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FIBROBLASTOID AND EPITHELIOID CELLS IN TISSUE CULTURE: DIFFERENCES IN SENSITIVITY TO OUABAIN AND TO PHOSPHOLIPID COMPOSITION

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Summary

Investigations on nine different mammalian cell lines revealed that permanently growing cells of one morphological class have numerous membrane properties in common which are different or even lacking in the other cell class. With electrophysiological methods it is shown that the sensitivity of the Na^+-K^+ pump to ouabain is three orders of magnitude higher in the ionically non-coupled epithelioid cells than in the ionically coupled fibroblastoid cells which respond like primary cultures. This is accompanied by considerably higher binding constants for ouabain of the epithelioid cells as was shown by [3H]ouabain binding and membrane potential measurements. The epithelioid cells also revealed a 50% lower relative amount of phosphatidylethanolamine and a 60-fold less net synthesis of phospatidylinositol. Finally, although primary cultures cannot proliferate without serum, permanent fibroblastoid cells have a reduced serum requirement and permanent epithelioid cells can proliferate without any serum.

Introduction

Permanently proliferating cells in vitro are subdivided into two morphologically different cell classes which comprise both malignant and non-malignant cell lines; the fibroblastoid and the epithelioid cells. Recent evidence enables these two cell classes to be distinguished by criteria more objective than morphology, including ionic coupling [1], bicarbonate-dependent permeability

changes of the plasma membrane [2], gap junctions [3] and serum requirement for proliferation [4].

In this contribution we report further differences that exist between the two cell classes, i.e. the sensitivity of the Na^+-K^+ pump to ouabain, the net synthesis of phosphatidylinositol, and the relative amount of phosphatidylethanolamine.

Materials and Methods

Cell cultures

Primary cultures of embryonic rat fibroblasts were prepared as already described [5] from the dorsal muscles of Marshall embryos at the 18th day of gestation. They were used for the experiments only from the second to the fourth passages and are called primary cells (SRE). Two spontaneous transformations to permanent proliferation from primary embryonic rat cells (Sprague-Dawley) have been passaged in vitro since 1967, the fibroblastoid REf and the epithelioid line REe. Three other permanently growing epithelioid cell lines HE (embryonic hamster), HeLa, and KB (human carcinoma) as well as the fibroblastoid 3T3 cells (mouse embryo) were kindly provided by Dr E. Friedrich-Freksa. The fibroblastoid BICR/MlR-K cells were derived from transplanted Marshall rat tumors, whereas the fibroblastoid BT5C2 cells are a tumor line which arose by in vitro culture of brain cells from ethylnitrosourea-treated rat fetuses. Both cell lines were kindly provided by Dr M.F. Rajewsky.

All cells were cultivated at 37°C in a modified Eagle-Dulbecco medium [6] with 10% calf serum under an atmosphere of 5% CO_2 in air at pH 7.4. The cells were passaged at the end of the log phase of growth by treating the monolayer with 0.25% trypsin in Ca-Mg-free isotonic phosphate-buffered salt solution. For stock cultures the cells were seeded into Roux glass flasks, and for experiments into Falcon plastic Petri dishes.

Fluorimetric analysis of DNA

This was performed with a Phywe ICP 11 impulse cytophotometer [7]. The trypsinized cells were washed twice in phosphate-buffered salt solution, fixed in methanol, incubated with ribonuclease (1 mg/ml distilled water) for 1 h at 37°C, and stained with ethidium bromide (10 µg/ml Tris buffer, pH 7.5; Serva, Heidelberg). The fluorescence intensity of ethidium bromide increases with the DNA content of a cell and, therefore, indicates the relative number of cells in the different cell cycle phases. The data are plotted as a DNA histogram. Between 50 000 and 200 000 cells have been counted for the histograms.

Electrophysiology

The electrophysiological methods have been described in detail elsewhere [8]. Every point represents more than 20 impalements in cells from different areas of the 50-mm dishes. Such measurements were completed within 15 min.

Ouabain binding

This was carried out with ^{3}H-labeled ouabain (NEN Corp.) which was supplied in ethanol-benzene. After evaporating the solvent in a stream of N_2

the ouabain was redissolved for the stock solution of 10^{-5} M in a K^+-free Eagle-Dulbecco medium supplemented with 10% calf serum as described by Boardman et al. [9]. The final ouabain concentrations of 10^{-6} M–10^{-8} M were obtained by dilution with K^+-free Eagle-Dulbecco medium; the concentrations of 10^{-4} M–10^{-2} M were prepared by addition of unlabelled ouabain medium to the stock solution.

The cells were grown to confluency with 2 ml Eagle-Dulbecco medium. This medium was changed to 0.9 ml of the [³H] ouabain medium. After a 2-h incubation period this [³H] ouabain medium was used again for a second experiment with the same cell line. The cells were washed four times in ice-cold K^+-free medium. The cells were removed from the dish by 1 ml distilled water using a rubber policeman and dried overnight at 50°C under vacuum in scintillation flasks. After dissolving the cells in 1 ml Nuclear Chicago Solvent + 1 drop distilled water, they were counted in a Packard Tricarb scintillation counter using toluene scintillation fluid (4 g PPO/1 l toluene). After quench-correction the counts were correlated to the cell number obtained from five parallel dishes and thus the number of ouabain molecules bound per cell was determined.

Incorporation of [³H] thymidine

Tylocine (GIBCO) was added to the culture medium in order to eliminate the possibility of mycoplasma contamination. After one day the medium was changed to medium containing various ouabain concentrations, and 24 h later [³H] thymidine incorporation into the acid-insoluble material during a pulse-labelling period of 1 h (1 μCi/ml medium, spec. act. 5 Ci/mmole, Amersham) was determined [10].

Phospholipid content and incorporation of inorganic ³²P into phosphatidyl-choline and phosphatidylinositol

The culture medium was removed 24 h after inoculation and replaced by phosphate-free medium. After 1 h inorganic ³²P (6 μCi/ml, carrier-free, NEN Corp.) was added for 30 min and the phospholipids were extracted and separated by thin-layer chromatography. The specific activity of phosphatidyl-choline and phosphatidylinositol and the total phospholipid composition were established on the basis of quantitative phosphate determinations [11].

