Biochimica et Biophysica Acta 863 (1986) 318–324
Elsevier

BBA 70362

BBA Report

Microelectrode measurement of cell membrane potential in isolated hepatocytes attached to collagen

E. Petzinger [a] and H. Bigalke [b]

[a] *Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, 4600 Dortmund 1 and* [b] *Zentrum für Pharmakologie und Toxikologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 3000 Hannover 61 (F.R.G.)*

(Received 7 July 1986)
(Revised manuscript received 2 October 1986)

Key words: Membrane potential; Microelectrode; K^+ channel; Ouabain; (Hepatocyte)

Isolated spherical rat hepatocytes attached to collagen-coated cover slips generate a mean membrane potential (E_m) of -78 ± 9 mV as measured with high-resistance microelectrodes. The recordings were biphasic and were stable for upto 20 minutes. The correlation between external potassium concentration and E_m was not linear. Several potassium-channel blockers did not effect the membrane potential. Addition of ouabain added to the incubation solution slowly depolarized the cells. The results indicate a high potassium permeability of the isolated spherical hepatocytes attached to collagen.

Estimation of the membrane potential E_m of hepatocytes is required for any calculation of electrical driving forces either for uptake or secretion of compounds which are actively transported by hepatocytes. Secretion of organic anions into bile is favoured by an inside negative membrane potential, whereas uptake is stimulated if sodium ions are cotransported together with the organic anion. There is considerable evidence that E_m is involved in the transcellular transport of bile acids [1–6].

Measurements of E_m of hepatocytes were made by either noninvasive indirect determination or by invasive direct puncture of the cells. Noninvasive determination with TPP^+ [7], sodium isothiocyanate [8,31], and $^{36}Cl^-$ [9,31] gave average E_m values for a cell population, of -100 mV, -17 mV/-34.4 mV, and -35.5 mV/-38.8 mV, respectively. The membrane potential of an individual cell is then calculated from the distribution ratio of the charged indicator according to the Nernst equation. However, complex mathematical corrections are required if the indicator is toxic (e.g. cyanide dyes), if the indicator is unevenly distributed within cellular compartments (especially TPP^+ in mitochondria), or if transport mechanisms disturb the passive distribution, e.g. chloride. The possibility that the cell population might be too heterogeneous because of damaged or dead cells for the calculation of a representative individual cell membrane potential must also be considered. Direct measurements with impaled microelectrodes reflect the individual membrane potentials of single cells. This kind of recording, however, has yielded widely differing results in different laboratories (Table I). In liver cells, either in vivo or in situ perfused liver, the E_m was found not to exceed -50.7 mV. After isolation of hepatocytes (and disruption of intercellular interconnections), values of E_m between -9 and -40

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPP^+, tetraphenylphosphonium cation.

Correspondence: Dr. E. Petzinger, Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, 4600 Dortmund 1, F.R.G.

TABLE I

MEAN RESTING MEMBRANE POTENTIALS OF HEPATOCYTES FROM LIVERS OF EITHER GUINEA PIG OF RAT DETERMINED DIRECTLY BY MICROELECTRODES (LITERATURE DATA)

Data of E_m below −20 mV are not referred.

Conditions	Mean E_m (mV)	Ref.
Liver	−33	16
(in vivo; in situ)	−35	17
	−27 to −42	18
	−36.2	19
	−38	20
	−41	21
	−42	22
	−43.8	23
	−45.8	24
	−47.5	25
	−49.8	26
	−50.7	27
Isolated hepatocytes	−9.7	28
(attached for culture)	−23.6	28
	−19	29
	−31	29
	−40.4	30

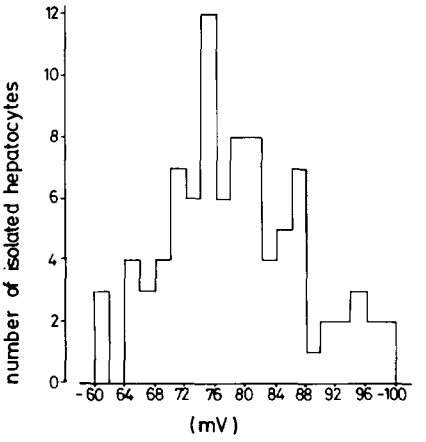


Fig. 1. Distribution of 90 recordings of E_m in isolated rat hepatocytes prepared from 19 rats. Hepatocytes were isolated according to Refs. 11 and 12, transferred on collagen-coated cover slips and incubated during 30 min in Dulbecco's MEM-medium supplemented with 10 mM Hepes until they became attached. Recordings of E_m were made at 37°C in Tyrode solution with 2.7 mM potassium chloride.

