells as determined by exclusion of 0.3% Trypan blue was greater than 90%. Cells were stored on ice for 0 to 3 h before use. Dry cell weight was calculated by drying a known volume of cells at 70°C for 24 h and subtracting the dry weight of an equal volume of medium.

Determination of accumulation of $^{86}Rb^+$, [^{3}H]TPMP$^+$, and $^{36}Cl^-$ by hepatocytes. Hepatocytes were incubated in 20-ml stoppered glass vials in medium containing 105.8 mM NaCl, 5.2 mM KCl, 25 mM $NaHCO_3$, 0.41 mM $MgSO_4$, 10.42 mM Na_2HPO_4, 2.5 mM $CaCl_2$, 10 mM glucose, 0.001% (w/v) Phenol red and 2.25% (w/v) defatted BSA. Stock 9% BSA was dialysed against 153 mM NaCl, 10.8 mM KCl and defatted by the method of Chen [25]. The medium was equilibrated to pH 7.4 with a mixture containing 95% air and 5% carbon dioxide. In media containing different K^+ concentrations, NaCl was replaced in part or completely with the same concentration of KCl and the BSA was replaced in part or completely with BSA dialysed against 150 mM KCl, to give final calculated concentrations of 5.6, 45, 86 and 125 mM K^+.

2 ml of cells (15–20 mg wet wt./ml) were routinely preincubated, in the presence of 1.0 μM carrier TPMP bromide, 0.1 mg/ml carrier inulin and radiochemicals (1 μCi [^{3}H]TPMP$^+$/ml and 5 μCi $^{86}Rb^+$/ml or 0.1 μCi $^{36}Cl^-$/ml and 2.5 μCi ^3H_2O/ml), for 10 min to allow the cells to establish ion gradients after being stored on ice [26]. At zero time inhibitors and ionophores (valinomycin (0–200 μM), myxothiazol (3 μM) and oligomycin (10 μg/ml)) were added. Cells were then incubated for 20 min unless otherwise indicated. For the time course experiments in Figs. 2 and 3 the radiochemicals were added at different times during the cell incubation to vary the length of time with radiochemical but keep the total length of the experiment constant. At the end of incubations duplicate 700 μl samples were removed and pipetted into 1.5 ml microfuge tubes at room temperature containing 100 μl of 2% (v/v) Triton X-100/0.25 M sucrose overlaid with 350 μl of oil (42% (v/v) dinonyl phthalate/58% silicone fluid D.C. 550) and centrifuged in an Eppendorf microfuge for 2 min. A 200-μl sample of the supernatant was removed and placed in a scintillation vial which was covered to prevent evaporation. The remaining supernatant and oil were aspirated and the sides of the microfuge tube were wiped clean. The pellet was resuspended by vortex mixing, the tube was cut through and the bottom of the tube containing the pellet mixture was placed in a scintillation vial and 3.0 ml of scintillant (optiphase 'HiSafe' II, LKB Scintillant Products, Loughborough, Leics., U.K.) was added to this and the 200 μl of supernatant. Parallel determination of intra- and extracellular pellet spaces were performed with each experiment. Cells were incubated with 2.5 μCi ^3H_2O/ml and 0.1 μCi hydroxy[^{14}C]methylinulin/ml for measurement of total pellet space (^3H_2O space) and extracellular space of pellet ([^{14}C]inulin space). ^3H_2O was added to cells at the start of incubation, hydroxy[^{14}C]methylinulin was added immediately prior to sampling. We found that under standard incubation conditions the time of incubation of cells with hydroxy[^{14}C]methylinulin made no difference to the apparent extracellular space. Radioactivity in supernatants and pellets was determined by dual-channel liquid scintillation counting for ^{3}H and ^{86}Rb, ^{3}H and ^{36}Cl, and ^{3}H and ^{14}C by using quench and crossover corrections as appropriate. The apparent volume of pellet available to each isotope (its space in μl) was calculated as (dpm in total pellet)/(dpm per μl of supernatant sample). The accumulation ratios ($[\text{cation}]_{in}/[\text{cation}]_{out}$) for $^{86}Rb^+$ and [^{3}H]TPMP$^+$ were calculated as (cation space − [^{14}C]inulin space)/(^3H_2O space − [^{14}C]inulin space). For greater precision correction for total pellet volume was made in each pellet containing $^{36}Cl^-$, thus $^{36}Cl^-$ distribution ($[Cl^-]_{in}/[Cl^-]_{out}$) was calculated as [(^3H_2O space − [^{14}C]inulin space) − (^3H_2O space − $^{36}Cl^-$ space)]/(^3H_2O space − [^{14}C]inulin space).

