Biochimica et Biophysica Acta, 1111 (1992) 221–230

BBAMEM 75787

Measurement of plasma membrane potential in isolated rat hepatocytes using the lipophilic cation, tetraphenylphosphonium: correction of probe intracellular binding and mitochondrial accumulation

Shuichi Saito, Yusuke Murakami, Seiji Miyauchi and Naoki Kamo

Department of Biophysics and Physicochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo (Japan)

(Received 22 April 1992)
(Revised manuscript received 4 August 1992)

Key words: Plasma membrane; Membrane potential; Hepatocyte; Mitochondrion; Tetraphenylphosphonium; Amphotericin B

The lipophilic cation tetraphenylphosphonium (TPP^+) has been extensively utilized as the probe for the membrane potential (V_m) in various cells. For application to mammalian cells, however, two serious problems require resolution: (1), correction of TPP^+ binding to intracellular constituents and (2), estimation of the considerable TPP^+ accumulation in mitochondria. We propose here a simple corrective method for the TPP^+ binding and its accumulation. TPP^+ distribution is assumed as: (1), two compartments (a cytosolic and a mitochondrial space); (2), a proportional relationship between TPP^+ bound amount and its unbound concentration in each compartment. We theoretically derived the simple equation:

$$V_m = -\frac{RT}{F}\ln\left(\frac{C/M_{phys}\ \text{ratio}}{C/M_{abol}\ \text{ratio}}\right)$$

where R, T and F have their usual thermodynamic significance. Here, the C/M ratio is defined as the ratio of TPP^+ concentration of apparent intracellular to extracellular space. The suffixes phys and abol respectively, mean the physiological and solely V_m-abolished conditions. This equation was checked with hepatocytes, because estimating hepatocytes V_m with TPP^+ distribution is not considered possible because of the relatively high mitochondrial content. The selective V_m abolition was achieved by permeabilization with 20 μM of amphotericin B. The V_m value was, thus, estimated to be -38.6 ± 0.3 mV, compatible with those obtained with microelectrodes in other laboratories. V_m in hepatocytes is composed of transmembrane K^+ diffusion potential (-20.6 ± 0.3 mV) and electrogenic Na^+/K^+-ATPase (-19.6 ± 0.4 mV). Addition of rheogenic L-alanine caused a transient but significant depolarization (from control to -34 ± 0.3 mV). These results taken together indicate that hepatocyte V_m can be accurately determined with the present simple method, so that it may possibly be applicable to the evaluation of V_m in other mammalian cells.

Introduction

The transmembrane electrical potential difference is called a plasma membrane potential (V_m) and V_m regulation is well-recognized to be of great importance in cell functions [1–3]. V_m measurements have been made by various methods. These include invasive direct puncture of the cell with a microelectrode and non-invasive indirect methods like measurement of the accumulation of the radiolabeled lipophilic cations $TPMP^+$ and TPP^+ [4–10], the exclusion of permeant anions like thiocyanate [11] and ^{36}Cl [12,13], or the fluorescence change of non-toxic potential sensitive fluorophores [14–18].

In direct measurements with microelectrodes, single cells must be immobilized in order for a microelectrode to be inserted. This is usually done by allowing cells to attach to a substratum, such as a glass plate or collagen matrix in the culture medium. Use of direct measurements, thus, poses some difficulties in fragile

Correspondence to: N. Kamo, Department of Biophysics and Physicochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.

Abbreviations: AMB, amphotericin B; CA, cholic acid; DIG, digitonin; EC_{50}, half effective concentration; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; L-Ala, L-alanine; NYS, nystatine; OUA, ouabain; PCG, benzylpenicillin; BSP, bromosulfophthialein; ROT, rotenone; TCA, taurocholic acid; $TPMP^+$, trimethylphosphonium; TPP^+, tetraphenylphosphonium; V_m, plasma membrane potential.

and/or small cells. Electrode insertion has the possibility of damaging cell integrity and imperfections in the cell-sealing process around the leak conductance introduced by the electrode are likely to cause significant errors in V_m determination. Hence, an ideal technique is one in which a microelectrode pierces the cell without either cell damage or leak conductance.

On the other hand, indirect ion distribution methods are more suitable for V_m measurement in delicate and/or small cells, since these are very difficult to impale on microelectrode. These methods also have the advantage that the average V_m of many cells can be determined easily and non-invasively. In principle, V_m is estimated from the transmembrane distribution of any freely permeable ion. Lipophilic cations, TPP^+ and its analogues in particular, have been extensively used for V_m estimation in various cells [4–10] and several microorganisms [19–21]. Two problems, however, must be overcome in order to accurately estimate V_m in mammalian cells using the TPP^+ distribution method. First, the much greater potential (approx. −180 mV) across the mitochondrial membrane results in most intracellular TPP^+ being highly concentrated in the mitochondrial matrix despite the relatively small capacity of this compartment [11]; the potential estimated utilizing TPP^+, thus, does not reflect V_m, but the mitochondrial membrane potential in mammalian cells. This obstacle is especially recognized in hepatocytes [11], due to their relatively high mitochondria content. Second, these lipophilic cations nonspecifically bind to intracellular components [22–25], leading to an additional problem of estimating unbound TPP^+ cytosolic concentration. The indirect distribution method with TPP^+ and its analogues, thus, for the time being, seems inapplicable for estimation of V_m in mammalian cells.

In the present study, we have developed a convenient method to correct TPP^+ binding inside hepatocytes and its accumulation into mitochondria. It permits accurate evaluation of V_m in hepatocytes utilizing TPP^+ distribution, an estimation generally considered impossible. It is possible that this technique might be applicable to the evaluation of V_m in other mammalian cells.

Materials and Methods

Chemicals. ^3H_2O (5 mCi/ml) and [^{14}C]inulin (3.22 μCi/mg) were purchased from New England Nuclear (Boston, MA). [^{3}H]TPP^+ (23 Ci/mmol) was purchased from Amersham (Buckinghamshire, England). AMB, DIG, firefly lantern extract, L-Ala, NYS, OUA and ROT were purchased from Sigma (St. Louis, MO). TPP^+ was purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents were commercial products of analytical grade.

Animals. Male Wistar rats (Hokkaido Dobutu, Muroran, Japan) (180–220 g) were used and given free access to food and water.

Monitoring of TPP^+ distribution into hepatocytes utilizing a TPP^+-selective electrode. Procedures for isolating rat hepatocytes were the same as previously reported [26,27]. The viability of isolated hepatocytes was checked by the Trypan blue exclusion test; the value obtained usually ranged from 95 to 98%.

A TPP^+-selective electrode [20,21,25] was used to measure TPP^+ uptake by intact, metabolically-inhibited or permeabilized hepatocytes. The electromotive force between the TPP^+-selective and calomel reference electrodes in the sample solution was measured by an electrometer (Model TR-8651, Takeda Riken, Tokyo) connected to a pen recorder. The construction and properties of the TPP^+-selective electrode are described in Ref. 25. The extracellular TPP^+ concentration (C_{ext}) was continuously monitored with the TPP^+-selective electrode and TPP^+ accumulation into hepatocytes was calculated utilizing the law of mass conservation. The TPP^+-selective electrode was calibrated before the beginning of each experiment by successive TPP^+ additions (concentration range: 0.1–5 μM) to Hepes buffer consisting of 137 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM Hepes and 5 mM glucose (pH 7.4). No pharmacological agent used in this study had any effect on TPP^+ monitoring by the selective electrode. The temperature was maintained at 37°C and the cells were maintained in suspension by a stirring device. The medium was continuously oxygenated with humidified gas (95% O_2, 5% CO_2).