⁴⁵Ca²⁺ uptake

The culture medium was removed after 24 h inoculation and replaced by a medium containing $^{45}Ca^{2+}$(10^{-3} M, spec. act. 1.2 Ci/mole, Amersham) and a growth-stimulating protein fraction from fetal calf serum. This protein was obtained after precipitation at pH 4.9 and removal of the lipoproteins [5] and was used instead of calf serum in order to avoid any Ca^{2+} contamination by the serum. The total amount of calcium within the cells was estimated from the equilibrium concentration of $^{45}Ca^{2+}$ [12] with the assumption that 10^6 cells have a volume of 2 μl.

Results

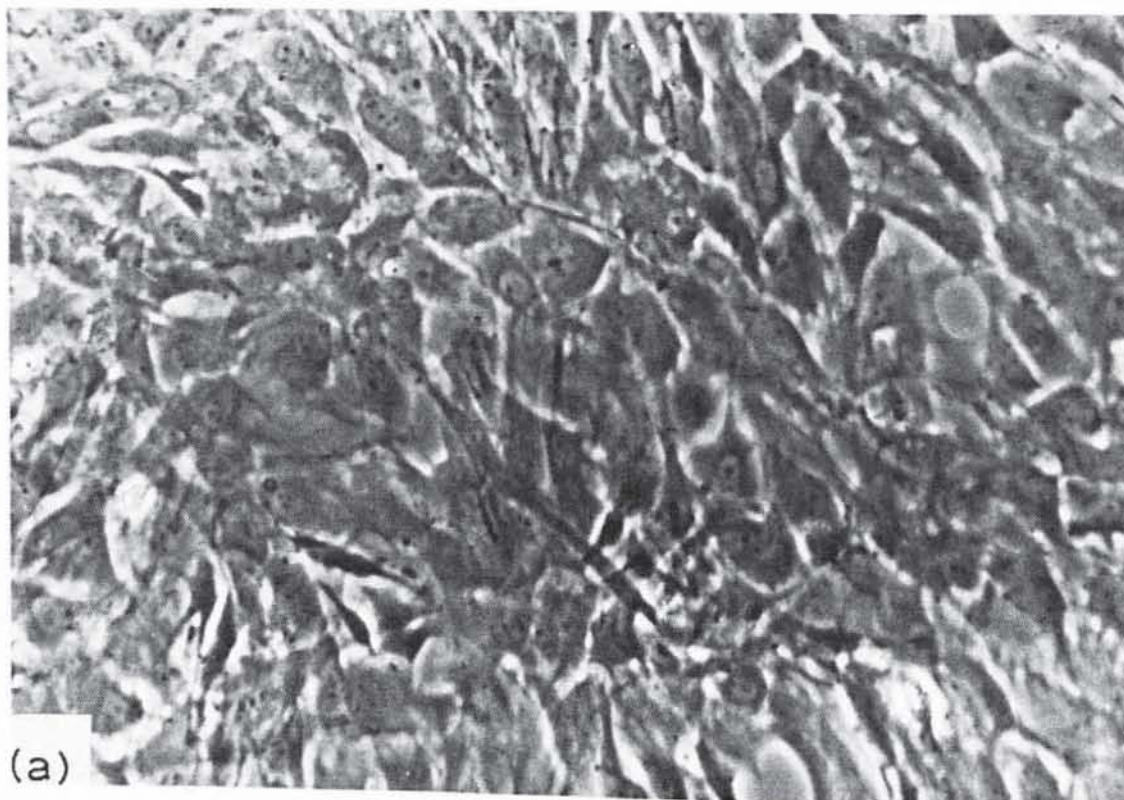Primary cultures of embryonic cells grow with either epithelioid or fibro-

blastoid appearance, but this difference has so far not been correlated with any of the investigated membrane properties. However, the two morphological classes of permanent cell lines are clearly separated by numerous membrane properties. The clear morphological differences can be seen in Fig. 1 for three different embryonic rat cell strains, a primary culture (Fig. 1a), a permanent fibroblastoid (Fig. 1b) and a permanent epithelioid (Fig. 1c) cell line.

Primary cultures of rat cells normally stop proliferation in vitro after 4—6 weeks and only after as yet unknown transformation steps do they gain the ability for unlimited proliferation. These untransformed cells characteristically depend upon serum for proliferation and accumulate in the G_1 phase of their cell cycle when kept in serum-free medium. Addition of serum or specific serum proteins will stimulate these cells to proceed through the cycle [13], an effect also observed to a lesser degree in permanent fibroblastoid cells (Ristow, H.-J., unpublished results).

Permanent epithelioid cells, however, are not so dependent upon serum and are able to proliferate for at least 72 h without serum [13]. The effect of a 24 h serum depletion on exponentially growing embryonic rat cells of the three types is illustrated by comparison of their DNA histograms (Fig. 2).