E_{Cl} and E_K were calculated from the $^{36}Cl^-$ distribution and the $^{86}Rb^+$ accumulation ratio using the Nernst equation.

$$E_{Cl} = +61.5 \log[Cl]_{in}/[Cl]_{out}$$

$$E_K = -61.5 \log[Rb]_{in}/[Rb]_{out}$$

Microscopy. Rhodamine 6G was dissolved in dimethylsulphoxide at a concentration of 0.1 mg/ml. This was stored at 4°C for up to 2 weeks. Cells were incubated as described in the legend to Fig. 6 to abolish mitochondrial membrane potential and loaded with rhodamine 6G (0.1 μg/ml) for 10 min. Rhodamine 6G will be concentrated into the cells under the influence of the plasma membrane potential under these conditions. A sample of the cell suspension was placed on a microscope slide and covered with a coverslip. Cells stained with rhodamine 6G were examined by epifluorescent illumination on a Leitz Diavert fluorescence microscope equipped with a high pressure Xenon lamp and a Leitz N filter block (530–570 nm excitation filter). Photographs were made using Kodak T-max (ASA400) film with an exposure time of 1 min. Phase contrast photo-

graphs of the same field of view were taken with the light switched on and an exposure time of 30 s. Kodak T-max film was developed for 6 min in Kodak HC110 (dilution B). The portion of cells which stain with Rhodamine 6G and so have a plasma membrane potential and the portion of cells that did not stain and are depolarised were counted from the photographs taken with fluorescence. Phase contrast photographs gave the total cell number.

To compare the portion of cells that exclude Trypan blue with the portion that stain with Rhodamine 6G, parallel incubations of cells were made as for the Rhodamine 6G loading experiment. At the end of incubation a sample of cell incubation was mixed with an equal volume of 0.6% (w/v) Trypan blue (dissolved in saline) and examined under a light microscope. Unstained cells and blue stained cells were counted.

ATP assays. Intracellular ATP was measured using Firefly Lantern extract in a Dupont 760 Luminescence Biometer as in [27].

Results

Effect of valinomycin on [^{3}H]TPMP$^+$ and ^{86}Rb$^+$ accumulation in hepatocytes

Hepatocytes were titrated with the potassium ionophore valinomycin (0–200 μM) in order to obtain a concentration of valinomycin that greatly increased K^+ flux across the plasma membrane and so brought the plasma membrane to E_K without collapsing the K^+ gradient. The K^+ gradient across the plasma membrane was monitored by measuring $^{86}Rb^+$ accumulation into cells. The distribution of [^{3}H]TPMP$^+$ was measured simultaneously. Mitochondrial uptake of [^{3}H]TPMP$^+$ was decreased by addition of the respiratory chain inhibitor, myxothiazol (3 μM) and the ATP synthase inhibitor, oligomycin (10 μg/ml). In the presence of oligomycin cells will be prevented from maintaining a mitochondrial membrane potential by the hydrolysis of ATP produced during glycolysis. For cells prepared from fed rats and respiring on 10 mM glucose cell ATP levels fell by only 10–15% over a 40-min incubation in the presence of myxothiazol and oligomycin (results not shown). These experiments were not possible with cells from starved rats since the inhibitors and ionophores caused substantial decreases in cell ATP levels and complete loss of the K^+ gradient (results not shown).