mV have been reported (Table I). However, such values differ considerably from the potassium equilibrium potential (E_K^+) (approx. −90 mV for an external [K^+] of 5.6 mM [10]) but are close to the chloride distribution potential (E_{Cl^-}), which is approximately −32 mV [10]. In our own measurements, performed repeatedly over the last three years, we have measured values of −60 to −99 mV after impalement of the isolated rat hepatocytes with microelectrodes (Fig. 1).

The hepatocytes were isolated by the collagenase perfusion method of Berry and Friend [11] with some modifications reported earlier [12]. Hepatocytes were then incubated in Tyrode buffer which contains 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 137 mM NaCl, 12.0 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, and 5.5 mM glucose. The pH was adjusted to 7.4 by HCl and all incubations were performed at 37°C under O_2/CO_2 (95%/5%) atmosphere. Primary monolayer cultures of the hepatocytes were prepared on collagen (rat tail) coated cover slips and maintained for 1–3 h in Dulbecco's MEM-medium buffered with 10 mM Hepes. The attached cells form bile canaliculi within 5–6 h and preserve their cellular polarity [13]. For the electrophysiological experiments the cover slips were transferred into small petri dishes which were refilled with Tyrode buffer and gased with O_2/CO_2. All recordings were performed at 37°C.

Microelectrodes of tip diameters of 0.2 μm were pulled with a Mecanex electrode pulling machine. They were filled with 4 M potassium acetate yielding a tip resistance of 80–120 MΩ. The mean membrane potential determined on 90 isolated hepatocytes from 19 cell preparations was −78.0 ± 9.0 mV with a range of −60 to −99 mV (Fig. 1). When the potential was occasionally measured in coupled cell-pairs no significant difference of E_m was found. A representative recording is shown in Fig. 2. Immediately after impalement the potential jumped to −20 mV. Thereafter, the potential increased steadily until it reached a plateau of −83 mV. Stable recordings were measured for up to 20 min. The E_m depolarized to −8 mV if isotonic KCl-Tyrode (containing 140 mM KCl) was superfused by a second micropipette and recovered to −78 mV within 6 min of cessation of superfusion (Fig. 2A). The level and duration of depolarization depended on the volume of ejected KCl solution. 3–5 μl evoked

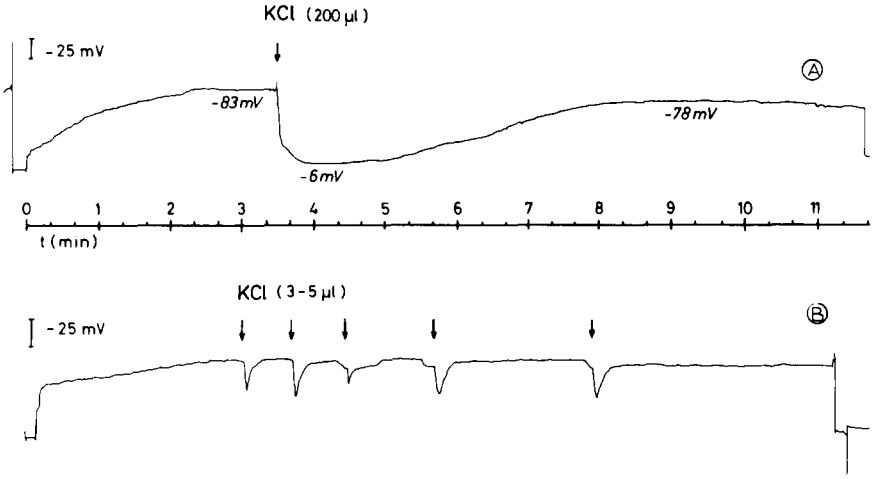


Fig. 2. Depolarization evoked by KCl superfusion of isolated rat hepatocytes. Membrane potentials of two isolated rat hepatocytes are recorded with microelectrodes filled with 4 M potassium acetate. Tip resistance of the electrodes was 80 MΩ. The impaled cell was superfused by a second micropipette with 139.7 mM KCl-Tyrode buffer which induced rapid depolarisations. After superfusion the local external potassium concentration decreased and the initial membrane potential recovered.