After serum depletion virtually all cells of the primary culture stopped their proliferation in the G_1 phase of the cell cycle. The ratio for the cell numbers $G_1 : G_2$ is about 2.8 in the normal distribution and about 9.7 in the serum-free sample. For the permanent fibroblastoid cells this serum effect is not so drastic, but nevertheless clearly observable with $G_1 : G_2$ ratios of about 2.4 for the normal distribution and of about 4.6 for the serum-free sample. The permanent epithelioid cells, however, have an almost unchanged DNA histogram after serum depletion, with $G_1 : G_2$ ratios of 1.6 and 2.1 respectively.
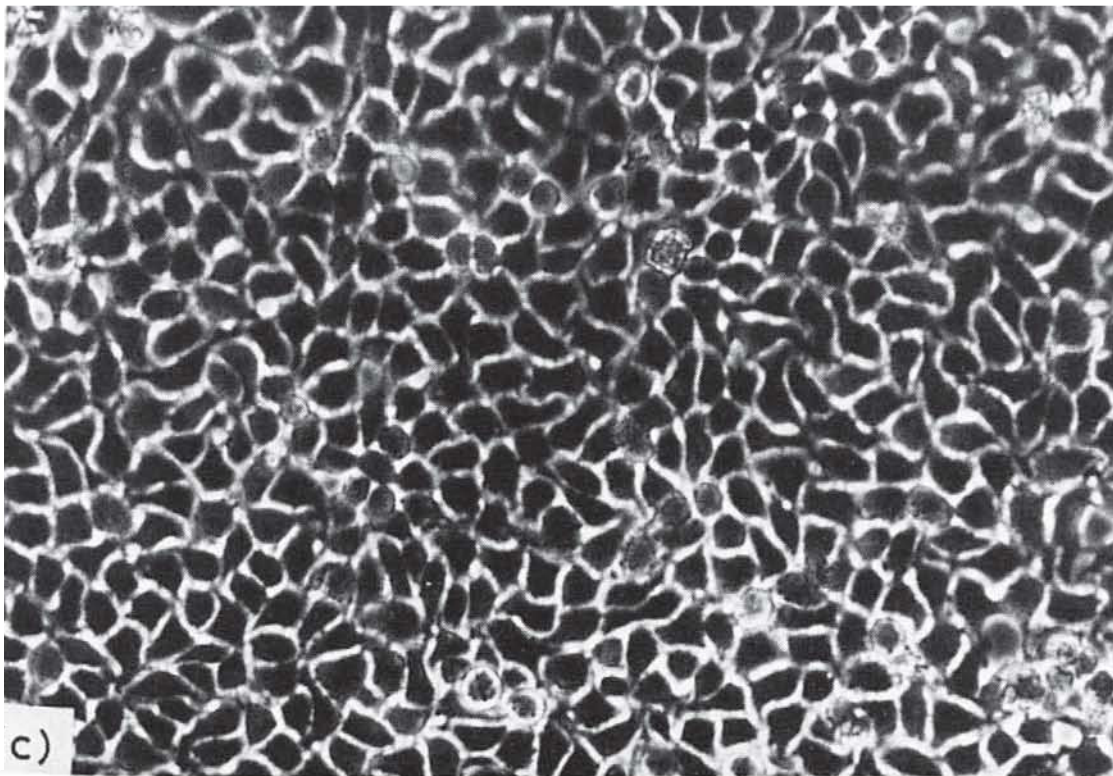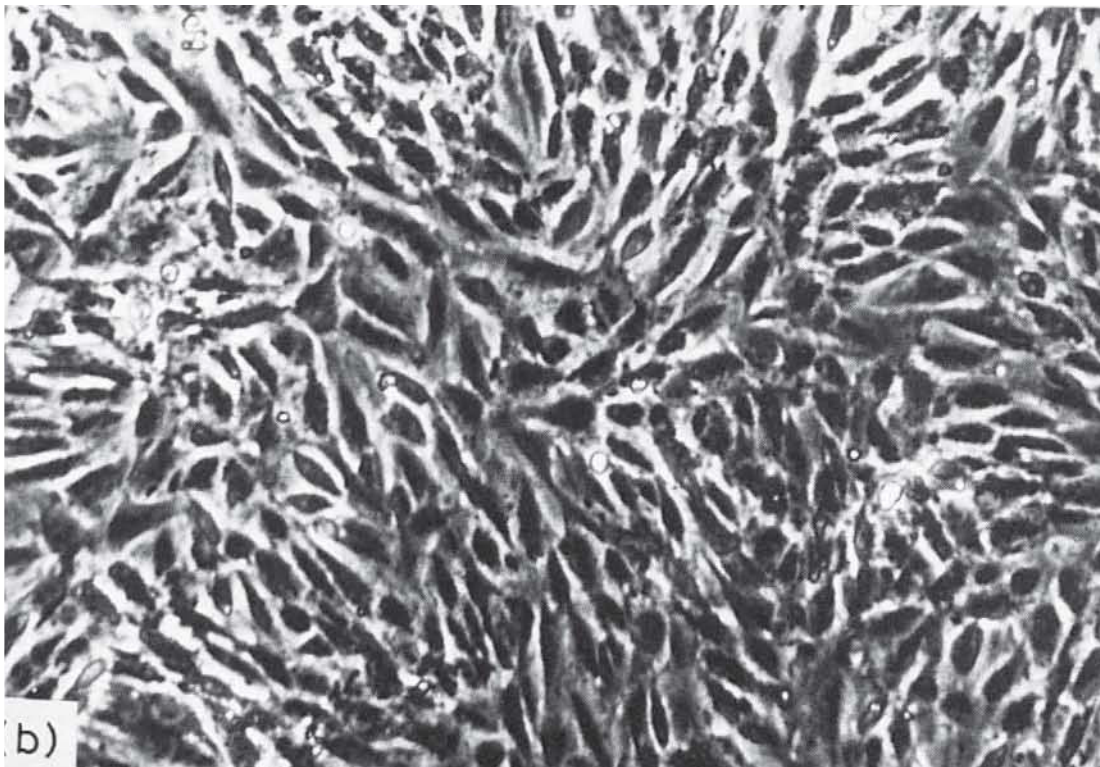

(a)

Fig. 1. Phase-contrast photographs of embryonic rat cells. (a) primary cells (SRE); (b) permanent fibro-blastoid cells (REf); (c) permanent epithelioid cells (REe). Magnification × 200.

