J. Membrane Biol. 86, 197–202 (1985)

The Journal of
Membrane Biology

Surface Charge of Mammalian Neurones as Revealed by Microelectrophoresis

S.L. Mironov and E.V. Dolgaya
A.A. Bogomoletz Institute of Physiology, Academy of Sciences of the Ukrainian SSR, Kiev-24, USSR

Summary. The surface charge of isolated rat dorsal root ganglion neurones was studied by microelectrophoresis technique. The increase of Ca concentration caused greater reduction of the electrophoretic mobility compared to that produced by an equivalent amount of divalent organic cations, dimethonium or hexamethonium. No charge reversal for Ca concentrations up to 80 mM was observed. These data fit the suggestion that two anion groups of the outer membrane surface can bind one Ca ion with apparent binding constant of about 50 M^{-1}. In solutions of low pH the electrophoretic mobility of cells decreased corresponding to titration of acidic groups with apparent pK = 4.2. Trypsin treatment in mild conditions markedly reduced the surface charge; however, neuraminidase and hyaluronidase did not change it. N-bromosuccinimide (a specific reagent for carboxylic groups of proteins) decreased the electrophoretic mobility about 60%. However, no increase of the surface charge after the action of specific reagents for amino groups (2,4,6-trinitrobenzenesulfonic acid and maleic anhydride) was observed. It was shown that the surface charge depends also on the intracellular metabolism. If 1 mM dibutyryl cAMP or theophilline was added to the culture medium (thus, raising the concentration of cAMP inside the cell) the surface charge increased. This effect developed slowly and reached its maximum on the third day of incubation. Treatment of cells by 5 mM tolbutamide (an inhibitor of some protein kinases) did not change cell mobility. Addition of 5 mM N-ethylmaleimide (an inhibitor of adenylate cyclase) to the culture medium produced some decrease of the surface charge. On the basis of data obtained it is suggested that the charge of the outer membrane surface of neurones studied is mainly determined by carboxylic groups of membrane proteins, and changes in intracellular cAMP concentration influence the synthesis and reconstruction of these membrane components.

Key Words surface charge · mammalian neurones · microelectrophoresis

Introduction

One of the parameters reflecting the state of the cell membrane is its surface charge, which interacts with ions present in aqueous solution to form an electrical double layer. This layer determines the local electric field in a layer of solution adjacent to the cell surface. In particular, it plays an important part in cell-to-cell interactions and determines the concentration profile of different biologically active substances (including permeant ions and charged substrates for cell receptors) in the immediate vicinity of the membrane.

In the present study we investigated in detail the outer membrane surface charge of rat dorsal root ganglion neurones using a microelectrophoresis method. As probes for testing the molecular structure of the surface we have used different divalent cations, enzymes and chemical modifying agents. In addition, we studied the electrophoretic mobility of neurones during their incubation in a medium containing drugs that affect the intracellular system of cyclic nucleotides to investigate the relationship between the surface charge of the neuronal membrane and intracellular metabolism.

Materials and Methods

In our experiments new-born rats were used. Dorsal root ganglia after isolation were transferred into isotonic Ca-free solution containing 2.5 mM KCl, 280 mM sucrose, 10 mM Tris-HCl (pH 7.3 to 7.4), 0.4% EDTA where they were incubated for 1 hour at room temperature. The ganglia were then washed, transferred into a new solution (*see below*) and neurones were mechanically isolated using thin metal needles. The absence of membrane damage was checked by staining neurones with trypane blue. In all cases the relative quantity of viable neurones (not stained by dye) exceeded 80%.

During the incubation of rat dorsal root ganglion neurones in sterile conditions at 37°C for 5 days their electrophoretic mobility was constant and did not differ from the value measured for freshly isolated neurones. Moreover, when we tried to isolate neurones using proteolytic treatment of ganglia by trypsin or pronase to soften the connective tissues before mechanical isolation a noticeable decrease in their mobility was observed (*see also* Results). However, during subsequent incubation in sterile conditions the surface of neurones apparently repaired and almost complete restoration of their surface charge occurred (as judged from mobility values).