After a 5 min preincubation of the medium at 37°C, TPP^+ accumulation into hepatocytes was initiated by adding a 500-μl aliquot of cell suspension ((2–3) · 10^7 cells/ml) to 4 ml of Hepes buffer containing 2 μM TPP^+. TPP^+ accumulation into intact hepatocytes approached equilibrium within 15 min (shown in Results). After equilibrium, AMB or DIG was added to the cell suspension (final concentration: 1–100 μM) followed by further addition of the metabolic inhibitor ROT (final concentration: 5 μM). A similar experiment was also performed, in which OUA at various concentrations (10 μM–2 mM) was added to cell suspensions with various K^+ concentrations (5–200 mM) after equilibrium.

Monitoring of TPP^+ distribution into hepatocytes utilizing [^{3}H]-TPP^+. To determine whether TPP^+ distribution into hepatocytes could be accurately estimated utilizing the TPP^+ selective electrode, uptake experiments with ^{3}H-TPP^+ were performed in the same way as with the electrode. After a 5 min preincubation of cell suspension ((2–3) · 10^6 cells/ml) at 37°C, an aliquot of TPP^+ solution containing [^{3}H]TPP^+ (0.1 μCi) was

added to start TPP^+ uptake by hepatocytes. Initial TPP^+ concentrations ranged from 0.01 to 5 μM. Treatment with pharmacological agents was performed as described above. An aliquot of cell suspension was taken at the designated time and placed on the top of a two-phase system in microfuge tubes, consisting of a 50 μl 3 M KOH bottom overlaid with 100 μl of silicone/mineral oil (density: 1.015). The tubes were then centrifuged for 10 s in a table-top microfuge (Beckman Instruments, Fullerton, CA). After the hepatocytes were dissolved in the alkaline solution, the bottom of the tube was sliced off, the content transferred into the scintillation vial and neutralized with 75 μl of 2 M HCl. 10 ml scintillation counting cocktail (Amersham) was then added to the vial. ^{3}H-radioactivity was determined with a liquid scintillation spectrophotometer (LSC 3600, Aloka, Tokyo, Japan).

The amounts taken up by hepatocytes were corrected for the adherent water film (2.2 μl/mg protein), and were expressed as the ratio of concentration in the intracellular space (cellular volume: 5.2 μl/mg protein) to that in the medium (C/M ratio). The adherent water volume and intracellular volume were determined using [^{14}C]inulin and ^3H_2O [27]. The counting coefficient of ^{3}H-radioactivity was determined by the channel ratio method employing an external standard to correct for ^{14}C-radioactivity spillover. Protein was determined with protein assay kits (Bio-Rad, Tokyo, Japan) using BSA as a standard.

Measurement of intracellular ATP. In experiments in which hepatocytes were treated with various agents, cells ($(2–3)\cdot 10^6$ cells/ml) were preincubated with those agents (time of preincubation and dose are shown in Table I) in Hepes buffer (pH 7.4) at 37°C. A 100-μl aliquot of cell suspension was then treated with 2 ml 5% perchloric acid, and cellular ATP was extracted. After neutralization by 1 M KOH and centrifugation at 3000 rpm for 15 min, the supernatant was used to determine ATP with a J4-7441 Chem-Glow Photometer (American Instrument, Silver Spring, MD) utilizing firefly lantern extract [28].

Theory

V_m calculation by TPP^+ distribution into hepatocytes

The lipophilic cation TPP^+ is accumulated not only across the plasma membrane, but also across the mitochondrial membrane. The much greater potential across the latter membrane leads to a considerable amount of TPP^+ in the mitochondrial compartment, despite its relatively small capacity [11]. Since this is the case, TPP^+ can be assumed to be distributed mainly in the cytosolic and mitochondrial spaces [11,22]. The amount of TPP^+ accumulation into hepatocytes is given by the following equation:

$$A_t = A_c + A_m \tag{1}$$

where A_t represents the total accumulation, A_c the amount in the cytosolic compartment and A_m the amount in the mitochondrial compartment. It was demonstrated earlier that a considerable amount of TPP^+ binds to constituents (membrane and proteins) of these two compartments [23]. The binding sites of the lipophilic cations in each compartment are soluble proteins and the interfaces between the membrane and aqueous solutions (the outer surface and the inner surface of the membrane) [29]. In the present study, the bound TPP^+ amount in each compartment is also assumed to be proportional to the unbound TPP^+ concentration in the corresponding compartment [22,24,29]. A_c and A_m are expressed as follows:

$$A_c = V_c C_{c,f} + A_{c,b}$$

$$= V_c C_{c,f} + K_o C_{ext} + K_i C_{c,f} + K_{c,p} C_{c,f} \tag{2}$$

where V_c represents the cytosolic compartment volume, $A_{c,b}$ the bound TPP^+ amount in the cytosol compartment, $C_{c,f}$ the unbound TPP^+ concentration in the cytosol compartment, K_o the proportionality constant at the external binding site of the plasma membrane, K_i the proportionality constant at the internal binding site of the plasma membrane and $K_{c,p}$ the proportionality constant at the protein-binding site in the cytosol. Similarly, we obtain:

$$A_m = V_m C_{m,f} + A_{m,b}$$

$$= V_m C_{m,f} + K_{m,o} C_{c,f} + K_{m,i} C_{m,f} + K_{m,p} C_{m,f} \tag{3}$$

where V_c represents the mitochondrial compartment volume, $A_{m,b}$ the bound TPP^+ amount in the mitochondrial compartment; $C_{m,f}$ the unbound TPP^+ concentration in the mitochondrial compartment; $K_{m,o}$ the proportionality constant at the external binding site of the mitochondrial membrane, $K_{m,i}$ the proportionality constant at the internal binding site of that membrane and $K_{m,p}$ the proportionality at the protein binding site in the mitochondria. Substituting Eqns. 2 and 3 into Eqn. 1 and rearranging gives

$$A_t = K_o C_{ext} + V_c(1+\alpha) C_{c,f} + V_m(1+\beta) C_{m,f} \tag{4}$$

$$\alpha = (K_{c,p} + K_i + K_{m,o})/V_c$$

$$\beta = (K_{m,p} + K_{m,i})/V_m$$

The principle that at equilibrium, a free ionic probe is distributed between the internal and external spaces in accordance with the Nernst equation, results in the following equation:

$$\Delta\psi = -(RT/zF) \ln(C_i/C_e) \tag{5}$$

where $\Delta\psi$ represents the membrane potential with respect to the outside, R the gas constant, T absolute

temperature, F the Faraday constant, z the valency of the ionic probe, C_i the internal concentration and C_e the external concentration of the probe. According to Eqn. 5, $C_{c,f}$ and $C_{m,f}$ are expressed as follows:

$$C_{c,f} = C_{ext} \exp(-(F/RT)\,\Delta\psi_{mem}) \quad (6)$$

$$C_{m,f} = C_{c,f} \exp(-(F/RT)\,\Delta\psi_{mit}) \quad (7)$$

where $\Delta\psi_{mem}$ and $\Delta\psi_{mit}$ are the membrane potentials across the plasma and mitochondrial membranes, respectively. Substituting Eqn. 6 into Eqn. 7 and rearranging yields

$$C_{m,f} = C_{ext} \exp\{-(F/RT)(\Delta\psi_{mem} + \Delta\psi_{mit})\} \quad (8)$$