Fig. 1 shows the effect of valinomycin on [^{3}H]TPMP$^+$ and $^{86}Rb^+$ accumulation in hepatocytes. In the absence of valinomycin the 14-fold accumulation of [^{3}H]TPMP$^+$ represents equilibration of this ion with the plasma membrane potential and any residual mitochondrial membrane potential in the presence of myxothiazol and oligomycin. The addition of 0.1 μM valinomycin resulted in a loss of [^{3}H]TPMP$^+$ from the cells. This is likely to be due to selective depolarisation of mitochondria by valinomycin without altering plasma membrane K^+ fluxes as observed with lymphocytes [28]. At higher concentrations of valinomycin [^{3}H]TPMP$^+$ accumulation was increased. This is due to hyperpolarisation of the plasma membrane as a result of increased membrane conductance to K^+. At concentrations of

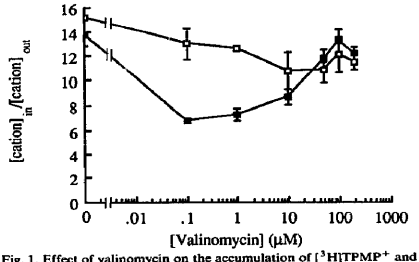

Fig. 1. Effect of valinomycin on the accumulation of [^{3}H]TPMP$^+$ and $^{86}Rb^+$ by hepatocytes. Cells were preincubated for 10 min at 37°C. At zero time valinomycin (0–200 μM), myxothiazol (3 μM), oligomycin (10 μg/ml), [^{3}H]TPMP$^+$ and $^{86}Rb^+$ were added. [^{3}H]TPMP$^+$ and $^{86}Rb^+$ in pellet and supernatant were measured at 50 min and the accumulation ratios were calculated as described in the Experimental section. [^{3}H]TPMP$^+$ accumulation ratios were corrected for binding of TPMP$^+$ to cytoplasmic components by determining the TPMP$^+$ accumulation into the cells, in the absence of a mitochondrial membrane potential, at several known values of plasma membrane potential. The plasma membrane potential was clamped by adding valinomycin and varying the external K^+ concentration. The binding correction factor for TPMP$^+$ was 0.21. This means that 21% of TPMP$^+$ in the cytoplasm is bound. The data shown are mean and range of two experiments: ■, [^{3}H]TPMP$^+$ accumulation ratio; □, $^{86}Rb^+$ accumulation ratio.

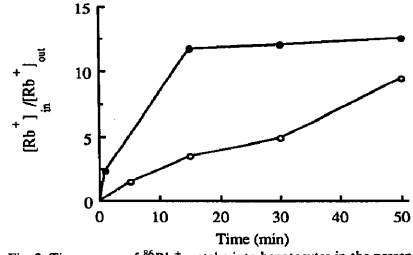

Fig. 2. Time course of $^{86}Rb^+$ uptake into hepatocytes in the presence and absence of valinomycin. After 10-min preincubation, cells were incubated for 50 min with myxothiazol (3 μM) and oligomycin (10 μg/ml) in the presence (●) or absence (○) of 100 μM valinomycin. $^{86}Rb^+$ was added to cells at the indicated times before termination and the experiment was terminated at 50 min. The $^{86}Rb^+$ accumulation ratio was calculated as described in the Experimental section. Results are means of duplicate points from a single experiment.

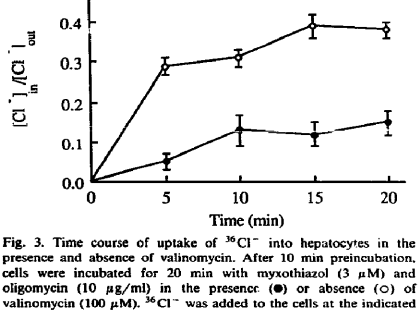

Fig. 3. Time course of uptake of $^{36}Cl^-$ into hepatocytes in the presence and absence of valinomycin. After 10 min preincubation, cells were incubated for 20 min with myxothiazol (3 μM) and oligomycin (10 μg/ml) in the presence (●) or absence (○) of valinomycin (100 μM). $^{36}Cl^-$ was added to the cells at the indicated times before termination and the experiment was terminated at 20 min. $^{36}Cl^-$ distribution was calculated as described in the Experimental section. Results represent mean and range of two experiments.

valinomycin above 50 μM the $^{86}Rb^+$ and [^{3}H]TPMP$^+$ come to equilibrium with the E_K. This was approximately -70 mV (negative inside) as measured from the [^{3}H]TPMP$^+$ and $^{86}Rb^+$ accumulation (see Fig. 1). Fig. 1 also shows that there is only a small drop in the $^{86}Rb^+$ accumulation with increasing valinomycin. From the $^{86}Rb^+$ accumulation ratio it can be calculated that the intracellular concentration of K^+ in the presence of 100 μM valinomycin is 70 mM ($[K^+]_{out} = 5.6$ mM).