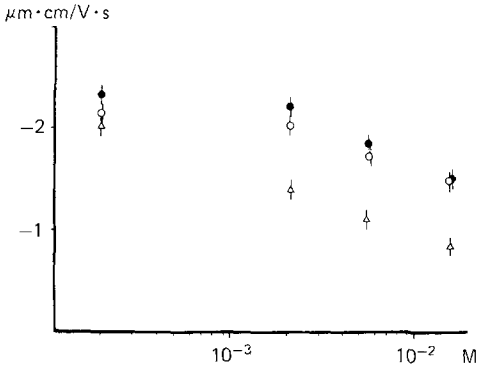

Fig. 1. Mean values of the electrophoretic mobility of rat dorsal root ganglion neurones versus the concentration of divalent cations (Ca^{2+} – △, dimethonium – ●, and hexamethonium – ○). Vertical bars correspond to SEM of measurements. Each solution contained a divalent cation (its concentration is indicated on abscissa), 10 mM Tris-HCl (pH 7.3 to 7.4) and the corresponding quantity of glucose. To remove possible contamination by traces of divalent inorganic cations, 1 mM EDTA was added to solutions containing organic divalent cations

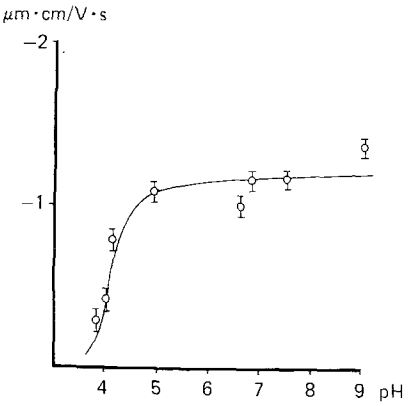

Fig. 2. Mean values of the electrophoretic mobility of rat dorsal root ganglion neurones versus pH value for cell suspension. pH value was kept by 10 mM Tris-HCl (pH 6 to 9) or Na-acetate (pH 3.5 to 6). Vertical bars correspond to SEM of measurements. Solid curve was drawn by eye

Electrophoretic measurements were made in a glass cylindric tube partially placed in water bath. This tube had internal diameter 3.5 mm, length 12 cm with a square hole (2 × 20 mm) in its upper part. Agar bridges (1 to 2 cm) were placed in the ends of the tube, then the remaining space was filled by cell suspension (about 1 ml) using a syringe and the hole was covered by a glass coverslip. Agar bridges connected the tube with two compartments containing Ag-AgCl electrodes in saturated KCl solution. For measurements DC supply (voltage 300 V) was used. Current passing through the tube ranged from 0.5 to 3 mA.

The tube was placed under a microscope. The heating effect of the incident light was partially compensated by using a heat filter (2.5% solution of $CuSO_4$). All experiments were done at room temperature (19 to 22°C) and their duration did not exceed 30 min. Control experiments made using a thermistor probe placed inside the cell filled by our basic solution have shown that while passing 2 mA current and switching on the microscope illumination the temperature increase in the tube during 30 min did not exceed 0.5°C.

Focusing the objective of the microscope on the bottom and the ceiling of the tube and using a micrometer screw the diameter of the cell was measured and then the objective was focused on the lower stationary level (Henry, 1938). To record the cell velocity the time needed for the cell to pass a given distance (usually 100 to 200 μm) in the presence of the electric field of different polarity was measured. Then the average velocity V was found and electrophoretic mobility W was calculated according to the formula $W = V/E = VgS/I$, where E is the strength of the electric field, S is the tube cross-section area, I is the current and g is the conductivity of solution measured separately.

All experiments were made "on line" with a computer which calculated the value of the electrophoretic mobility for each cell and then using experimental data of 50 to 100 measurements performed their statistic analysis (building of a histogram, calculation of mean values and standard error). In all experiments standard deviation never exceeded ±0.1 μm · cm/V · sec.

Test experiments performed for erythrocytes have shown that their mobility measured in solutions of different ionic composition agrees well with literature data (Seaman & Heard, 1960).