Substituting Eqns. 6 and 8 into Eqn. 4 and rearranging yields

$$A_t = C_{ext}\{K_o + V_c(1+\alpha)\exp(-(F/RT)\,\Delta\psi_{mem}) + V_m(1+\beta)\exp(-(F/RT)(\Delta\psi_{mem} + \Delta\psi_{mit}))\} \quad (9)$$

The value of A_t divided by V_c is designated as the apparent TPP^+ intracellular concentration (C_{app}) and rearranging to solve the ratio of its apparent intracellular concentration to the extracellular concentration (C/M ratio) yields

$$C/M \text{ ratio} = C_{app}/C_{ext} = K_o/V_c + \{1 + \alpha + V_m/V_c(1+\beta)\exp(-(F/RT)\,\Delta\psi_{mit})\} \times \exp(-(F/RT)\,\Delta\psi_{mem}) \quad (10)$$

Under conditions where V_m would be selectively abolished, Eqn. 10 is reduced to the following equation:

$$C/M_{abol} \text{ ratio} = 1 + K_o/V_c + \alpha + V_m/V_c(1+\beta) \times \exp(-(F/RT)\,\Delta\psi_{mit}) \quad (11)$$

where C/M_{abol} ratio is the C/M ratio value under conditions where V_m is selectively abolished. TPP^+-binding to the surface of hepatocytes seems negligible, since TPP^+ initial-adsorption in the accumulation experiment revealed minimal (Fig. 1). The parameter K_o/V_c in Eqns. 10 and 11 might, thus, be negligible. Putting Eqn. 11 into Eqn. 10, V_m can be calculated as follows:

$$\Delta\psi_{mem} = -(RT/F)\ln\left(\frac{C/M_{phys} \text{ ratio}}{C/M_{abol} \text{ ratio}}\right) \quad (12)$$

where C/M_{phys} ratio represents the C/M ratio under physiological conditions.

It should be noted that this calculation is based on selective V_m abolition. In the same manner, a change in V_m can also be calculated utilizing the C/M ratio value

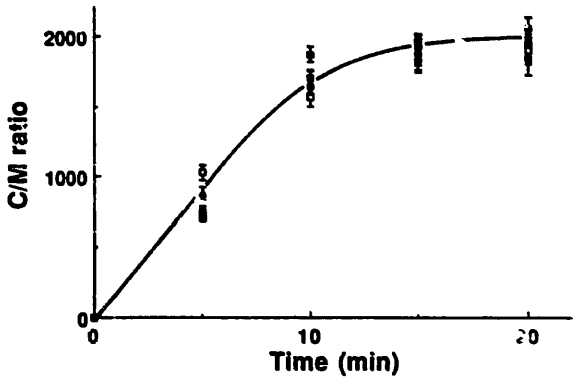

Fig. 1. TPP^+ accumulation by intact rat isolated hepatocytes. TPP^+ uptake determined utilizing TPP^+ selective electrode; Cell suspension ((2–3)·10^6 cells/ml) was incubated in Hepes buffer containing TPP^+ (1–5 μM) at 37°C. Extracellular TPP^+ concentration was monitored utilizing the TPP^+-selective electrode. TPP^+ accumulation into hepatocytes was calculated from extracellular TPP^+ utilizing the law of mass conservation. (●), 1 μM; (▲), 2 μM; (■), 5 μM. TPP^+ uptake determined utilizing [^{3}H]TPP^+; After cell suspension was incubated at 37°C for 5 min, a small aliquot of [^{3}H]TPP^+ was added to start TPP^+ uptake at various concentrations (0.01–5 μM) TPP^+ accumulation into hepatocytes was estimated from ^{3}H-radioactivity in cells. (○), 0.01 μM; (△), 1 μM; (□), 5 μM. TPP^+ distribution was expressed as C/M ratio. Each point represents the mean ± S.E. of five experiments.

under conditions before V_m perturbation. Assuming that V_m alone is altered under various experimental conditions, the change in V_m can be obtained using Eqn. 12:

$$\Delta\psi_{m,change} = \Delta\psi_{mem} - \Delta\psi'_{mem} = (RT/F)\ln\left(\frac{C/M \text{ ratio}}{C/M' \text{ ratio}}\right) \quad (13)$$

where $\Delta\psi_{m,change}$ represents a change in V_m; C/M ratio and C/M' ratio, TPP^+ distributions before and after V_m perturbation, respectively.

Results

TPP^+ distribution into isolated hepatocytes

The uptake time-courses of TPP^+ are shown in Fig. 1. TPP^+ distribution approached equilibrium between medium and hepatocytes within 15 min and the C/M ratio remained unchanged over incubation periods of 60 min. The uptake time-courses at various TPP^+ concentrations (0.01–5 μM) were superimposed on a single curve, suggesting that TPP^+ binding was proportional to its unbound concentration in hepatocytes. The uptake time-courses obtained with the TPP^+-selective electrode coincided with those with [^{3}H]TPP, this electrode, thus, proving to be an accurate TPP^+ detector. Cellular ATP levels were maintained at 3.7 to 4.3 mM over the incubation periods, indicating that the mitochondrial activity might remain unchanged (Table I).

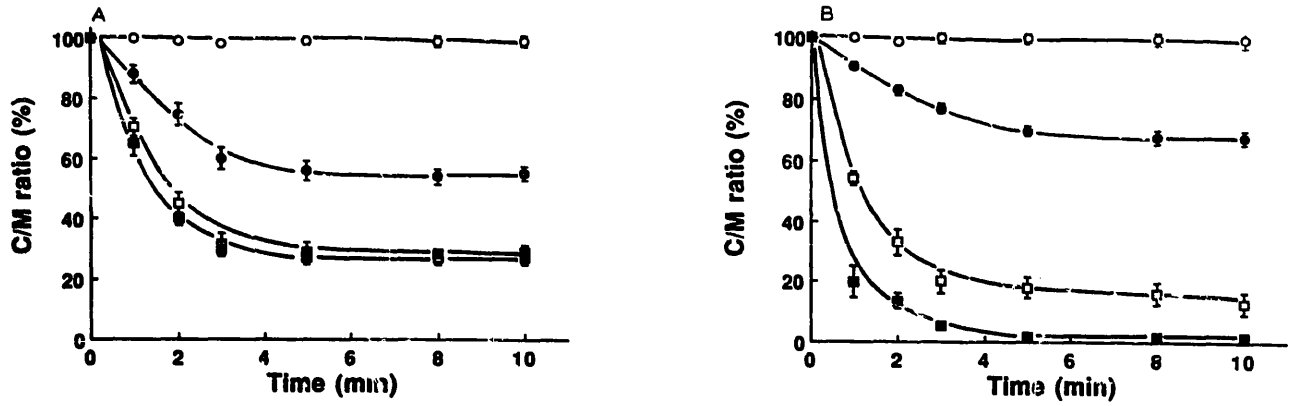

Fig. 2. Time profiles of TPP^+ release elicited by AMB and DIG. Panel A, TPP^+ release by AMB; Panel B, TPP^+ release by DIG. After hepatocytes ((2–3)·10^6 cells/ml) were incubated at 37°C in Hepes buffer containing 2 μM TPP^+ for 20 min, an aliquot of the membrane permeabilizer (AMB or DIG) was added at various concentrations (1–20 μM). TPP^+ distribution was expressed as % of C/M ratio in intact hepatocytes at equilibrium. (○), control; (●), 5 μM; (□), 10 μM; (■), 20 μM. Each point represents the mean ± S.E. of five experiments.