Time course of $^{86}Rb^+$ uptake

Fig. 2 shows that the rate of $^{86}Rb^+$ uptake into hepatocytes was increased in the presence of 100 μM valinomycin. Steady-state $^{86}Rb^+$ distribution with valinomycin was achieved within 15 min and control $^{86}Rb^+$ distribution, in the absence of valinomycin, had not reached steady state even after 50 min. These results show that the $^{86}Rb^+$ and thus the K^+ conductance of the plasma membrane was greatly increased in the presence of this concentration of valinomycin.

Time course of $^{36}Cl^-$ uptake

Fig. 3 shows the time course of $^{36}Cl^-$ distribution into hepatocytes in the presence and absence of valinomycin. The half-time for $^{36}Cl^-$ equilibration seems unaffected by the presence of valinomycin and in both cases it is equilibrated by about 10–15 min. The time of equilibration of $^{36}Cl^-$ may be less but for these experiments it was important that $^{36}Cl^-$ had reached steady state. Therefore 15 min was chosen for the following experiments to allow equilibration of both $^{36}Cl^-$ and $^{86}Rb^+$. A similar time course for the uptake of $^{36}Cl^-$ was presented by Bradford et al. [12]. The rapid equilibration of $^{36}Cl^-$ in hepatocytes shows that it is a suitable probe for measuring relatively short term changes in E_{Cl} in these cells.

Qualitatively the data presented in Fig. 3 suggests the $^{36}Cl^-$ distribution reflects the plasma membrane potential. In the absence of valinomycin $^{36}Cl^-$ equilibrates with the resting plasma membrane potential (approximately -27 mV from data presented in Fig. 3; compare with microelectrode measurements [1–3,5]). In the presence of valinomycin it equilibrates with a hyperpolarised membrane potential (approximately -54 mV from data presented in Fig. 3). This supports evidence that $^{36}Cl^-$ is passively distributed according to the plasma membrane potential [1,3,4,6,7,12].

Effect of increasing $[K^+]_{out}$ on cell volume

For cells incubated in standard NaCl medium, in the absence of ionophores and inhibitors, the cell volume was 1.7 ± 0.07 μl/mg dry wt. (mean of seven observations ± S.E.). In the same medium in the presence of valinomycin, oligomycin and myxothiazol (Fig. 4) it was 1.5 ± 0.10 μl/mg dry wt. (mean of three observations ± S.E.). This lack of a significant ($p > 0.1$) volume change contrasts with the results of Bear et al. [7] who observed a shrinking of cells of 16% in the presence of valinomycin. Fig. 4 shows that at increased $[K^+]_{out}$ the cells increased slightly in volume; this increase was taken into account in subsequent calculation of E_{Cl} and E_K.

Comparison of E_K and E_{Cl}

The results presented above show that valinomycin at a concentration of 100 μM greatly stimulated $^{86}Rb^+$

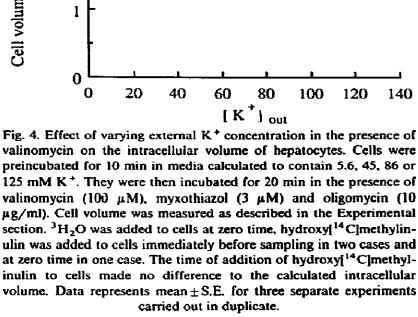

Fig. 4. Effect of varying external K^+ concentration in the presence of valinomycin on the intracellular volume of hepatocytes. Cells were preincubated for 10 min in media calculated to contain 5.6, 45, 86 or 125 mM K^+. They were then incubated for 20 min in the presence of valinomycin (100 μM), myxothiazol (3 μM) and oligomycin (10 μg/ml). Cell volume was measured as described in the Experimental section. ^3H_2O was added to cells at zero time, hydroxyl[^{14}C]methylinulin was added to cells immediately before sampling in two cases and at zero time in one case. The time of addition of hydroxyl[^{14}C]methylinulin to cells made no difference to the calculated intracellular volume. Data represents mean ± S.E. for three separate experiments carried out in duplicate.