For most experiments we used a basic solution containing (in mM): 2.5 KCl, 2 $CaCl_2$, 280 glucose and 10 Tris-HCl (pH 7.3 to 7.4). When we altered the ionic strength of the solution we used sucrose to keep the tonicity constant. In studies of mobility versus pH relationship we used solutions containing 10 mM buffer: Tris-HCl (pH 6 to 9) or Na-acetate (pH 3.5 to 6). The latter solution is not perfectly studied for such experiments because its ionic strength changes in the range of pH studied ($pK_{CH_3COOH} = 4.75$). However, it has certain advantages—it possesses a maximum buffer capacity in our experimental conditions and minimum affinity to Ca ions. We did not succeed in using an alternative procedure, namely, establishing the desired pH value for cell suspension by its titration, due to difficulties in controlling pH inside our microelectrophoresis cell. In low pH solutions the pH value for cell suspension was usually greater than the pH value of cell-free solution by 0.2 to 0.5 units but did not change before and after the experiments. Therefore in studying the dependence of the mobility on pH we used pH values measured for cell suspension.

The treatment of cells by N-bromosuccinimide corresponded to the procedure described by Seaman and Heard (1960) who used this agent for the identification of carboxylic groups of proteins located on the outer membrane surface of erythrocytes.

As recommended in literature (*see* e.g. Means & Feeny, 1971) the treatment of neurones by 2,4,6-trinitrobenzenesulfonic acid and maleic anhydride (specific reagents for amino groups) was done in alkaline solutions (pH = 9). In both cases these solutions contained 50 mM $Na_2B_4O_7$, 1 to 50 mM of the agent used and a corresponding quantity of glucose. Cells in these solutions were incubated for 0.5 to 3 hours at 37°C.

Enzymic treatment of rat dorsal root ganglion neurones by 0.1% trypsin ("Spofa") solution made on basic solution was carried out at 22°C. The time of treatment was fixed by a trypsin inhibitor ("Reanal") taken in excess (control experiments have shown that trypsin inhibitor did not change the electrophoretic mobility of neurones).

0.1 ml of neuraminidase (Vibrio Cholerae, "Serva," activity 5.5 U, 0.02 mg/ml) was diluted by 0.9 ml of stock solution with pH 5.5. A 0.3% solution of hyaluronidase ("Reanal," 500

Table 1. Electrophoretic mobility of dorsal root ganglion neurones after treatment of isolated cells by different drugs at 37°C

Agent[a]	$W(\mu m \cdot cm/V \cdot sec)$[c]
Control	1.4
Neuraminidase	1.3
Hyaluronidase	1.5
Trypsin[b]	0.6
N-bromosuccinimide	0.7
2,4,6-Trinitrobenzenesulfonic acid	1.4
Maleic anhydride	1.4

[a] The procedure of treatment is described in Materials and Methods, except that trypsin-given values correspond to the stationary level of mobility reached after treatment. All W values were measured in our basic solution containing 2 mM $CaCl_2$, 2.5 mM KCl, 280 mM glucose, 10 mM Tris-HCl (pH 7.3 to 7.4).
[b] Measured after 15 min of treatment (*see also* Fig. 3).
[c] For all experiments the value of standard deviation did not exceed ± 0.1 $\mu m \cdot cm/V \cdot sec$.

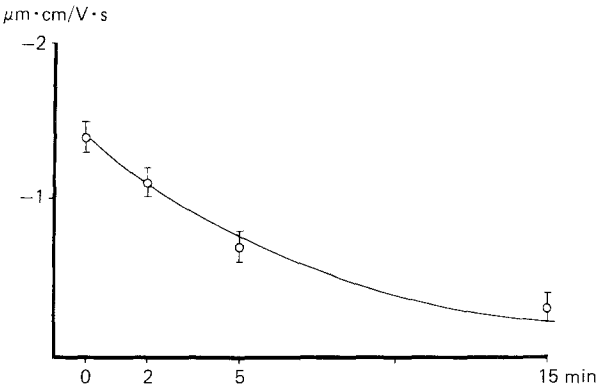

Fig. 3. Mean values of the electrophoretic mobility of rat dorsal root ganglion neurones after treatment of 0.1% trypsin solution at 22°C for different periods of time. Time of treatment was fixed by trypsin inhibitor taken in excess (control experiments have shown that it did not alter the mobility)

USP U/mg) was prepared using the stock solution. In both cases cells were incubated in these solutions at 37°C for 0.5 to 4 hr.