TABLE II

EFFECT OF OUABAIN (1 mM), Ca^{2+} (1.8 mM) AND Na^+ (142 mM) ON E_m OF ISOLATED RAT HEPATOCYTES ATTACHED TO COLLAGEN-COATED COVER SLIPS

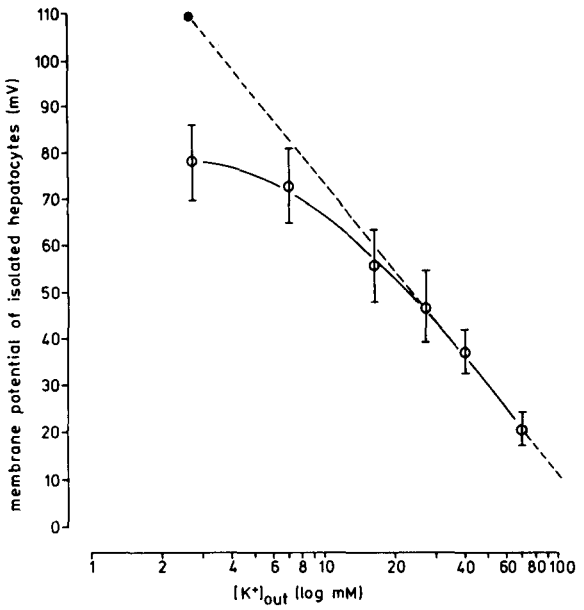
E_m in single cells after preincubation with 1 mM ouabain.

E_m (mV) after preincubation for			
20–45 min	60–75 min	90–120 min	Control after 120 min
−80	−47	−20	−76
−70	−62	−25	−76
−70		−13	
−85		−15	
		−14	

E_m in single cells after incubation in Ca^{2+}-free Tyrode buffer substituted with 1 mM EGTA, in Na^+-free Tyrode substituted with choline chloride, $LiHCO_3$ and KH_2PO_4 [a] or in normal Tyrode buffer in the presence of apamin (10 µg/ml) or quinine (350 µM).

E_m (mV) after incubation with					
Tyrode − Ca^{2+}		Tyrode − Ca^{2+} + EGTA	(a) Tyrode − Na^+ (b) Tyrode + sodium methylsulfate	Tyrode buffer + apamin	+ quinine
−30	−15	−24	(a) −75	−68	−80
−12	−21	−14	−70	−75	−76
−25	−40	−15		−85	−83
−43	−41		(b) −75		−85
−15	−22		−83		−87
−15	−24		−70		

[a] instead of NaCl, $NaHCO_3$ and NaH_2PO_4 in normal Tyrode buffer.



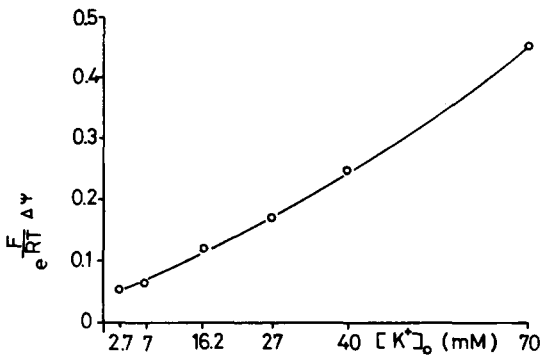


Fig. 3. (A) Relationship between the membrane potential of rat hepatocytes attached to collagen and the actual potassium concentration of the incubation buffer. Hepatocytes were briefly incubated in Tyrode solution with potassium chloride concentrations of 2.7, 7.0, 16.2, 40.0, and 70.0 mM. Osmolarity was kept constant by omitting NaCl. Each point represents 4–9 determinations in separate cell preparations. The mean E_m in control cells, incubated in 2.7 mM KCl-Tyrode solution was -76 ± 8 mV ($n = 60$ cells). From the slope of the regression line (dotted line) a factor of 63.9 was calculated for a 10-fold change in $[K^+]_{out}$. By the Nernst equation E_{K^+} was calculated from this value to be -109 mV and $[K^+]_{in}$ to be 169 mM. (B) Plot of the data of (A) on a graph of $\exp[E_m(F/RT)]$ versus the external potassium concentration. The nonlinear relationship indicates variability of either P_{K^+} or P_X depending on the external potassium concentration. P_X is the term for the permeability ratio of any other ion besides that for potassium in the Goldman-Hodgkin-Katz equation.

small depolarizations which recovered within a few seconds (Fig. 2B).