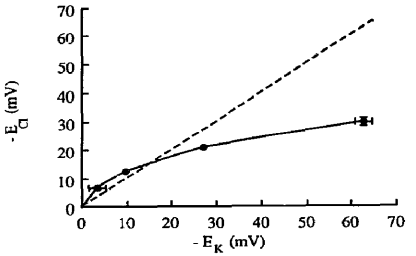

Fig. 5. Relationship between E_{Cl} and E_K. Cells were preincubated in media containing 5.6, 45, 86, 125 mM K^+ for 10 min and then incubated for 20 min in the presence of valinomycin (100 μM), myxothiazol (3 μM) and oligomycin (10 μg/ml). $^{36}Cl^-$ distribution and $^{86}Rb^+$ accumulation ratio were determined in parallel incubations by addition of $^{36}Cl^-$ or $^{86}Rb^+$ to cells at zero time. A parallel determination of intracellular volume was performed for each $[K^+]_{out}$. ^3H_2O was added to cells at the start of incubation; hydroxy[^{14}C]methylinulin was added to cells immediately before sampling in two cases and at the start of incubation in one case. E_{Cl} and E_K were calculated from the $^{36}Cl^-$ distribution and the $^{86}Rb^+$ accumulation ratio as described in the Experimental section. The dotted line shows the expected result if $^{36}Cl^-$ is passively distributed across the plasma membrane. Data are means ± S.E. of three experiments carried out in duplicate.

fluxes across the plasma membrane. The [^{3}H]TPMP$^+$ accumulation into cells at this concentration of valinomycin indicates that the plasma membrane potential is clamped at the K^+ diffusion potential. In the presence of valinomycin $^{36}Cl^-$ and $^{86}Rb^+$ distribution reached steady state within 15 min of addition of isotope to the cells.

We were then able to measure the distribution of $^{36}Cl^-$ at different clamped values of the plasma membrane potential (varied by changing extracellular K^+ concentration) to see whether E_{Cl} was the same as E_K in the presence of valinomycin i.e. whether the distribution of $^{36}Cl^-$ gives a quantitative measure of the plasma membrane potential. Fig. 5 shows that for values of E_K above about −20 mV E_{Cl} falls below that expected (indicated by the dotted line) if $^{36}Cl^-$ were passively distributed according to the Nernst equilibrium. The results show that the distribution of $^{36}Cl^-$ underestimates the plasma membrane potential and suggests that at higher potentials $^{36}Cl^-$ is accumulated to a greater extent than would be expected for a passive distribution of this ion.

This result might occur if there was an active inward movement of $^{36}Cl^-$ despite good evidence to suggest that $^{36}Cl^-$ is passively distributed across the plasma membrane [1,3,4,6,7,12,29]. It could also be caused by binding of $^{36}Cl^-$ to intracellular components, but there is no evidence that this occurs. Alternatively such a result might be obtained if $^{36}Cl^-$ was entering a space in the cell pellet that hydroxy[^{14}C]methylinulin, the extracellular marker, was unable to penetrate. This could happen if a portion of cells are depolarised and thus have $^{36}Cl^-$ evenly distributed across the plasma membrane but are still able to exclude hydroxy[^{14}C]methylinulin. We examined this possibility by comparing the proportion of cells that stain with a fluorescent probe of membrane potential, Rhodamine 6G [30,31], with the proportion of cells that are able to exclude Trypan blue. Here we are using Trypan blue exclusion as a measure of hydroxy[^{14}C]methylinulin exclusion. We believe this to be a fair assumption since cells take up Trypan blue when there is gross structural damage to the plasma membrane and inulin is a much larger molecule than Trypan blue. Rhodamine 6G is normally used as a probe for mitochondrial potential in cells [30,31]; here we are depolarising the mitochondria by addition of valinomycin (and myxothiazol and oligomycin) and observing the much smaller signal due to accumulation across the plasma membrane in response to the plasma membrane potential.