The membrane potentials of all cells are of the same order of magnitude, as can be seen from Table I. The membrane potential is mainly due to the distribution of K^+ between inside and outside the cells and it is maintained by the Na^+-K^+ pump. Inhibition of this pump by ouabain, which in turn causes a
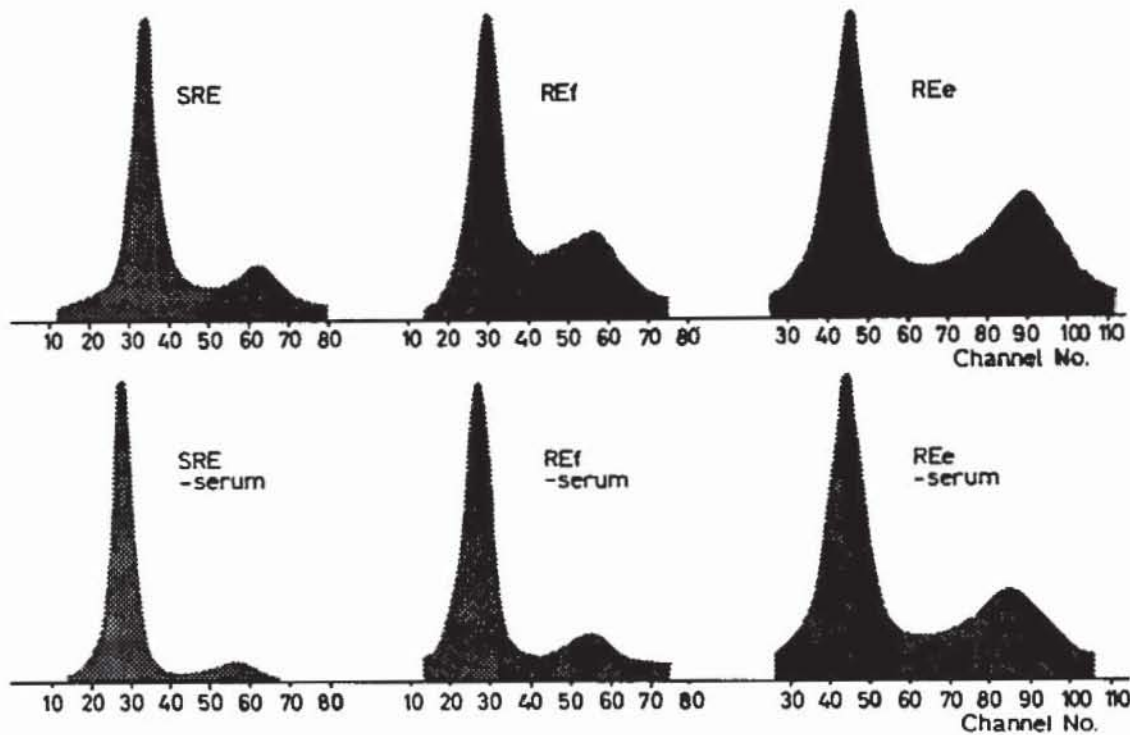
Fig. 2. DNA histograms of embryonic rat cells. Left, primary cells (SRE); middle, permanent fibroblastoid cells (REf); right, permanent epithelioid cells (REe). Cells cultured 48 h in medium + 10% calf serum (upper row) are compared with cells cultured for 24 h in medium + 10% calf serum and subsequent 24 h incubation in serum-free medium (lower row).

decrease of the potential difference, revealed that the two morphological classes differ in their sensitivities to ouabain. This is illustrated in Fig. 3. After 24 h incubation in Eagle-Dulbecco medium with various ouabain concentrations, the epithelioid cells almost equilibrated with the medium at a concentration of 10^{-6} M. Embryonic rat cells were the only epithelioid line that survived a 24 h incubation with 10^{-5} M ouabain. In contrast, both the fibroblastoid and the primary cells remained unaffected by concentrations of ouabain up to 10^{-4} M, and at 10^{-3} M ouabain the membrane potential was about 5% of the control.

TABLE I

MEMBRANE POTENTIALS OF THE INVESTIGATED PERMANENT CELL LINES AND THE PRIMARY CELLS (SRE)

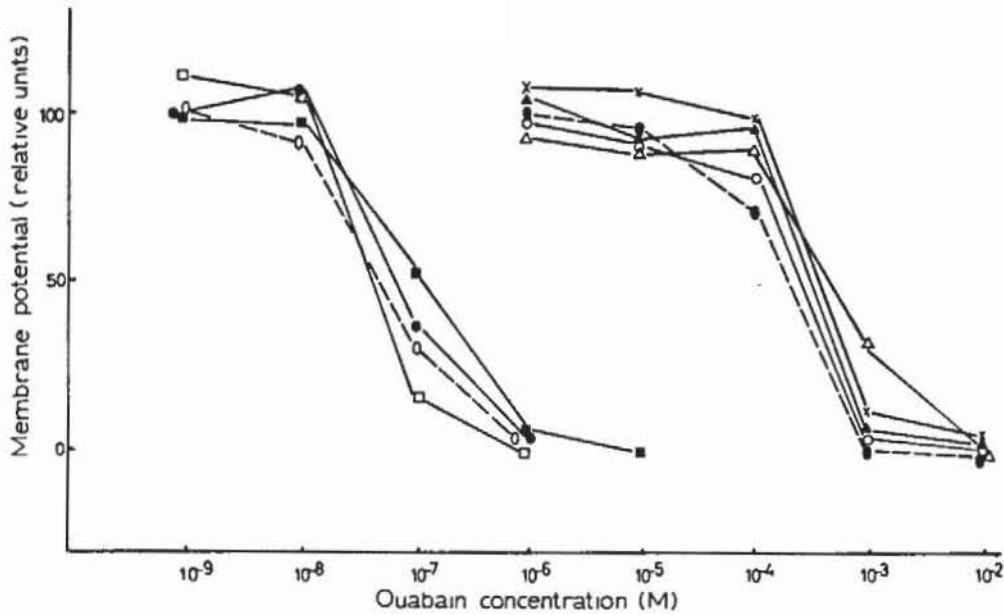| Cell line	Origin	Morphology	Membrane potential ± S.E. (mV)	Number of impalements
REe	Rat embryo	Epithelioid	42.5 ± 0.5	374
KB	Human carcinoma	Epithelioid	44.5 ± 0.8	140
HeLa	Human carcinoma	Epithelioid	29.5 ± 0.7	140
HE	Hamster embryo	Epithelioid	45.2 ± 0.9	62
REf	Rat embryo	Fibroblastoid	48.3 ± 0.5	226
BICR/M1R-K	Rat tumour	Fibroblastoid	50.6 ± 0.5	181
3T3	Mouse embryo	Fibroblastoid	31.8 ± 0.7	201
BT5C2	Rat tumour	Fibroblastoid	61.3 ± 0.5	189
SRE	Rat embryo	Fibroblastoid (epithelioid)	48.0 ± 0.8	216

Fig. 3. The effect of ouabain on Na$^+$-K$^+$-pump of cultured cells demonstrated by membrane potential measurements. After a 24 h incubation with ouabain the membrane potentials were determined and normalized to the control values which are listed in Table I. Each point represents more than 20 measurements with a standard error similar to that given in Table I for the respective cell line. Epithelioid cells depolarize at concentrations of 10^{-7}M, fibroblastoid and primary rat cells at 10^{-3}M ouabain. □———□, HeLa; ○———○, REf; ○-----○, HE; △———△ BT5C2; ■———■, REe; ●-----●, 3T3; ●———●, KB; ▲———▲, SRE; X———X, BICR/M1R-K.