Effects of AMB and DIG on TPP^+ accumulation into hepatocytes

As aforementioned, the method to correct the intracellular TPP^+ binding and its mitochondrial accumulation mainly involves the estimation of TPP^+ distribution under selective V_m abolition. To determine TPP^+ distribution under selectively V_m abolisned conditions, we managed to selectively permeabilize the plasma membrane by polyene macrolide antibiotics (AMB) and saponin extract (DIG) at various concentrations. After equilibrium of TPP^+ distribution, the addition of both agents to the cell suspension was followed by a rapid TPP^+ release within 5 min (Fig. 2). Both TPP^+ releases induced by AMB and DIG revealed a concentration-dependence, the maximum of which was achieved at more than 20 μM. TPP^+ release caused by more than 20 μM AMB remained constant at 60% of TPP^+ accumulation, whereas DIG completely released the TPP^+ accumulated in hepatocytes (Fig. 3). TPP^+ unreleasable by AMB was released by 5 μM ROT, indicating that this unreleasable TPP^+ involves TPP^+ accumulation in mitochondria. TPP^+ release caused by the other polyene macrolide antibiotic NYS revealed a similar concentration-dependence (data not shown).

Intracellular ATP levels were measured to determine whether these agents selectively permeabilized the plasma membrane without damage to mitochondria. There was no effect of 20 μM AMB on the intracellular ATP levels (Table I), suggesting that AMB might selectively abolish V_m without damaging mitochondria. In fact, the mitochondria activity estimated from oxygen consumption proved that the AMB effect on mitochondrial respiration was minimal (data not shown). By contrast, 5 μM ROT and 20 μM DIG decreased the intracellular ATP levels by at least 75%, indicating damage to mitochondria. Further, the marker-enzyme releases for cytosol and mitochondria were determined (lactate dehydrogenase and citrate synthase, respectively, data not shown). DIG augmented release of both enzymes from hepatocytes in a concentration-dependent manner, the maximum being

TABLE I

Effect of pharmacological agents on intracellular ATP levels

Values are mean ± S.E. of five cell preparations. After hepatocytes ((2–3)·10^6 cells/ml) were incubated in Hepes buffer, pharmacological agents were added to cell suspension. ATP level was measured as described previously [28], following a 10 min incubation. Comparisons were by paired t-test. * $P < 0.01$ vs. control; NS, not significantly different from control.

Agent	Intracellular ATP (mM)
Control	4.1 ± 0.12
1 mM OUA	4.0 ± 0.24 NS
1 mM OUA + 150 mM K^+	3.9 ± 0.35 NS
20 μM AMB	3.8 ± 0.23 NS
20 μM DIG	1.1 ± 0.38 *
5 μM ROT	0.6 ± 0.23 *

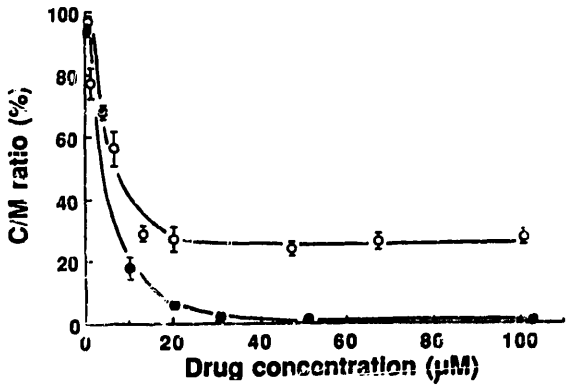

Fig. 3. Dependence of TPP^+ release from isolated rat hepatocytes on AMB and DIG. Cells were treated with the given substances as detailed in Materials and Methods. TPP^+ distribution was expressed as % of C/M ratio in intact hepatocytes at equilibrium. (○), AMB; (●), DIG.

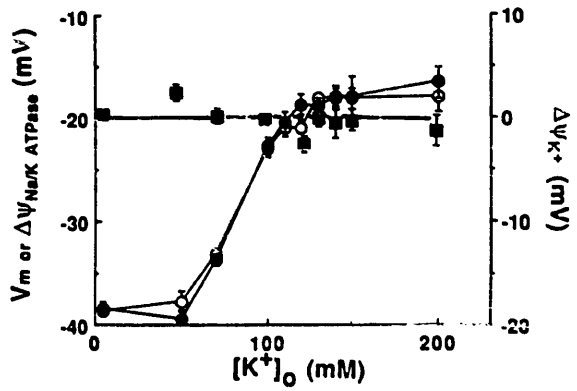

Fig. 4. Dependence of V_m, $\Delta\psi_{Na^+/K^+\text{-}ATPase}$ and $\Delta\psi_{K^+}$ upon $[K^+]_o$. V_m and $\Delta\psi_{Na^+/K^+\text{-}ATPase}$ were calculated utilizing TPP^+ release elicited by 20 μM AMB and 1 mM OUA, respectively. $\Delta\psi_{K^+}$ was calculated by subtracting $\Delta\psi_{Na^+/K^+\text{-}ATPase}$ from V_m in this figure. The null-point of the relationship between $\Delta\psi_{K^+}$ and $[K^+]_o$ indicates the point where $[K^+]_o$ is identical to $[K^+]_i$. The 120 mM point represents $[K^+]_i$ obtained by extrapolation to the null-point. (○), V_m; (■), $\Delta\psi_{Na^+/K^+\text{-}ATPase}$; (●), $\Delta\psi_{K^+}$.

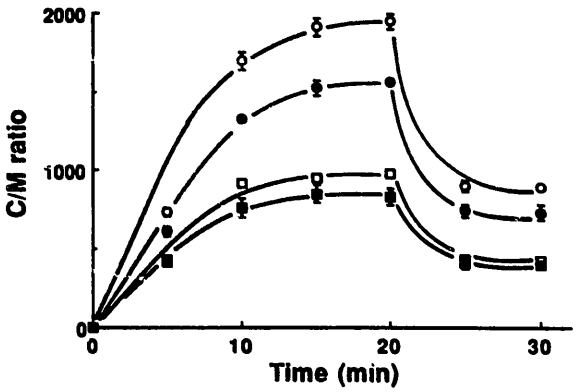

Fig. 6. Effect of $[K^+]_o$ and OUA on TPP^+ accumulation. After hepatocytes ((2–3)·10^6 cells/ml) were incubated in Hepes buffer containing 2 μM with various $[K^+]_o$ (5–200 mM), an OUA aliquot was added to cell suspension. $[K^+]_o$ was varied by substituting external NaCl with an equivalent KCl concentration. After a 20 min incubation period, an aliquot of OUA solution was added to the cell suspension (final concentration 1 mM). TPP^+ accumulation was expressed as C/M ratio. Each point represents the mean ± S.E. of five experiments. (○), $[K^+]_o$ = 5 mM; (●), $[K^+]_o$ = 70 mM; (□), $[K^+]_o$ = 120 mM; (■), $[K^+]_o$ = 150 mM.

obtained at 15 μM. No marker-enzyme release was observed with AMB, however, despite its high concentration (more than 20 μM), confirming that the permeabilization with 20 μM AMB was appropriate.