Comparison of the proportion of Rhodamine 6G stained cells with the proportion of cells excluding Trypan blue

Cells were loaded with Rhodamine 6G and photographs were taken as described in the Experimental section. Fig. 6(a) shows that cells in standard NaCl medium without inhibitors take up Rhodamine 6G and appear bright. The proportion of these cells stained with Rhodamine 6G is similar to the proportion excluding Trypan blue (see Table I). Fig. 6(b) shows that cells that are depolarised (incubated in high K^+ medium in the presence of valinomycin) stain poorly with Rhodamine 6G and appear dull on the photographs. Fig. 6(c) shows cells incubated in NaCl medium with valinomycin, myxothiazol and oligomycin. It can clearly be seen that there are two distinct populations of cells, some bright (polarised) and some dull (depolarised). A greater proportion of the cells appear dull under these conditions. The phase contrast photograph of the same field of view of cells taken of fluorescence Fig. 6(d) allows a total cell count to be made. This deleterious effect on the plasma membrane potential of some of the cells is caused by valinomycin, since only a small proportion of the cells appear depolarised when incubated in NaCl medium in the absence of valinomycin but in the presence of myxothiazol and oligomycin (Fig. 6(e) and (f)).

Table I gives the proportion of cells which exclude Trypan blue and stain with Rhodamine 6G under these three conditions. For cells incubated in NaCl medium in the presence of valinomycin there is a large difference between % Trypan blue exclusion and % cells that are polarised. If we assume that the portion of cells excluding Trypan blue represents the portion of cells excluding hydroxy[^{14}C]methylinulin then 20.5% of the cell

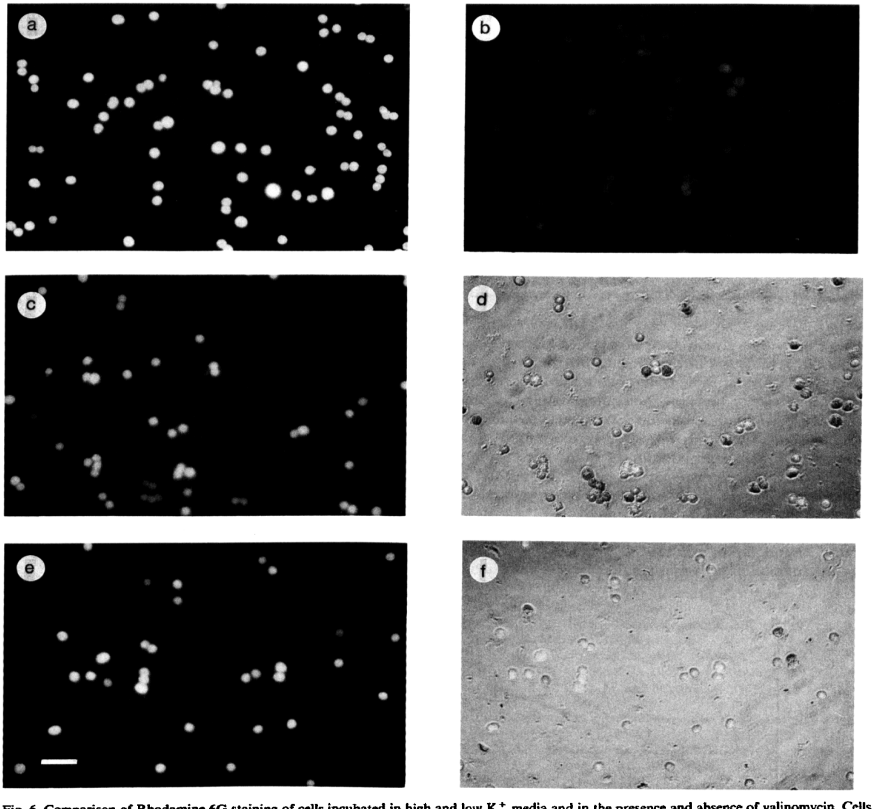

Fig. 6. Comparison of Rhodamine 6G staining of cells incubated in high and low K^+ media and in the presence and absence of valinomycin. Cells were incubated in standard NaCl medium, $[K^+] = 5.6$ mM (a), KCl medium (125 mM) (b) or standard NaCl medium in the presence of valinomycin (100 μM), myxothiazol (3 μM) and oligomycin (10 μg/ml), (c) and (d), or in standard NaCl medium with myxothiazol and oligomycin only, (e) and (f). Cells were preincubated for 10 min before the addition of inhibitors and then incubated for 10 min. Rhodamine 6G (0.1 μM) was then added to cells and after a further 10 min a sample of cells was placed on a microscope slide, covered with a cover slip and immediately photographed as described in the Experimental section. Photographs showing fluorescence of Rhodamine 6G are shown in (a), (b), (c) and (e). Phase contrast photographs of the same field of view of cells in (c) and (e) are shown in (d) and (f), respectively. Size bar on (e) = 50 μm. All other photographs were of the same magnification.