Some of the cells show even higher membrane potentials than their controls as long as they are incubated with doses lower than 10^{-8}M in the case of epithelioid cells and 10^{-4}M in the case of fibroblastoid cells. Ionic coupling was found in ouabain-treated fibroblastoid and primary cells as long as the cells retained a membrane potential.

The concentration of ouabain sufficient to inhibit the Na$^+$-K$^+$ pump also influences DNA synthesis (Fig. 4). Primary cells again respond in the same
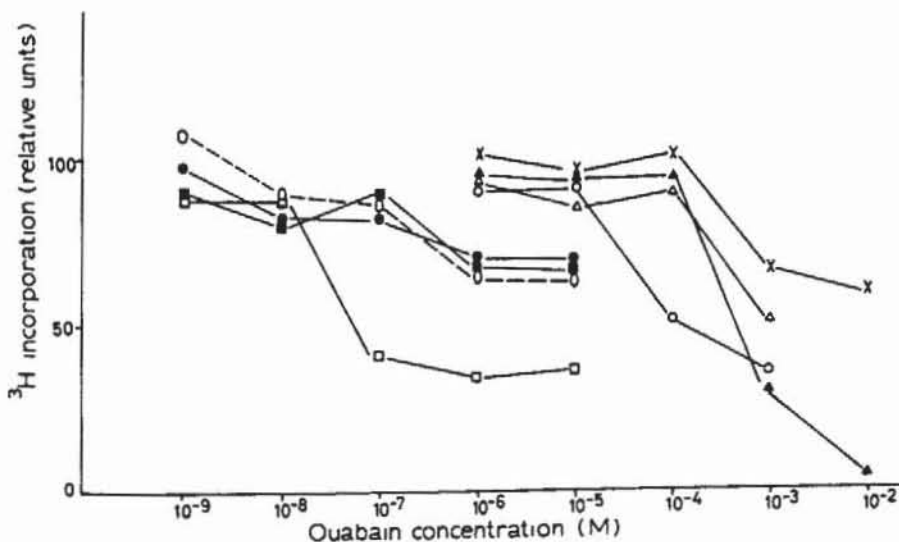

Fig. 4. The effect of ouabain on the [^{3}H]thymidine incorporation into acid-insoluble material of cultured cells. Each point was determined at least in duplicate. Both cell classes respond with a decreased incorporation at the same concentrations at which the cells also depolarize (see Fig. 3). Where not indicated, no further decrease had been observed. □———□, HeLa; X———X, BICR/M1R-K; ○-----○, HE; ○———○, REf; ■———■, REe; △———△, BT5C2; ●———●, KB; ▲———▲, SRE.

manner as the fibroblastoid cells. Cell counting and the DNA histograms obtained by fluorimetric analysis also indicated that the run through the cell cycle is slowed down.

The striking difference between the two morphological classes in their sensitivity to ouabain could be due to different affinities of the receptor sites of the surface membrane for the glycoside. Therefore, we determined the number of ouabain molecules that bind to the cells as a function of ouabain concentration in the culture medium (Fig. 5). At concentrations of 10^{-7}M and 10^{-6}M, which decreased the membrane potential and influenced DNA synthesis, the binding of ouabain to epithelioid cells reached a plateau of about $2 \cdot 10^6$ molecules per cell. With higher ouabain concentrations the number of bound molecules per cell increased linearly, as was found for fibroblastoid cells throughout the whole range of ouabain concentration.

This difference in ouabain binding can also be seen from the different recovery of the membrane potentials after washing ouabain off. The cells were incubated at 37°C until membrane potentials were to be measured. In Fig. 6, the membrane potentials are seen to decrease at a somewhat faster rate in the fibroblastoid than in the epithelioid cells under the influence of 10^{-3}M and 10^{-6}M ouabain respectively. Experiments with epithelioid rat cells using 10^{-3}M ouabain indicated that this was probably due to the considerably higher
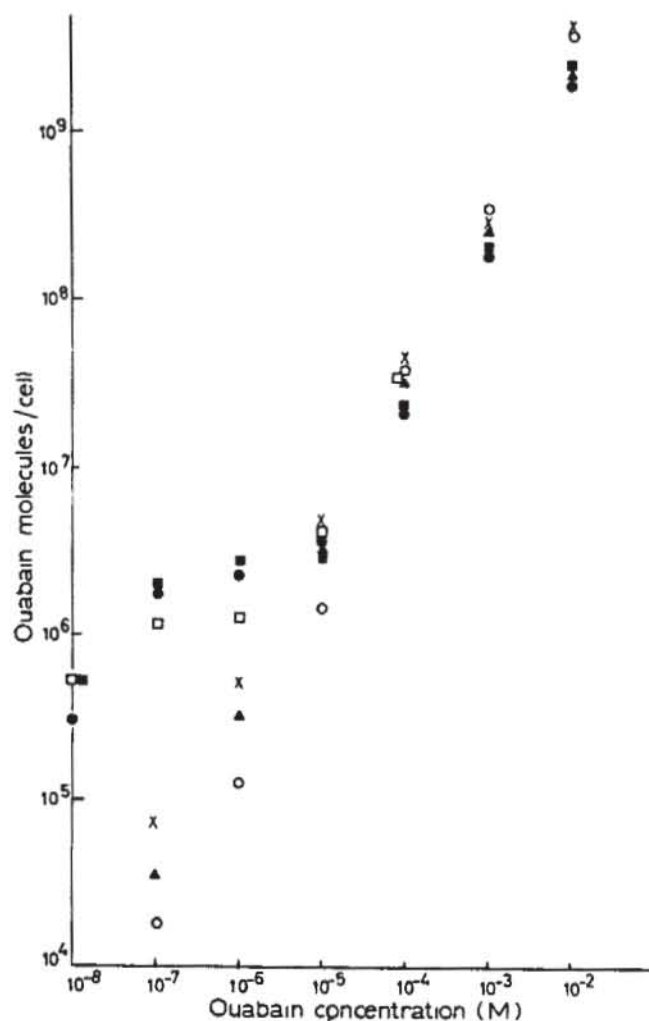