Assuming that TPP^+ accumulation into hepatocytes permeabilized with 20 μM AMB corresponds to that under V_m-selective abolition conditions, V_m was calculated to be -38.6 ± 0.3 mV (mean ± S.E., $n = 10$), utilizing Eqn. 12. To establish further the validity of our V_m evaluation, we measured the effects of increasing extracellular KCl concentration on V_m, assuming that the transmembrane K^+-gradient contributes to rat hepatocyte V_m. V_m plotted vs. extracellular K^+ concentration, $[K^+]_o$, as shown in Fig. 4, decreased in response to increasing $[K^+]_o$. Despite a high K^+ concentration (200 mM), V_m revealed some values (approx. −15 mV), indicating that V_m production in rat hepatocytes involves a mechanism other than transmembrane K^+-gradient.

Effect of K^+-gradient and Na^+/K^+-ATPase on TPP^+ accumulation

After equilibrium of TPP^+ distribution, OUA addition to cell suspension (OUA concentration 10–2300 μM) was followed by a rapid TPP^+ release within 5 min (Fig. 5A). OUA augmented TPP^+ release in a concentration-dependent manner, with the maximum obtained at 1 mM OUA (Fig. 5B). The EC_{50} of TPP^+ release by OUA was approx. 200 μM. The effects of $[K^+]_o$ on TPP^+ accumulation at initial equilibrium and after 1 mM OUA addition are depicted in Fig. 6; accumulation decreased in response to increasing

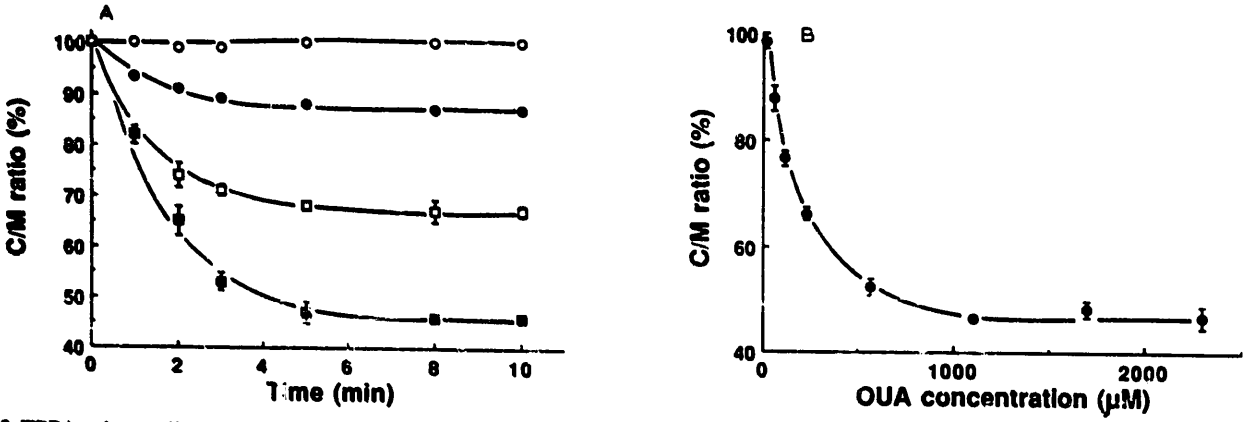

Fig. 5. TPP^+ release elicited by OUA. Panel A, Time-courses of TPP^+ release elicited by OUA. TPP^+ distribution was expressed as % of C/M ratio in intact hepatocytes at equilibrium. (○), control; (●), 50 μM; (□), 230 μM; (■), 1000 μM. Panel B, Dependence of TPP^+ release OUA concentration. TPP^+ distribution was expressed as % of C/M ratio in intact hepatocytes at equilibrium. After hepatocytes ((2–3)·10^6 cells/ml) were incubated at 37°C in Hepes buffer containing 2 μM TPP^+ for 20 min, an OUA aliquot was added at various concentrations (10–2300 μM). Each point represents the mean ± S.E. of five experiments.

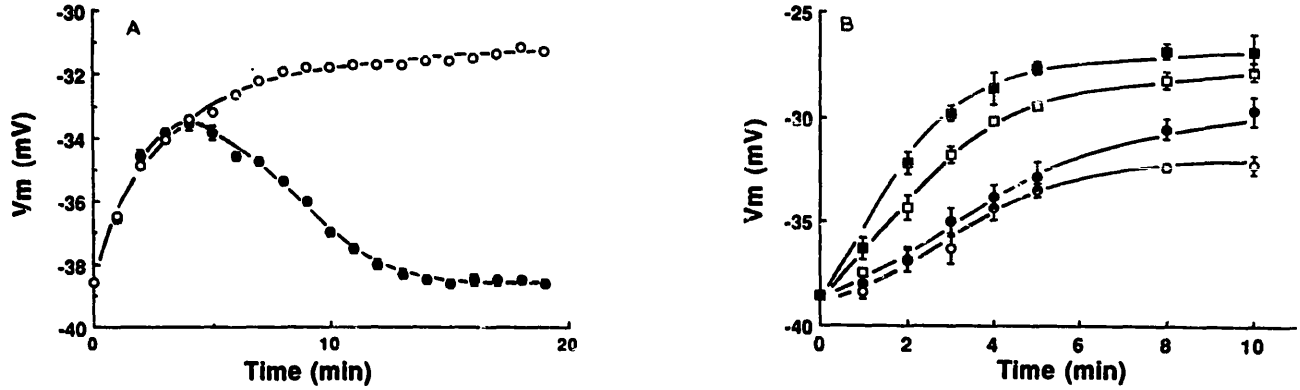

Fig. 7. Effect of L-Ala and organic anions on rat hepatocyte V_m. Panel A, Effect of L-Ala (10 mM) on V_m. Open and closed circles represent effects of L-Ala on V_m in the presence and the absence of 1 mM OUA, respectively. Panel B, Effect of various organic anions (10 μM) on V_m. (■), BSP; (○), CA; (●), PCG; (□), TCA. After hepatocytes ((2–3)·10^6 cells/ml) were incubated in Hepes buffer containing 2 μM TPP^+ for 20 min, an aliquot of compound was added to cell suspension. Each point represents the mean ± S.E. of four experiments.

$[K^+]_o$. The TPP^+ release elicited by 1 mM OUA addition decreased with increasing $[K^+]_o$, whereas the ratio of C/M ratio before OUA addition to that after OUA remained unchanged, irrespective of $[K^+]_o$. Assuming that TPP^+ release elicited by 1 mM OUA involves the inhibition of electrogenic Na^+/K^+-ATPase, the membrane potential produced by Na^+/K^+-ATPase ($\Delta\psi_{Na^+/K^+\text{-ATPase}}$) was calculated utilizing Eqn. 13. The $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ values remained constant (approx. −20 mV), irrespective of $[K^+]_o$ (Fig. 4), indicating that the electrogenic Na^+/K^+-ATPase contributes to V_m production independent of $[K^+]_o$. Furthermore, we determined the membrane potential attributed to the transmembrane K^+-gradient ($\Delta\psi_{K^+}$) under various K^+ concentrations by subtracting the $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ values from the V_m values. The relationship between $[K^+]_o$ and $\Delta\psi_K$ is depicted in Fig. 4. $[K^+]_o$ can be considered identical to intracellular K^+ concentration ($[K^+]_i$) at the null-point of $\Delta\psi_{K^+}$. Extrapolating the $\Delta\psi_{K^+}$ values to the null-point, we, thus, determined $[K^+]_i$ to be approx. 120 mM. Utilizing TPP^+ distribution under conditions of the transmembrane K^+-gradient elimination ($[K^+]_o = 120$ mM), the $\Delta\psi_{K^+}$ values were then determined to be -20.6 ± 0.3 mV, compatible with those obtained by subtracting $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ from V_m.