The correlation between external potassium concentration ($\log[K^+]_o$) and E_m was not linear. The shape of the curve resembled very much that found by Heller and Van der Kloot [26] on guinea pig hepatocytes in a perfused liver system, although the absolute values of the measured potentials in rat hepatocytes were more than double such values. When the data of Fig. 3A were taken for a plot of $\exp[E_m(F/RT)]$ versus $[K^+]_o$ the correlation was not linear (Fig. 3B). This indicates that the permeability ratio term in the Goldman-Hodgkin-Katz equation was not constant for all medium potassium concentrations used. However, the data are consistent with the assumption that the potassium permeability is high in these cells.

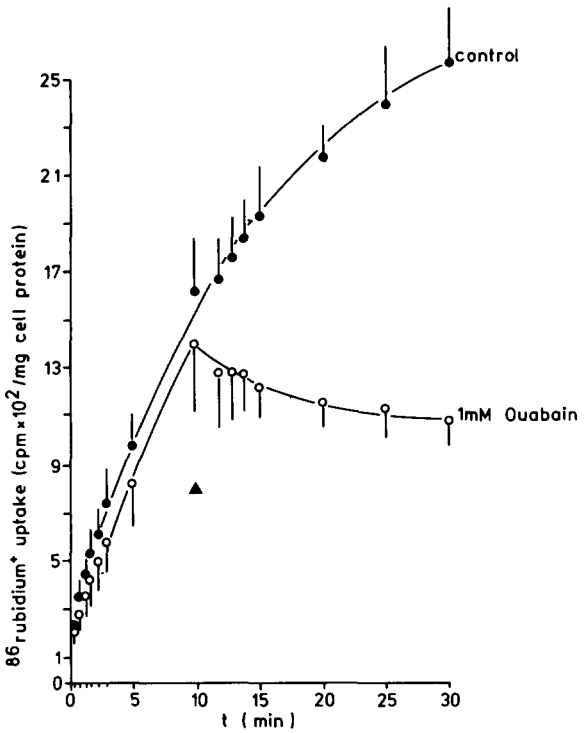


Fig. 4. Effect of 1 mM ouabain on the transport of rubidium-86 across the membrane of isolated rat hepatocytes. Hepatocytes were incubated in the presence of 1–5 μCi $^{86}Rb^+$ at 37°C under O_2/CO_2 (95/5) atmosphere in Tyrode solution. Ouabain, final concentration 1 mM, was added after 10 min. Immediately, $^{86}Rb^+$ uptake was blocked and a slow efflux observed. The velocity of $^{86}Rb^+$ release correlated with the slow depolarization of E_m measured by microelectrodes (see also Table II).

The deviation from linearity in Fig. 3A can be explained either by permeabilities of ions other than potassium or by a change in the permeability properties for potassium in hepatocytes.

Ouabain slowly depolarized isolated rat hepatocytes. The rate of depolarization by 1 mM ouabain, during the recording from a single cell, was 20 mV within 7 min. When liver cells were preincubated with 1 mM ouabain and E_m determinations were made at various intervals, E_m slowly decreased with time. After 120 min, the mean membrane potential of ouabain-treated cells was −17 mV (Table II). In separate experiments ouabain evoked a slow $^{86}Rb^+$ efflux out of isolated liver cells, subsequent to a blockade of $^{86}Rb^+$ uptake, which indicates complete inhibition of the $(Na^+ + K^+)$-ATPase (Fig. 4).

Triton X-100 (final concentration 1%) caused cell lysis and E_m decreased to residual values of −5 to −10 mV (not shown).