During long-term incubation of cells after their isolation they were transferred in sterile conditions to solution containing 9 parts of Hanks medium 199 and 1 part of bovine serum solution. This medium contained also 5000 U of benzyl-penicillin and one of the reagents listed below: 1 mM dibutyryl cAMP or theophiline, 5 mM N-ethylmaleimide or tolbutamide (all reagents from "Serva").

After each treatment cells were washed twice by centrifugation in basic solution for 10 min at 2500 rpm and immediately afterwards the measurements were made.

Results

We first studied the effect of Ca ions on the electrophoretic mobility of rat dorsal root ganglion neurones. An increase in Ca^{2+} concentration reduced the mobility of neurones (Fig. 1). Two effects may explain this observation—an increase of screening of the surface charge due to increase of the ionic strength of the solution and partial neutralization of the surface charge which reflects the process of Ca-binding by surface anion groups. To test these possibilities we substituted Ca ions by equivalent quantity of organic divalent cations dimethonium (ethane-1,2-bis-ethyldimethylammonium) or hexamethonium (hexane-1,6-bis-trimethylammonium). In addition, these solutions contained 1 mM EDTA to remove residual divalent cations. Data shown in Fig. 1 indicate that all three divalent cations produced qualitatively similar effects. However, organic divalent cations caused smaller reduction of the electrophoretic mobility than Ca ions. Alvarez et al. (1983) have shown that bis-ammonium cations exert only screening effect on the surface charge of phospholipid vesicles. Thus, it seems probable that our results directly demonstrate Ca-binding by surface anion groups present on the outer membrane of mammalian neurones.

A rough estimate of the apparent Ca-binding constant could be made by finding the inverse value of Ca concentration needed for a twofold reduction in the mobility and comparing it to the equivalent quantity of hexamethonium or dimethonium ions. This estimate gave a value of $K_{Ca} = 50\ M^{-1}$.

The surface charge of neurones depended also on the pH of the solution. Fig. 2 demonstrates that the mobility did not change for pH values from 5 to 9 and sharply decreased in low pH solutions (pH < 5). This effect may be attributed to the titration of surface anion groups with apparent $pK_H = 4.2$ determined as the pH value when the mobility is decreased twice.

Table 1 lists the experimental results of action of some agents on the surface charge of rat dorsal root ganglion neurones. Enzymic treatment of cell by 0.1% trypsin solution at 22°C strongly decreased their mobility, this effect being dependent on the time of the treatment (Fig. 3). Moreover, if trypsin was acting for more than 30 min, damage of many cells was observed.

On the contrary, neuraminidase and hyaluronidase did not change either the viability or the surface charge of neurones studied. In accordance with data of Haydon and Seaman (1967) in our experiments neuraminidase in similar conditions reduced the mobility of erythrocytes from -1.8 ± 0.1 to -1.0 ± 0.1 $\mu m \cdot cm/V \cdot sec$ and this effect reached the steady level after two hours of treatment.

Action of N-bromosuccinimide at 37°C for 30

Table 2. Electrophoretic mobility (in μm · cm/V · sec) for rat dorsal root ganglion neurones during their incubation in a culture medium containing different agents[a]

Agent	Day of incubation			
	1	2	3	4
Control	−1.4	−1.4	−1.4	−1.4
Dibutyryl cAMP	−1.5	−1.4	−1.7	−1.8
Theophiline	−1.3	−1.5	−1.7	−1.8
N-ethylmaleimide	−1.2	−1.1	−1.4	−1.3
Tolbutamide	−1.4	−1.3	−1.3	−1.3

[a] N = 4. Standard deviation did not exceed ±0.1 μm · cm/V · sec. All mobility measurements were made in basic solution.

min markedly reduced the surface charge of neurones. On the contrary, neither 2,4,6-trinitrobenzenesulfonic acid nor maleic anhydride changed the mobility (Table 1).