Effect of L-Ala and organic anions on hepatocyte V_m

10 mM L-Ala evoked an electrogenic response in isolated hepatocytes (Fig. 7A). When hepatocytes were exposed to 10 mM L-Ala, hepatocyte V_m decreased within 5 min from control to -34 ± 0.3 mV (mean ± S.E., $n = 4$). After the initial depolarization event, the plasma membrane repolarized. 1 mM OUA, on the contrary, inhibited the repolarization, suggesting that this action involved Na^+/K^+-ATPase. However, the rate of depolarization in response to L-Ala was somewhat slower than that obtained with the microelectrode [30–34]. We further examined the effect of the organic anions BSP, CA, PCG and TCA on hepatocyte V_m, since it has been confirmed that they are transported into hepatocytes by the carrier-mediated transport systems. These organic anions also induced plasma membrane depolarization with 5 min, after which it remained stable (Fig. 7B).

Discussion

The indirect ion-distribution method has been commonly used to estimate V_m. The indirect distribution method with a cationic probe TPP^+, however, seems inapplicable at least at present to the accurate estimation of V_m in mammalian cells, because of the cation's binding to the intracellular constituents and its high level of accumulation in mitochondria. The goal of this study was to determine whether V_m in hepatocytes can be accurately estimated utilizing the TPP^+ distribution method with a TPP^+-selective electrode.

Attempts have often been made to correct for TPP^+ intracellular binding and mitochondrial accumulation by subtracting the accumulation after selective V_m abolition [6,7]. However, these attempts are theoretically flawed, since changes in V_m will change the intracellular TPP^+ concentration and so alter the total amount within mitochondria, even if the $\Delta\psi_{mit}$ value remains unchanged. In contrast, we here corrected for TPP^+ binding and accumulation in mitochondria by dividing by TPP^+ accumulation into hepatocytes after V_m abolition (shown in Eqn. 12). It should be noted that we took into account TPP^+ redistribution between the mitochondria and cytosol after selective V_m abolition. The method seems to us to be without flaw and superior to previous techniques. Assuming that TPP^+ is distributed mainly in the cytosolic and mitochondrial spaces and that it binds proportionally to intracellular contents [22–24], V_m could, thus, be expressed as a simple equation, Eqn. 12. As far as we know, this

method of correction is the first to be presented. Assuming a selective V_m abolition by hepatocyte exposure to 20 μM AMB, V_m was calculated to be -38.6 ± 0.3 mV, compatible with results in other laboratories (-35 to -37 mV) [30–32,35,36]. Accordingly, this correction method might be applicable to the accurate evaluation of V_m in other mammalian cells.

As stated, this means of correcting the intracellular TPP^+ binding and its mitochondrial accumulation is based primarily on whether TPP^+ distribution can be accurately estimated under conditions of selective V_m abolition. To selectively abolish hepatocyte V_m, we attempted to permeabilize the plasma membrane utilizing DIG and AMB without damage to mitochondria. These pharmacological agents interact specifically with sterols and thereby permeabilize, but do not solubilize, cells. This interaction with cholesterol gives them a major advantage over other permeabilizing agents such as glycerol, toluene or Triton X-100, which are relatively non-specific in their effects on different types of cell membrane. In general, organelles do not have high molar cholesterol/phospholipid ratios [37]. In contrast, the high molar ratio of cholesterol to phospholipids in plasma membranes of eukaryocytes provides a certain selectivity of plasma membrane permeabilization with these agents. This is why we first chose treatments with these agents for the purpose of selective V_m abolition.

However, while AMB seemed to selectively abolish hepatocyte V_m, DIG unexpectedly seemed to fail (Fig. 3). What mechanism is involved in this difference of action? Initially, DIG interacts selectively with cholesterol and pulls it out of the plasma membrane, forming a large hole which permits large molecules access to intracellular sites [38,39]. Usually, DIG can only be applied within a narrow range of time and concentration [38–41]. The permeabilization by continuously exposing hepatocytes to DIG may have been extreme and may have resulted in mitochondrial damage. This may be why the permeabilization with DIG exposure failed to achieve the selective V_m abolition in this study. AMB, on the contrary, selectively interacts with cholesterol and forms a small, channel-like pore, which allows the leakage of monovalent cations, such as K^+ and Na^+, but is impermeable to glucose and large molecules [42–45]. Indeed, no marked enzyme release was observed with AMB following 20 min incubation, despite the use of a high AMB concentration (50 μM). Furthermore, AMB has been shown to inhibit Na^+/K^+-ATPase at concentrations above 5 μM [46], suggesting that $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ is simultaneously abolished. Consequently, AMB succeeded in selectively abolishing hepatocyte V_m without mitochondrial damage. The permeabilization with AMB would be rather moderate and more suitable for selective V_m abolition.

The Na^+/K^+-ATPase-specific inhibitor OUA elicited a rapid TPP^+ release from hepatocytes in a concentration-dependent manner (Fig. 5), the maximum of which was obtained at approx. 1 mM. In rodent hepatocytes, Na^+/K^+-ATPase inhibition requires a relatively high OUA concentration (inhibition constant 0.2 mM) [47,48], compatible with the concentration-dependent manner of TPP^+ release by OUA. Accordingly, TPP^+ release by OUA involved Na^+/K^+-ATPase inhibition. Utilizing Eqn. 13, we then calculated $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ to be -19.6 ± 0.4 mV, indicating that the electrogenic Na^+/K^+-ATPase activity is one of the major V_m determinants in rat hepatocytes. Other investigators also demonstrated that OUA partially depolarizes the plasma membrane in hepatocytes [11,35,39], although it is frequently stated that the quantitative contribution of the Na^+/K^+-ATPase activity to V_m is small.

The TPP^+ release by OUA, however, might be explained by another possibility: the depolarization is attributable to a disappearance of the transmembrane K^+-gradient or mitochondrial damage. Using the ^{86}Rb-distribution technique, we demonstrated that the OUA effect on the transmembrane K^+-gradient was minimal during the experimental period (unpublished data). Moreover, intracellular ATP level also remained unchanged in the presence of 1 mM OUA, suggesting no mitochondrial damage. Taken together, these resulted in our assumption that TPP^+ release elicited by OUA might involve primarily the plasma membrane depolarization caused by the electrogenic Na^+/K^+-ATPase inhibition.