water space that is impermeable to inulin has $^{36}Cl^-$ evenly distributed across it in the presence of valinomycin.

The heterogeneous cell population for Rhodamine 6G staining of cells incubated in NaCl medium in the presence of valinomycin, myxothiazol and oligomycin may be the result of a fall in cell ATP levels. As indicated above cell ATP levels fell by 10–15% in the presence of oligomycin and myxothiazol alone over a 30-min incubation as compared to control incubations.

TABLE I

Comparison of % of cells staining with Rhodamine 6G with % of cells excluding Trypan blue

The proportion of cells staining with Rhodamine 6G was determined from photographs as shown in Fig. 6. The proportion of cells that exclude Trypan blue was determined by parallel incubation of cells as for determination of Rhodamine 6G staining. Trypan blue was added to cells immediately before they were observed under a standard light microscope. Unstained cells were scored as a percentage of unstained and blue stained cells. Results are weighted means ± S.E. with the number of cell preparations as indicated in the parentheses. *: significantly different by Student's *t*-test from cells incubated in NaCl medium only ($P < 0.005$). †: not significantly different by Student's *t*-test from cells incubated in NaCl medium. For statistical analysis arc sine transformations were performed on percentage data.

Conditions of incubation	Cells stained with Rhodamine 6G (%)	Total cells counted (phase contrast)	Cells excluding Trypan blue (%)	Total cells counted	Difference (%)
NaCl medium only	87.5 ± 1.0 (4)	762	91.1 ± 1.0 (3)	800	3.6 ± 1.5
NaCl medium + oligomycin + myxothiazol	84.8 ± 2.0 (4) †	323	88.5 ± 1.0 (2) †	600	3.7 ± 2.4
NaCl medium + valinomycin + oligomycin + myxothiazol	67.9 ± 3.0 (4) *	713	88.4 ± 1.0 (3) †	800	20.5 ± 1.8

This implies that glycolysis from supplied glucose maintains cell ATP levels in the absence of oxidative phosphorylation. Under these conditions we did not observe a large difference between rhodamine staining of cells and Trypan blue exclusion. In the presence of valinomycin ATP levels fell steadily reaching 30% of control at 30 min after addition of valinomycin (results not shown). We suggest that the greater proportion of depolarised cells in the presence of valinomycin is due to a fall in cell ATP levels.

We have recalculated the $^{36}Cl^-$ distribution and $^{86}Rb^+$ accumulation and thus E_{Cl} and E_K to account for the fraction of cells that are depolarised but exclude

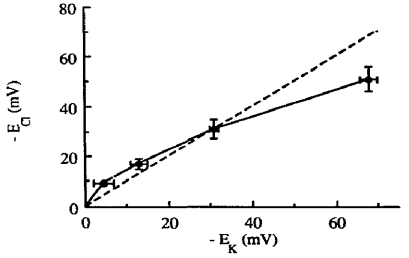

Fig. 7. Relationship between E_{Cl} and E_K after correction for depolarised intact cells. The accumulation ratios used to produce Fig. 5 were recalculated by reducing the measured cell volume by 20.5% under each condition. This value of 20.5% was obtained from the difference between the portion of cells that have a plasma membrane potential and so stain with Rhodamine 6G and the portion that exclude Trypan blue (assumed also to exclude the extracellular marker of pellet water space, hydroxy[^{14}C]methylinulin) as shown in Table I. The dotted line having a slope of 1 is the expected result if $^{36}Cl^-$ is passively distributed across the plasma membrane. The slope of the lines fitted by linear regression for each of the three experiments (not shown) were significantly different from 1.