Fig. 5. The binding of ouabain to cultured cells. The cells had been incubated for 2 h in K-free medium with [^{3}H] ouabain. Each point represents means of 2—5 determinations. □————□, HeLa; X————X, BICR/M1R-K; ■————■, REe; o————o, REf; ●————●, KB; ▲————▲, SRE.
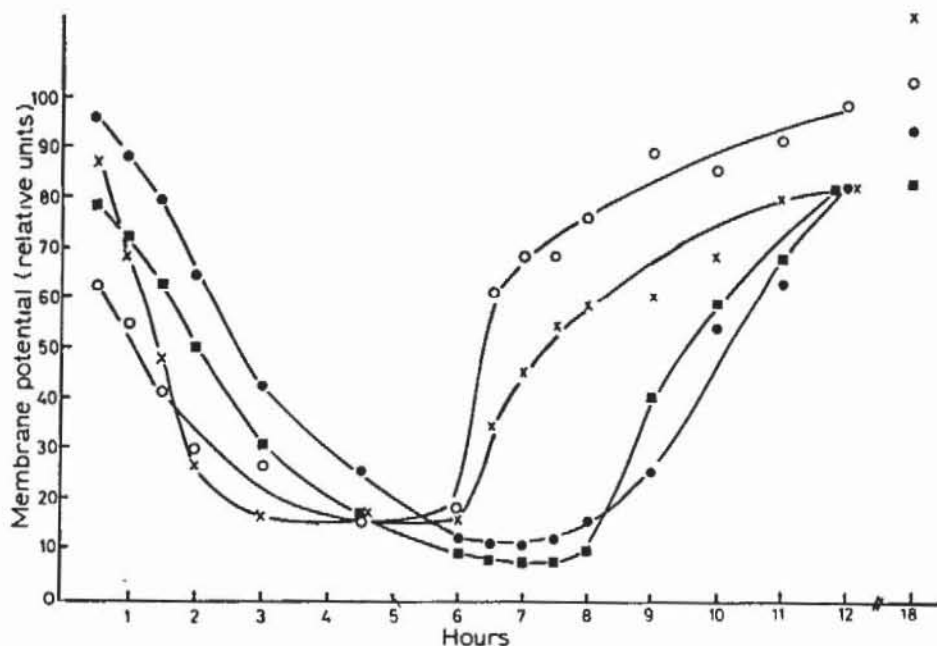
Fig. 6. Kinetics of cell depolarizations by 10^{-3}M ouabain for the fibroblastoid cell lines BICR/M1R-K (X———X) and REf (o———o) and by 10^{-6}M ouabain for the epithelioid cell lines KB (●———●) and Ree (■———■). After 6 h the medium was changed to ouabain-free medium which resulted in an immediate recovery of the membrane potentials in the case of the fibroblastoid cells, whereas the epithelioid cells have a lag phase of about 2 h before recovery.

ouabain concentration used for the fibroblastoid cells. However, fibroblastoid cells quickly reestablished their membrane potential after the ouabain was washed off, whereas the epithelioid cells remained inhibited for about 2 h before recovering at almost the same rate as the fibroblastoid cells. Medium exchange during the measurement of the membrane potential in the fibroblastoid BT5C2 cells demonstrated that the potential difference increased within 2 min from 5 mV to 40 mV, which is about 65% of the control. At this level, however, no further increase could be detected during the following 30 min.

The activity of the Na^{+}-K^{+}-activated ATPase as well as of numerous other membrane-bound enzymes are dependent on the presence of phospholipids [14]. Therefore, we investigated the phospholipid composition of the different cell lines in question (Table II). There is a difference only in the relative amounts of phosphatidylethanolamine of epithelioid and fibroblastoid cells. However, the net synthesis of phosphatidylinositol is about 60 times decreased in epithelioid cells. These results are summarized by the ratio of the net synthesis of phosphatidylcholine:phosphatidylinositol which is about 12 for epithelioid cells and about 0.2 for fibroblastoid and primary cells (Table III).

Incubation of epithelioid cells in phosphatidylethanolamine- or phosphatidylinositol-containing medium (8—800 µg/ml) had no effect on the morphology neither on the ouabain sensitivity nor were the cells recoupled. Fibroblastoid cells also remained unaffected by the addition of phosphatidyl serine, which has been shown to influence ouabain binding to Na^{+}-K^{+}-activated ATPase [15,16].

Morphological changes in cultured cells, however, have been effected by lipophilic acids [17] and by dibutyryl adenosine $3' : 5'$ cyclic monophosphate [18]. An 18 h incubation with $2 \cdot 10^{-3}$M butyric acid had a drastic effect on

TABLE II

COMPOSITION OF PHOSPHOLIPIDS (% OF TOTAL PHOSPHOLIPIDS) IN PERMANENT FIBRO-
BLASTOID (BICR/M1R-K, BT5C2, REf), PERMANENT EPITHELIOID (REf, KB, HE) AND PRIMARY
RAT (SRE) CELLS

Phospho-lipid	Cell line						
	BICR/M1R-K	BT5C2	REf	SRE	REe	KB	HE
Sphing-omyelin	5.6 ± 0.6	8.4 ± 0.7	10.8 ± 1.5	7.1 ± 1.0	8.2 ± 2.2	10.3 ± 0.7	11.8 ± 0.7
Phos-phatidyl-choline*	47.1 ± 2.1	45.2 ± 3.8	43.9 ± 2.4	48.3 ± 1.0	57.1 ± 8.1	45.4 ± 1.2	44.0 ± 2.9
Phos-phatidyl-inositol*	10.1 ± 0.4	9.0 ± 1.3	8.6 ± 1.0	9.4 ± 0.1	8.9 ± 0.7	9.7 ± 0.6	11.4 ± 1.4
Phos-phatidyl-serine*	6.1 ± 0.3	5.3 ± 0.1	6.7 ± 1.2	4.4 ± 0.4	5.2 ± 1.5	8.4 ± 0.8	8.7 ± 1.2
Phos-phatidyl-ethanol-amine*	26.5 ± 2.0	28.4 ± 1.8	25.5 ± 1.1	27.0 ± 1.2	18.3 ± 0.4	19.0 ± 0.8	18.7 ± 0.9
Cardiolipin	4.5 ± 0.3	3.7 ± 0.1	4.7 ± 0.7	4.0 ± 1.4	4.8 ± 1.4	7.2 ± 1.0	6.1 ± 1.6