We further calculated the $\Delta\psi_{K^+}$ value by subtracting the $\Delta\psi_{Na^+/K^+\text{-ATPase}}$ value from the V_m value (Fig. 4). The plot of $\Delta\psi_{K^+}$ vs. $[K^+]_o$ shows that $\Delta\psi_{K^+}$ decreases with increasing $[K^+]_o$, thereby substantiating the validity of the potential recording. In Fig. 4, the $[K^+]_i$ value can be considered identical to the $[K^+]_o$ value at the null-point of $\Delta\psi_{K^+}$. The $[K^+]_i$ value was, thus, estimated to be approx. 120 mM by extrapolation of $\Delta\psi_{K^+}$ to the null-point, comparable to the values obtained with perfused liver [50] and isolated hepatocytes [51]. Combining Na^+/K^+-ATPase inhibition and transmembrane K^+-gradient elimination ($[K^+]_o = 120$ mM), V_m was then estimated to be 38.9 ± 0.6 mV. This was comparable to that obtained with AMB, indicating that selective V_m abolition can also be achieved by simultaneous Na^+/K^+-ATPase inhibition and transmembrane K^+-gradient elimination.

On the other hand, the ratio of $[K^+]_i$ to $[K^+]_o$ is approx. 24. If the electrogenic permeability to K^+ were high, the $\Delta\psi_{K^+}$ value of -80 mV would be expected from $[K^+]_i$ and $[K^+]_o$ values, being far removed from the observed value of $\Delta\psi_{K^+}$ (-20 mV). This indicates that $\Delta\psi_{K^+}$ in hepatocytes is not simply a consequence of the transmembrane K^+-diffusion gradient. When added to hepatocytes at low concentrations, valinomycin specifically increases the permeability of the

plasma membrane to K^+ and causes membrane hyperpolarization [10,35], suggesting that the permeability for K^+ is not high. Whereas K^+ conductance contributes in part to $\Delta\psi_{K^+}$, the curvilinear relationship between $\Delta\psi_{K^+}$ and $[K^+]_o$ (Fig. 4) is not a Nernstian function, as would be expected of a K^+-selective membrane. Thus, membrane conductances of other ions, notably Na^+ and/or Cl^-, also may contribute to $\Delta\psi_{K^+}$.

In our experiments, we used the TPP^+-electrode, which can monitor the V_m change continuously. As shown in Fig. 7A, 10 mM L-Ala produced the biphasic response. The addition of L-Ala causes an immediate and transient depolarization, followed by repolarization. Similar findings were reported by other laboratories when a microelectrode was utilized. Thus, the electrode can follow a relatively rapid V_m change.

The repolarization-hyperpolarization of hepatocytes during L-Ala uptake (Fig. 7A) is confirmed to be involved by mechanisms that regulate its osmotic and electrical equilibrium [52–55]. In response to the cotransport of Na^+ and L-Ala, cells must compensate for both solute load and increased positive charge to maintain their volume and V_m, respectively. When hepatocytes are incubated with 10 mM L-Ala, there is an immediate decrease in V_m and an increase in intracellular Na^+ and cell volume. Subsequent increases in K^+ conductivity and Na^+/K^+-ATPase pump activity restore V_m [44]. We found that this activated Na^+/K^+-ATPase pump might be inhibited by 1 mM OUA, leading to an impairment of V_m repolarization (Fig. 7A). The electrogenic response evoked by TCA was similar to that stimulated by L-Ala uptake, although repolarization was not observed (Fig. 7B). TCA-stimulated depolarization was also observed by Fitz and Scharschmidt [30] and Bear et al. [33], using microelectrodes. They concluded that Na^+-coupled TCA uptake by hepatocytes occurs through an electrogenic system which transports more than one Na^+ with each TCA molecule. It is of great interest that other organic anions, BSP, CA and PCG, produced a similar electrogenic response, suggesting the feasibility that these anions might be taken up by hepatocytes through an inwardly positive charged electrogenic transport system. Despite these organic anions having been confirmed to be transported via a carrier-mediated system [56–59], the electrogenicity remains unknown and is worthy of investigation in the future.

Two assumptions were made in this paper for the sake of the simple modeling of TPP^+ distribution in hepatocytes. First, TPP^+ has been assumed to be distributed mainly into two compartments, cytosolic and intramitochondrial. However, strictly speaking, a hepatocyte consists of a multicompartment system, so that TPP^+ distribution in hepatocytes must be described utilizing multicompartments. On the other hand, a hepatocyte contains a relatively high content of mitochondria, into which most intracellular TPP^+ may be accumulated by its very negative V_m (−180 mV), in spite of the relatively small capacity of this compartment. Indeed, TPP^+ accumulation into mitochondria was calculated to be more than 90% of its total content in hepatocytes, indicating a minimal TPP^+ distribution into other organelles. The time courses of TPP^+ accumulation into hepatocytes were expressed by three exponential equations, suggesting that hepatocytes might have two main TPP^+ distribution pools (data not shown). Therefore, the first assumption that TPP^+ distributes mainly into the cytosol and mitochondria seems appropriate. Second, a proportional TPP^+ binding has been assumed, the amount of bound TPP^+ in each compartment being proportional to unbound TPP^+ concentration. We previously reported that TPP^+ binding to mitochondria and intracellular constituents revealed saturation [23]. The relationship can be expressed as Langmuir adsorption, the binding of which was almost linear up to as high as 800 μM. In the present study, TPP^+ mitochondria did not exceed 800 μM and TPP^+ C/M ratio remained unchanged irrespective of a wide range of TPP^+ concentrations (0.01–5 μM), thus, indicating a proportional TPP^+ binding. Consequently, this assumption might also be judged appropriate. However, there is the question of whether or not the binding parameters might depend on hepatocyte V_m. When the amounts of bound probes are increased, we cannot deny the possibility that some probes may be located in the middle part of the membranes and that the binding parameters may depend on V_m. This point should be further investigated, although previous analysis show the independence of binding parameters on V_m [23].

The measurement of V_m and its change with TPP^+-selective electrode involves the estimation of TPP^+ redistribution in hepatocytes following thermodynamic equilibrium. Accordingly, a rapid TPP^+ redistribution between hepatocytes and medium is prerequisite to monitoring V_m and its modifications under different experimental conditions, especially any rapid and transient change. As shown in Figs. 2 and 5, the TPP^+ redistribution caused by various inhibitors and ligands was achieved within a few minutes, sufficient for monitoring a rapid V_m change. Indeed, we were able to monitor a rather rapid V_m change under various conditions (Fig. 7). However, the rate of depolarization in response to L-Ala in the present study was relatively slower than that obtained with the microelectrode [30–34], probably as a result of the delay in TPP^+ redistribution elicited by V_m alteration. The response rate of the TPP^+-selective electrode might, thus, be somewhat slower than that of the microelectrode, so that the interpretation of monitoring data on a very fast transient V_m change (less than 1 min) should be made cautiously.

In the present paper, we demonstrate that it is possible to correct both TPP^+ binding inside cells and its accumulated mitochondria provided we can obtain TPP^+ cellular distribution for cells whose V_m is selectively abolished. The rat hepatocyte V_m values determined in the present study were in agreement with those in other laboratories (−35 mV to −37 mV) [30–32,35,36]. Furthermore, the V_m transient depolarization caused by L-Ala was similar to those demonstrated with microelectrode by other investigators [30–34], suggesting that this electrode can monitor a rather rapid change in V_m. These results taken together suggest that this method using a TPP^+-selective electrode is of value and applicable to estimating V_m of any other mammalian cell which may be difficult to measure with a microelectrode.