The time-course of the membrane potential obtained after cell puncture was biphasic. The maximum value was achieved only slowly during the first minute. We assume that this is due to a sealing process around the impaled microelectrode. Induced electrode vibrations after impalement prevented the sealing, leading to a stepwise depolarization. The increase in E_m with time might have been also the result of an activation of potassium channels by influx of Ca^{2+} subsequent to cell puncture. However, potassium-channel blockers such as quinine (350 μM) (Table II), apamin (10 μg/ml) (Table II), 2,4-diaminopyridine (250 μM) (not shown), and dentrotoxin (10 μg/ml) (not shown) failed to influence the E_m. In the absence of external Ca^{2+} or in the presence of 1 mM EGTA (in absence of Ca^{2+}), however, the mean potential was below −20 mV (Table II), indicating that the presence of external Ca^{2+} was essential for the maintenance of a high E_m. The mechanisms by which Ca^{2+} maintains liver cell E_m in rats remains unknown. It should be noted that rat hepatocytes in contrast to guinea pig hepatocytes have been reported to lack Ca^{2+}-dependent K^+ channels [14,15].

Whereas Ca^{2+} was necessary in maintaining liver cell E_m, sodium and chloride were not. In the absence of sodium (NaCl was substituted by choline chloride, $NaHCO_3$ by $LiHCO_3$, and NaH_2PO_4 by KH_2PO_4) potentials of −70 to −75 mV were measured. Substitution of Cl^- by methylsulfate gave potentials of −70 to −83 mV (Table II). The results are thus consistent with the assumption that the membrane potential of isolated rat hepatocytes is mainly due to a potassium diffusion potential, with only minor contributions by sodium and chloride.

The finding of a high membrane potential in isolated hepatocytes, attached to collagen, was unexpected in view of previously reported literature (see Table I). After a relatively long observation period (3 years) we would like to emphasize the importance to these studies of two important factors. The first is the quality of the microelectrodes. Such electrodes should have a small tip diameter in order to avoid injury of the cell and a short shank to reduce vibrations. When the impaled electrode was sealed into the membrane a membrane resistance of about 35 MΩ could be calculated. If cell swelling occurred immediately after impalement the recorded potentials were below −15 mV. Such observations were often made with early studies when electrodes with lower resistance were used. If liver cells started to swell at the end of a recording the potential decreased rapidly.

The second important factor was the procedures involved in the maintenance of cultured hepatocytes. After isolation, hepatocytes were incubated for at least 30 min in suspension before they were placed on cover slips. Over this period membrane damage and potassium ion loss during the isolation procedure was reversed. Attachment of hepatocytes on collagen-coated cover slips during a further 30-min incubation resulted in immobilisation of the cells and thus facilitated the impalement with microelectrodes. Within $2\frac{1}{2}$ hours of isolation they retained a spherical morphology, after which they started to spread and flatten. Only spherical cells were used in this study, as a 'good seal' was difficult to obtain with flattened cells. Completely flattened hepatocytes, growing as monolayers, have been reported to possess membrane potentials of −10 to −25 mV [28]. Interestingly, also in this monolayer culture biphasic membrane potentials were observed on hepatocytes, although the measured potentials were markedly low. In that study it was concluded

that the slow increase in the membrane potential after cell puncture, was the result of either a sealing process for a shunt pathway around the electrode or a reduction of any other influx of depolarizing cations (Na^+ or Ca^{2+}) into the cells. As the measured potentials were only about -20 mV it was assumed that low potentials were the result of a current flow through an electrode shunt which affects normal membrane resistance [28]. With respect to spherical hepatocytes, attached to collagen, the high membrane potential might be unique as these cells are not electrotonically coupled. However, our observation of identical potentials in coupled cell-pairs might indicate that either coupling does not alter this membrane potential, or that functional coupling was impaired in these cells as result of the isolation procedure.

From these reported observations we conclude that the high membrane potentials measured in the present experiments are the result of a high potassium permeability of the membrane of isolated spherical hepatocytes. The potassium permeability is probably not induced by Ca^{2+}-activated potassium channels. Neither blockers of Ca^{2+}-activated K^+ channels, such as apamin or quinine (see Ref. 14) nor of other K^+ channel inhibitors such as 2,4-diaminopyridine or dentrotoxin [32] altered the potentials. However, the absence of external Ca^{2+} reduced our potential recordings (Table II), possibly indicating that the function of external calcium is to seal off the membrane at the punctured lesion.

The importance of E_m in bile acid secretion was believed to be small as the resting membrane potential of hepatocytes was repeatedly reported not to exceed -50 mV, and such a low potential would be insufficient to drive bile acids against their 10-fold electrochemical gradient, which is presumed to exist across the canalicular pole of hepatocytes [6]. However, in order to maintain bile acid secretion against such an inward-directed concentration gradient a potential of -78 mV would be an adequate driving force.