* May contain alkyl- and alcenyl-lipids in addition to the diacyl compounds.

the morphology of epithelioid cells which became typical fibroblastoid cells, as
illustrated in Fig. 7 for HeLa cells. However, after this procedure the sensitivity
to ouabain remained unchanged and the cells were still non-coupled ionically,
as investigated with the three epithelioid cell lines KB, HeLa and REe. Fibro-
blastoid cells were unaffected by butyric acid. On the other hand, an 18 h
incubation in 1 mM dibutyryl adenosine $3' : 5'$ cyclic monophosphate en-
hanced the elongation of fibroblastoid cells, but did not influence the morpho-

TABLE III

RATIO OF NET SYNTHESIS OF PHOSPHATIDYLCHOLINE: PHOSPHATIDYLINOSITOL IN PER-
MANENT EPITHELIOID (REe, HE, KB), PERMANENT FIBROBLASTOID (BT5C2, BICR/M1R-K, REf)
AND PRIMARY (SRE) CELLS

Means ± standard error; n = number of determinations.

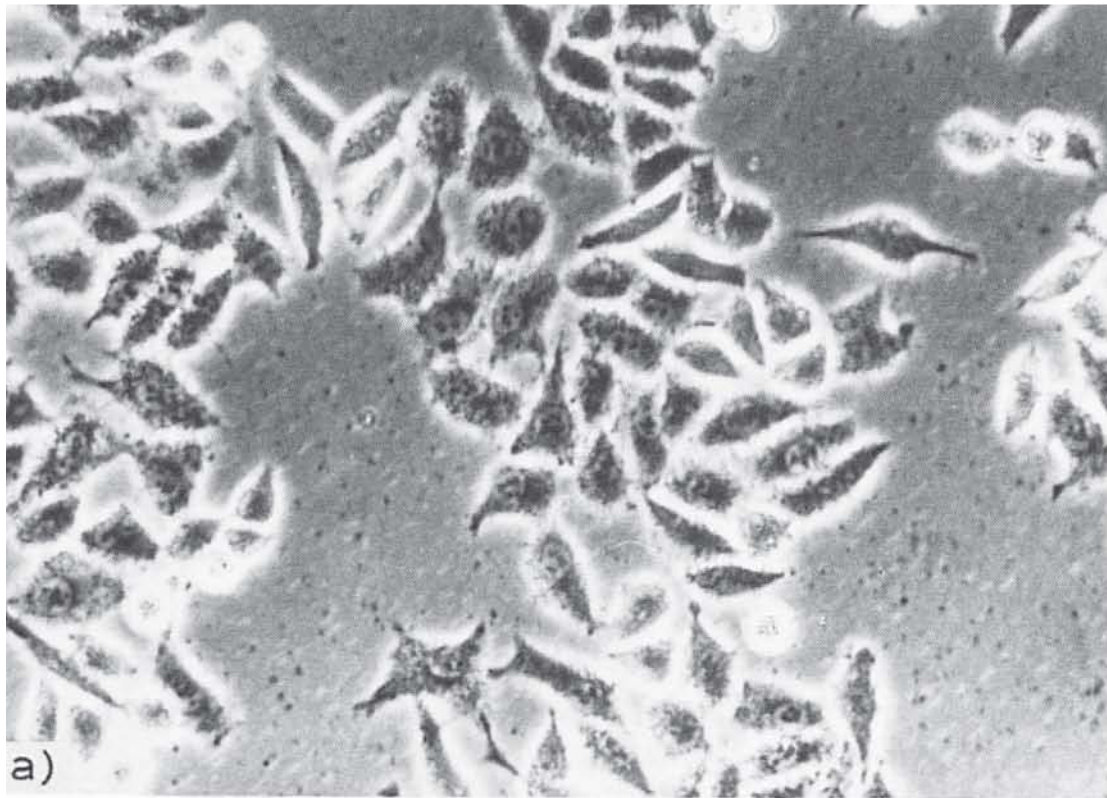| Cell line	Phosphatidylcholine / Phosphatidylinositol	n
REe	13.3 ± 1.6	8
HE	12.6 ± 0.5	8
KB	11.3 ± 2.3	8
BT5C2	0.17 ± 0.01	8
BICR/M1R-K	0.17 ± 0.02	8
REf	0.43 ± 0.08	7
SRE	0.13 ± 0.01	6

Fig. 7. Phase-contrast photograph of HeLa cells. (a) control; (b) after 18 h incubation in 2mM butyric acid. The morphology changed from epithelioid to fibroblastoid, but no functional changes could be observed. Magnification × 200.

logy of epithelioid cells. The electrophysiologically detectable properties remained unaffected.

Since it is known that Ca^{2+} concentration affects the Na^+-K^+-activated ATPase [19] and high concentrations of Ca^{2+} have been implicated as re-

TABLE IV

TOTAL Ca^{2+} CONCENTRATION IN PERMANENT FIBROBLASTOID (BICR/M1R-K, BT5C2, REf), PERMANENT EPITHELIOID (REe, KB) AND PRIMARY RAT (SRE) CELLS

Mean values ± standard deviations of 3—4 independent analyses.