References

1 Martonosi, A.N. (ed.) (1985) The Enzymes of Biological Membranes, Vol. 3, Membrane Transport, 2nd Edn., Plenum Press, New York.
2 Stein, W.D. (1986) Transport and Diffusion Across Cell Membranes, Academic press, Orlando.
3 Moule, S.K. and McGivan, J. (1990) Biochim. Biophys. Acta 1031, 383–397.
4 Heinz, E., Geck, P. and Pietzyk, C. (1975) Ann. N.Y. Acad. Sci. 264, 428–441.
5 Lichtshtein, D., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 650–654.
6 Yorek, M.A. and Dunlop, J.A. (1991) Biochim. Biophys. Acta 1061, 1–8.
7 Kiefer, H., Blume, A.J. and Kaback, H.R. (1980) Proc. Natl. Acad. Sci. USA 77, 2200–2204.
8 Kuroki, M., Kamo, N., Kobatake, Y., Okimasu, E. and Utsumi, K. (1982) Biochim. Biophys. Acta 693, 326–334.
9 Ehrhardt, V. (1984) Biochim. Biophys. Acta 775, 182–188.
10 Bear, C.E., Petrunka, C.N. and Strasberg, S.M. (1985) Hepatology 5, 383–391.
11 Hoek, J.B., Nicholls, D.G. and Williamson, J.R. (1980) J. Biol. Chem. 255, 1458–1464.
12 Akerman, K.E.O. and Jarvisalo, J.O. (1980) Biochem. J. 192, 183–190.
13 Nobes, C.D. and Brand, M.D. (1989) Biochim. Biophys. Acta 987, 115–123.
14 Bashford, C.L., Chance, B. and Prince, R.C. (1979) Biochim. Biophys. Acta 545, 46–57.
15 Waggoner, A.S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–68.
16 Grinvald, A., Hildesheim, R., Farber, I.C. and Anglister, L. (1982) J. Biophys. 39, 301–308.
17 Bashford, C.L. and Pasternak, C.A. (1984) J. Membr. Biol. 79, 275–294.
18 Reers, M., Smith, T.W. and Chen, L.B. (1991) Biochemistry 30, 4480–4486.
19 Harold, F.M. and Papineau, D. (1972) J. Membr. Biol. 8, 27–44.
20 Kamo, N., Kacanelli, T. and Packer, L. (1983) Methods Enzymol. 88, 356–360.
21 Kamo, N., Demura, M. and Kobatake, Y. (1986) J. Membr. Sci. 27, 233–239.
22 Olavarria, J.S., Galindo, C., Montero, M., Baquero, Y., Vitorica, J. and Satrustegui, J. (1988) Biochim. Biophys. Acta 935, 322–332.
23 Demura, M., Kamo, N. and Kobatake, Y. (1987) Biochim. Biophys. Acta 894, 355–364.
24 Rottenberg, H. (1984) J. Membr. Biol. 81, 127–138.
25 Kamo, N. and Kobatake, Y. (1986) Methods Enzymol. 125, 46–58.
26 Miyauchi, S., Sugiyama, Y., Iga, T. and Hanano, M. (1988) J. Pharm. Sci. 77, 688–692.
27 Chung, Y.B., Miyauchi, S., Sugiyama, Y., Harashima, H., Iga, T. and Hanano, M. (1990) J. Hepatol. 11, 240–251.
28 Mori, Y., Matsumoto, K., Ueda, T. and Kobatake, Y. (1986) Protoplasma 135, 31–37.
29 Demura, M., Kamo, N., Kobatake, Y. (1985) Biochim. Biophys. Acta 820, 207–215.
30 Fitz, J.G. and Scharschmidt, B. (1987) Am. J. Physiol. 252, G56–G64.
31 Wondergem, R. and Castillo, L.B. (1989) Am. J. Physiol. 254, G795–G801.
32 Weinman, S.A., Graf, J. and Boyer, J.L. (1989) Am. J. Physiol. 256, G826–G832.
33 Bear, C.E., Davison, J.S. and Shaffer, E.A. (1987) Biochim. Biophys. Acta 903, 388–394.
34 Ballatori, N., Wondergem, R. and Boyer, J.L. (1988) Biochim. Biophys. Acta 946, 261–269.
35 Bradford, N.M., Hayes, M.R. and McGivan, J.D. (1985) Biochim. Biophys. Acta 845, 10–16.
36 Edmondson, J.W., Miller, B.A. and Lumeng, L. (1985) Am. J. Physiol. 249, G427–G433.
37 Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
38 Fiskum, G., Craig, S.W., Decker, G.L. and Lehninger, A.L. (1980) Proc. Natl. Acad. Sci. USA 77, 3430–3434.
39 Murphy, E.M., Coll, K., Rich, T.L. and Williamson, J.R. (1980) J. Biol. Chem. 255, 6600–6608.
40 Wilson, S.P. and Kirshner, N. (1983) J. Biol. Chem. 258, 4994–5000.
41 Ahnert-Hilger, G., Bhakdi, S. and Gratzl, M. (1985) J. Biol. Chem. 260, 12730–12734.
42 Ermishkin, L.N., Kasumov, Kh.M. and Potseluyev, V.M. (1977) Biochim. Biophys. Acta 470, 375–367.
43 Gale, E.F. (1984) in Macrolide Antibiotics. Chemistry, Biology and Practice (Omura, S., ed.), p. 425–455, Academic Press, New York.
44 Bolard, J. (1986) Biochim. Biophys. Acta 864, 257–304.
45 Raatikainen, O., Kauppinen, R.A., Komulainen, H., Taipale, H., Pirttila, T. and Tuomisto, J. (1991) Biochem. Pharmacol. 41, 1345–1350.
46 Vertut-Doi, A., Hannaert, P. and Bolard, J. (1988) Biochem. Biophys. Res. Commun. 157, 629–697
47 Bakkeren, J.A.J.M. and Bonting, S.L. (1968) Biochim. Biophys. Acta 150, 460–466.
48 Anner, B.M. (1985) Biochem. J. 227, 1–11.
49 Wondergem, R. and Harder, D.R. (1980) J. Cell. Physiol. 104, 53–69.
50 Claret, M. and Mazet, J.L. (1972) J. Physiol. 223, 279–295.
51 Graf, J., Henderson, R.M., Krumpholtz, B. and Boyer, J.L. (1987) J. Membr. Biol. 95, 241–254.
52 Bakker-Grunwald, T. (1983) Biochim. Biophys. Acta 713, 239–242.
53 Kristensen, L.O. and Folke, M. (1984) Biochem. J. 221, 265–268.
54 Kristensen, L.O. (1986) Am. J. Physiol. 251, G575–G584.
55 Howard, L.D. and Wondergem, R. (1987) J. Membr. Biol. 100, 53–61.
56 Schwarz, L., Burr, R., Schwenk, M., Pfaff, E. and Greim, H. (1975) Eur. J. Biochem. 55, 617–623.
57 Anwer, M.S., Kroker, R. and Hegner, D. (1976) Hoppe-Seyler's Z. Physiol. Chem. 375, 1477–1486.
58 Schwenk, M., Burr, R., Schwarz, L. and Pfaff, E. (1976) Eur. J. Biochem. 64, 189–197.
59 Tamai, I., Tetsuya, T. and Tsuji, A. (1985) J. Antibiotics 38, 1774–1780.