Table 2 lists data about the long-term action of different drugs on the surface charge of rat dorsal root ganglion neurones during their incubation in sterile conditions at 37°C for 5 days along with control values (culture medium contained no drug). Dibutyryl cAMP and theophiline in concentration of 1 mM increased the electrophoretic mobility compared to control values, this effect being developed slowly and reaching its maximum on the third day of incubation. On the contrary, N-ethylmaleimide decreased the mobility of neurones on the first two days of incubation and the effect then increased and the mobility approached the control values. However, no statistically significant changes of the mobility of neurones when incubated with tolbutamide was observed.

Discussion

On the basis of data obtained in the present work some suggestions about the molecular nature of the groups that determine the surface charge of rat dorsal root ganglion neurones can be made. It is interesting to compare these data with those obtained for erythrocytes.

For example, the value of the electrophoretic mobility for our basic solution measured for erythrocytes (−1.8 ± 0.1 μm · cm/V · sec) is somewhat more negative than for mammalian neurones (−1.4 ± 0.1 μm · cm/V · sec), which indicates that the surface charge density of erythrocytes is, probably, slightly greater than that of neurones.

Seaman et al. (1969) have studied the effect of Ca ions on the electrophoretic mobility of erythrocytes and leukocytes. Their data indicate the presence of two types of acid groups on the surface which can interact with Ca ions, namely, the residues of neuraminic (sialic) acid and nonidentified acid groups of peripheral membrane proteins which are presumably carboxylic groups. Using data given in the quoted paper we calculated Ca-binding constant for these anion groups. The corresponding apparent Ca-binding constants are within the range 15 to 40 M^{-1} which is close to our estimate of K_{Ca} = 50 M^{-1} obtained for outer membrane surfaces of neurones.

Another important question is the problem of stoichiometry of complexes formed by Ca ions on the membrane surface. McLaughlin et al. (1981) for vesicles from phosphatidylserine (anion) and phosphatidylcholine (neutral) have shown that their electrophoretic mobility changed sign when the concentration of Ca in solution was raised to 10 to 80 mM. Ca concentration needed for such charge reversal depended on the ratio between these two membrane components and its upper limit (i.e. 80 mM) corresponded to vesicles from pure phosphatidylserine, i.e. when they possessed maximum possible surface charge density. From these data McLaughlin et al. concluded that on the surface of vesicles mainly complexes of one Ca ion with one phospholipid molecule were formed. In our experiments, however, the mobility did not change sign when Ca concentration was raised to 80 mM. For erythrocytes and leukocytes Seaman et al. (1969) also could not observe the charge reversal for Ca concentration up to 50 mM. All these facts, probably, indicate that the mechanism of interaction of Ca ions with phospholipid vesicles and cell membranes are different; on the surface of mammalian neurones and blood cells neutral complexes of one Ca ion with two anion groups are mainly formed. Alternatively, the intrinsic association constant of the 1 : 1 complexes could be <10 M^{-1}. In this case charge reversal would not be observed until [Ca] > 0.1 M.

On the basis of titration curves of the electrophoretic mobility of red blood cells treated by different chemical agents Haydon and Seaman (1967) concluded that 40% of their surface charge corresponded to α-carboxyl groups of proteins with pK_H = 3.4 and 60% of it was determined by carboxylic groups of sialic acid with pK_H = 2.6. Moreover, the alkaline hydrolysis of the outer erythrocyte surface revealed the acidic groups with pK_H = 3.95 which, probably belonged to γ-carboxylic groups of glutamic acid. Our estimate of pK_H = 4.2 obtained for rat dorsal root ganglion neurones is close to pK_H for peripheral proteins of the outer membrane surface of erythrocytes.

It should be stressed that the titration curve of the electrophoretic mobility for neurones is much steeper than the Langmiur isotherm. Most probably, this effect reflects possible surface shrinking during the neutralization of its charge that contributes to the electrophoretic mobility. (A decrease of the ionic strength in our low pH solution could not be responsible for this effect. When we corrected the measured mobility values for this change in the ionic strength the apparent pK_H value changed not more than 0.1 and the resulting curve became even steeper.)