Cell line	Ca^{2+} concentration (mM)
BICR/M1R-K	3.0 ± 0.6
BT5C2	3.3 ± 0.5
REf	0.5 ± 0.03
SRE	2.0 ± 0.5
REe	2.0 ± 0.3
KB	2.4 ± 0.5

sponsible for the breakdown of ionic coupling [20], a surface membrane property which permanent epithelioid cells are lacking [1], the total amount of Ca^{2+} was measured in some cell lines. In all investigated cell lines the total amount of Ca^{2+} was about 10^{-3} M regardless of the cell morphology (Table IV). The highest ($3.3 \cdot 10^{-3}$ M) and the lowest ($5 \cdot 10^{-4}$ M) Ca^{2+} concentration was found in cells that belong to the permanent fibroblastoid cells. The two cell classes are, therefore, indistinguishable with respect to total Ca^{2+} concentration.

TABLE V

CORRELATION OF PLASMA MEMBRANE PROPERTIES WITH MORPHOLOGY OF CULTURED MAMMALIAN CELLS

Morphology when forming a confluent monolayer	Fibroblastoid	Epithelioid	Refs.
Lowest ouabain concentration for complete cell depolarization	10^{-3} M	10^{-6} M	This paper
Bound ouabain molecules per cell (10^{-7} M ouabain in medium)	$\sim 4 \cdot 10^4$	$\sim 2 \cdot 10^6$	This paper
Ratio of net synthesis of phosphatidylcholine: phosphatidylinositol	~ 0.2	~ 12	This paper
Relative amount of phosphatidyl-ethanolamine	$\sim 27\%$	$\sim 19\%$	This paper
Total Ca^{2+} concentration	0.5—$3.3 \cdot 10^{-3}$ M	2.0—$2.4 \cdot 10^{-3}$ M	This paper
Ionic coupling	+	−	[1, 24—27]
Gap junctions	+	−	[3, 26—29]
Permeability change with bicarbonate concentration	+	−	[2]
Serum requirement	+	−	[4]
Morphology change by butyric acids	−	+	[17]
Morphology change by dibutyryl adenosine 3'-5' cyclic monophosphate	+	−	[18]
Malignancy after reimplantation in vivo	+ −	+ −	

Discussion

Differences in the behaviour of permanently cultured epithelioid and fibroblastoid cell lines have already been described and the most remarkable properties are listed in Table V together with our results presented in this paper. It seems that the cell morphology is the expression of a drastic transformation of the surface membranes, since most of the described properties differ between the two morphological classes by several orders of magnitude or they are completely lacking in one cell class. We must underline, however, that the differentiation by morphology is only valid for permanently growing cells and not for untransformed primary rat cells which undergo only a limited number of cell divisions. These primary cultures show all the properties listed in Table V for the permanent fibroblastoid cells.

The most prominent differences between the two morphological classes of permanent cells seem to be the sensitivity of the Na^+-K^+ pump to ouabain, and the absence of both ionic coupling and gap junctions in epithelioid cell lines. Interestingly the epithelioid rat line was somewhat more resistant to ouabain than the other epithelioid cells from human and hamster origin. This may indicate that also in the transformed state some species-specific differences in the sensitivity for ouabain may be expressed [21]. Since most of the fibroblastoid cell lines originate also from the rat, the difference of three orders of magnitude must, however, account for other plasma membrane properties.

The higher affinity of epithelioid cells for ouabain at low concentrations may be explained by a different fluidity of the membranes, caused by different phospholipid compositions. As already mentioned, the activity of numerous membrane-bound enzymes is dependent on certain phospholipids. Since only phospholipids adjacent to the appropriate enzymes are effective, the overall phospholipid composition of the membrane is less important and it seemed unlikely that a difference in the total amount of phospholipids could be detected. Both cell classes were, however, significantly distinguishable by the net synthesis of phosphatidylinositol and by the relative amount of phosphatidylethanolamine. Therefore, we investigated the effect of phospholipids on the membrane properties. The cells remained unaffected when phospholipids were added to the culture medium.

It is possible, however, to alter the morphology of the cells with dibutyryl cyclic AMP, testosterone, colcemid and cytochalasine B [18] and with lipophilic acids [17]. We have confirmed the results of Ginsburg et al. [17] who described morphological changes with butyric acid specifically for epithelioid cells, while fibroblastoid cells remained unaffected. Dibutyryl adenosine 3' : 5' cyclic monophosphate on the other hand only influenced our fibroblastoid lines, resulting in elongation to extremely spindle-like cells, while epithelioid cells remained unaffected. In no case could functional alterations be detected, as measured by ionic coupling and by sensitivity to ouabain. These morphological changes of the cells have been interpreted as intracellular interactions with the microtubular-microfibrillar system [18] and may, furthermore, be different in epithelioid and fibroblastoid cells [17].

Fibroblastoid cells are ionically coupled by low-resistance junctions, whereas epithelioid cells are non-coupled [1]. Based on measurements of the

Ca^{2+} concentration in squid and crab it has been suggested that low-resistance junctions are present only when low Ca^{2+} concentrations ($< 10^{-6}$M) are maintained. Consequently it has been suggested that high cytoplasmic Ca^{2+} is responsible for the breakdown of these intercellular junctions [20]. In both morphological cell classes, however, the total amount is between 0.5 and $3.3 \cdot 10^{-3}$M Ca^{2+}, which is in agreement with the literature [22]. Fibroblastoid cells would, therefore, need to bind a considerably higher amount of Ca^{2+} than epithelioid cells and hence have a lower amount of free Ca^{2+} if the hypothesis of the Ca^{2+}-dependent ionic coupling is true. It has been reported, however, that only about 40% of the total amount of Ca^{2+} is found as free Ca^{2+} within a cell [23]. It is impossible, therefore, that 10^{-6}M of the free cytoplasmic Ca^{2+} can be obtained in fibroblastoid cells, but it cannot be excluded that different amounts of Ca^{2+} are bound in the two cell classes. This question, however, remains unanswered, as long as intracellular measurements with Ca^{2+}-sensitive electrodes are lacking.

Our results indicate that it may be pointless to compare cell lines which have been transformed to permanent proliferation in vitro, e.g. the malignant epithelioid HeLa cells and the non-malignant fibroblastoid 3T3 cells, with respect to growth. Both cell lines certainly differ in numerous properties, but many of them are not due to cell malignancy and result only from the different surface membranes which are characteristic for the two morphological classes of permanently growing cells.

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