Trypan blue in the presence of valinomycin. The recalculated data is presented in Fig. 7. (The $^{86}Rb^+$ accumulation and $^{36}Cl^-$ distribution in the presence of valinomycin presented in Figs. 2 and 3, respectively, is corrected for the fraction of cells that are depolarised under these conditions.) $^{36}Cl^-$ distribution and $^{86}Rb^+$ accumulation were recalculated for each $[K^+]_{out}$ although we only compared Rhodamine 6G staining of cells with Trypan blue exclusion for cells incubated in low K^+ medium in the presence of valinomycin (E_K of about −70 mV). However, the recalculation had little effect at higher $[K^+]_{out}$ and we believe this extrapolation from low to high $[K^+]_{out}$ is reasonable. The relationship between E_{Cl} and E_K indicates that for measurements of membrane potential from −4 to −40 mV the distribution of $^{36}Cl^-$ gives a quantitative measure of the plasma membrane potential. However, when the plasma membrane was hyperpolarised to about −70 mV, by imposing a K^+ diffusion potential in the presence of valinomycin and low $[K^+]_{out}$, calculation of plasma membrane potential from the $^{36}Cl^-$ distribution underestimated the membrane potential. Since $^{36}Cl^-$ is excluded from the cells measurement of high plasma membrane potentials with this probe is necessarily inaccurate due to errors in the measurement of cell volume and of extracellular space (see also Refs. 10 and 29). Also, any small electroneutral Cl^- transport that might take place would have larger effects at high $^{36}Cl^-$ exclusion.

Discussion

In the present paper we provide a quantitative assessment of the use of $^{36}Cl^-$ to measure plasma membrane potential in hepatocytes. We confirm previous observations [12] that $^{36}Cl^-$ equilibrates across the plasma membrane in 10–15 min. The quantitative analysis is carried out comparing the membrane potential calcu-

lated from the $^{36}Cl^-$ distribution (E_{Cl}) with that of E_K clamped by the presence of valinomycin and different extracellular K^+ concentrations and measured using $^{86}Rb^+$ accumulation. We assume that if E_{Cl} reflects E_K under these conditions then electrogenic Cl^- conductance is great enough to allow Cl^- to equilibrate with the plasma membrane potential under other, more physiological, conditions.

In the presence of valinomycin, E_{Cl} and E_K are approximately equal only from 0 to about -20 mV. However, the experiments with Rhodamine 6G and Trypan blue showed that valinomycin causes a proportion of the cells to lose their membrane potential but remain inulin-impermeable. Correction for this effect improves the linearity of the relationship between E_{Cl} and E_K so that it now extends to about -40 mV. Since the effect is caused by valinomycin, correction should not normally be necessary when measuring plasma membrane potentials using $^{36}Cl^-$ distribution in the absence of valinomycin.

Even after this correction the results show that the distribution of $^{36}Cl^-$ slightly underestimates the plasma membrane potential when it is hyperpolarised to -70 mV. This underestimation at high membrane potential may be due to the inability of present methods to measure cell volume and extracellular space accurately, as suggested by other workers [10,29]. This is because $^{36}Cl^-$ is excluded and at low $[Cl^-]_{in}$ in slight underestimation of the cell volume or overestimation of the extracellular space of the pellet will increase the calculated intracellular $^{36}Cl^-$ concentration and result in an underestimation of the plasma membrane potential. Small heterogeneities in the cell population of the sort produced by addition of valinomycin will also be much more apparent at high membrane potential, as will any concentration-independent binding of Cl^- to cellular components.

Despite these problems in the use of $^{36}Cl^-$ distribution to measure plasma membrane potential at high potentials, our results show that it is a good quantitative method over the normal range of potentials found in unstimulated cells. Only above about -40 mV does it become inaccurate; at these higher potentials it should be used only qualitatively unless rigorous calibration can be carried out. The results also show that Cl^- transport pathways such as $Na^+/K^+/2Cl^-$ transport do not contribute significantly to chloride distribution in hepatocytes at least for membrane potentials below about -40 mV.

Acknowledgements

We thank Professor Michael Berry for his help with this work and Mark Leach for technical assistance. This work was supported by a grant from the Science and Engineering Research Council. C.D.N. was supported by a grant from the Department of Medical Biochemistry at Flinders University of South Australia.

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