In our experiments neither neuraminidase nor hyaluronidase changed the surface charge of neurones. On the other hand, neuraminidase reduced the electrophoretic mobility of erythrocytes by about 60%, which agrees with the results obtained by Haydon and Seaman (1967). Similar treatment of neurones even twice as long did not reduce their mobility. Thus, contrary to red blood cells, sialic acid residues do not contribute to the surface charge of rat neurones. Bolognani et al. (1981) studied mollusc neurones and came to a similar conclusion.

Recently we have studied (Dolgaya et al., 1984) the surface charge of murine neuroblastoma cells C-1300, which were cloned from transformed sympathetic neurones. It appeared that the main features of the surface charge of normal and transformed neurones were qualititatively similar. However, the mobility of neuroblastoma cells was markedly reduced after neuraminidase treatment.

Trypsin in mild conditions removed a significant portion (more than 50%) of acidic groups from the outer membrane surface of neurones. We suggest that these acid groups may belong to protein-like surface molecules. Seaman and Heard (1960) could not remove more than 25% of acidic groups from the outer surface of erythrocytes by trypsin treatment, even in more severe conditions. In addition, incubation of red blood cells in the trypsin solution did not produce any changes in their properties. In our experiments, however, we found that after trypsin treatment for 30 min or longer many neurones were lysed.

N-bromosuccinimide (Table 1) strongly reduced the mobility of neurones. Treatment of erythrocytes in similar conditions (Seaman & Heard, 1960) induced only a 25% reduction in their mobility. As this reagent is a specific one for carboxylic groups this fact (as well as above-mentioned experimental data—titration curves, action of different enzymes) indicates that the surface charge of neurones is mainly determined by carboxylic groups belonging to peripheral membrane proteins.

Neither 2,4,6-trinitrobenzenesulfonic acid nor maleic anhydride (specific reagents for amino groups) changed the electrophoretic mobility of neurones. This fact indicates that the contribution of amino groups (if any) to the surface charge of neurones is small.

The results of our experiments concerning the action of some agents influencing the intracellular system of cyclic nucleotides on the surface charge of rat dorsal root ganglion neurones indicated that surface charge is closely connected with intracellular processes.

It is known that dibutyryl cAMP and theophilline (Rassmussen & Goodman, 1977) may penetrate the cell membrane and cause the increase of intracellular concentration of cAMP. The first compound acts by replacing cAMP in all its reactions and the second one induces similar effects by inhibiting phosphodiesterases that hydrolyze cAMP to AMP. The results of our experiments (Table 2) indicate that both agents increased the surface charge by about 30%. We should note that this effect develops slowly and reaches a steady level on the third day of incubation.

Reilly and Blecher (1983) have shown that N-ethylmaleimide inhibits adenylate cyclase, the enzyme which synthesizes cAMP inside the cell. Incubation of neurones in a medium containing N-methylmaleimide induced a decrease of their surface charge (Table 2).

Thus, we conclude that all these effects obtained during long-term incubation of rat dorsal root ganglion neurones in the presence of different drugs support the conclusion (*see* e.g. Rasmussen & Goodman, 1977) that cAMP plays the important role in structure and functioning of cell membranes. The observed effects may reflect changes in synthesis and reconstruction of different protein-like membrane components induced by changes of intracellular cAMP concentration.

Tolbutamide is known (Kanamori et al., 1976) as inhibitor of some protein kinases which proceed a cAMP-dependent phosphorylation of membrane and/or intracellular proteins. However, we could not observe statistically significant changes of the surface charge of rat dorsal root ganglion neurones during long-term incubation in a medium containing 5 mM tolbutamide. The absence of any effect may be due to high specificity of this drug. Indeed, Kanamori et al. (1976) have shown that tolbutamide is not a universal inhibitor of all protein kinases.

We thank Prof. P.G. Kostyuk for helpful discussions and the critical reading of the manuscript, Drs. M.I. Losinski and V.E. Gmiro for providing us with dimethonium and hexamethonium, respectively, and Mrs. L.I. Bogdanova for technical assistance.

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Received 7 February 1984; revised 22 August 1984