The authors wish to thank Professors Drs. F. Dreyer, G. Isenberg, J.B. Stokes, and E. Heinz for helpful discussions and I. Nickola for her support in establishing short-time cell cultures. E. Petzinger thanksfully acknowledges the encouragement by Professor Dr. M. Frimmer, Giessen and Professor Dr. H. Fasold, Frankfurt. This work was supported by the Deutsche Forschungsgemeinschaft grant FA 48-23, Sonderforschungsbereich 169.

References

1 Ruifrok, P.G. and Meijer, D.K.F. (1982) Liver 2, 28–34
2 Inoue, M., Kinne, R., Tran, T. and Arias, I.M. (1982) Hepatology 2, 572–579
3 Duffy, M.C., Blitzer, B. and Boyer, J.L. (1983) J. Clin. Invest. 72, 1470–1481
4 Petzinger, E. and Frimmer, M. (1984) Biochim. Biophys. Acta 778, 539–548
5 Meier, P.J., Meier-Abt, A.S., Barrett, C. and Boyer, J.L. (1984) J. Biol. Chem. 259, 10614–10622
6 Blitzer, B.L. and Boyer, J.L. (1982) Gastroenterology 82, 346–357
7 Ehrhardt, V. (1984) Biochim. Biophys. Acta 775, 182–188
8 Hoek, J.B., Nicholls, D.G. and Williamson, J.R. (1980) J. Biol. Chem. 255, 1458–1464
9 Bradford, N.M., Hayes, M.R. and McGivan, J.D. (1985) Biochim. Biophys. Acta 845, 10–16
10 Mazet, J.L., Claret, M. and Claret, B. (1974) J. Membrane Biol. 18, 335–350
11 Berry, M.S. and Friend, D.S. (1969) J. Cell Biol. 43, 506–529
12 Petzinger, E. and Seeger, R. (1976) Naunyn-Schmideberg's Arch. Pharmacol. 295, 211–213
13 Nickola, I. and Frimmer, M. (1986) Cell Tissue Res. 243, 437–440
14 Burgess, G.M., Claret, M. and Jenkinson, D.H. (1981) J. Physiol. 317, 67–90
15 Cook, N.S. and Haylett, D.G. (1985) J. Physiol. 358, 373–394
16 Wondergem, R. and Harder, D.R. (1980) J. Cell Physiol. 102, 193–197
17 Claret, M., Coraboeuf, E. and Favier, M. (1970) Arch. Int. Physiol. Biochem. 78, 531–535
18 Friedman, N., Somlyo, A.V. and Somlyo, A.P. (1971) Science N.Y. 171, 400–402
19 Haylett, D.G. and Jenkinson, D.H. (1972) J. Physiol. 225, 72–750
20 Graf, J. and Petersen, O.H. (1974) Proc. Royal Soc. Lond. B 187, 363–367
21 Chapman, L.M. and Wondergem, R. (1984) J. Cell Physiol. 121, 7–12
22 Friedman, N. and Dambach, G. (1980) Biochim. Biophys. Acta 596, 180–185
23 Williams, J.A., Withrow, C.D. and Woodburg, D.M. (1971) J. Physiol. (Lond) 212, 101–115
24 Friedman, N. and Dambach, G. (1973) Biochim. Biophys. Acta 307, 399–403
25 Olson, J.R., Hosko, M.J. and Fujimoto, J.M. (1979) Life Sci. 25, 2043–2050
26 Heller, P. and Van der Kloot, W. (1974) J. Physiol. 243, 577–598
27 Schanne, O. and Coraboeuf, E. (1966) Nature 210, 1390–1391

28 Wondergem, R. (1981) Am. J. Physiol. 241, C209–C214
29 Graf, J., Gautam, A. and Boyer, J.L. (1984) Proc. Natl. Acad. Sci. USA 81, 6515–6520
30 Bear, C.E. and Shaffer, E.A. (1985) Hepatology 5, 1028
31 Edmondson, J.W., Miller, B.A. and Lumeng, L. (1986) Am. J. Physiol. 249, G427–G433
32 Penner, R., Petersen, M., Pierau, F.-K. and Dreyer, F. (1986) Pflügers Arch. 407, 365–369