

The Action of Blood Serum and its Components on Potential-dependent Sodium Channels in Neuroblastoma Cells

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Abstract. Blood plasma, serum and its fractions containing components of different molecular weights as well as some identified serum constituents were tested for their action on sodium currents of voltage-clamped, internally dialyzed neuroblastoma cells. Only components with a molecular weight over 50 kDa produced a persistent increase in sodium channel currents (stimulatory effect) and shifts in activation and inactivation curves along the voltage axis towards more negative or positive potentials, respectively (modifying effect). Both modulations taken together provide a somewhat higher level of sodium electro-excitability system activity. Among the identified serum components tested, including those possessing high physiological activity, albumin was the only one which reproduced the effects of whole blood serum both qualitatively and quantitatively. The data obtained allow to assume albumin to play a role of an active substance responsible for the blood plasma or serum effects on the potential-dependent transporting system in the neuroblastoma cell membrane. Albumin seems to be involved in holding the functional activity of sodium channels on a suitable level rather than being involved in any regulatory processes.

Key words: Blood serum — Albumin — Sodium channel — Modulation

Introduction

We have shown earlier (Zubov and Salnikov 1984; 1986) that blood serum added to previously serum-deprived neuroblastoma cells specifically modulated sodium currents.

Blood serum is shown to have a strong biological activity in relation to cultured cells. The trigger and regulation of different cellular functions as a rule are accompanied with transcriptional independent “early” events including those related to the activity of ion channels. For example, modulation of membrane potential in

resting cells under the action of growth factors or serum can be explained as a result of altered permeabilities of plasma membrane for Na^+ , Ca^+ and K^+ ions (Moolenaar et al. 1979, 1981; Rothenberg et al. 1982).

It has been shown that epidermal growth factor (EGF) increases the internal concentration of calcium ions in A 431 cells probably by opening plasma membrane calcium channels (Moolenaar et al. 1986; Tilly et al. 1988). The existence of such mechanism was proved directly in A 431 cells by the patch-clamp method (Mozhayeva et al. 1989). Modulation of functions of electrically excitable channels, in particular the calcium and potassium channels, by neurotransmitters, hormones and other physiologically active substances of blood plasma or serum is a mechanism underlying the regulation of different processes (for review see Reuter 1983; Dunlap et al. 1987; Rosenthal and Schultz 1987; Hoffman et al. 1987).

As it is seen from the above examples modulating factors are, as a rule, highly active specific substances such as growth factors, hormones and neurotransmitters. Thus, the answer to the question concerning the physiological meaning of the modulations observed by us and the extent to which they reflect the natural regulation of the potential-dependent sodium system, would probably depend also on the kind of serum factors which cause these effects. This prompted us to identify serum components responsible for the changes in the activity of neuroblastoma sodium channels.

Materials and Methods

Cell cultivation: Mouse neuroblastoma cells N18A-1 (Nisman et al. 1987) were grown in Eagle media, supplemented with 10 vol% heat inactivated bovine serum as a monolayer in a humidified atmosphere of 5% CO_2 in air at 37°C . Cells were plated at $5 \cdot 10^3$ cells/cm². For electrical measurements cells grown exponentially for 2-4 days were used and they received fresh media 24 hours prior to beginning an experiment. Neuro 2a cells were grown in Eagle media supplemented with 10 vol% foetal calf serum instead of bovine serum (Gibco).

Electrical measurements: Currents through sodium channels (Fig. 1) were measured by the method of intracellular dialysis adapted for neuroblastoma cells (Kostyuk et al. 1975). The cells were suspended by pretreatment with 0.7 mmol/l EDTA in Hanks solution and harvested. The cell pellet was resuspended in a solution containing 140 mmol/l NaCl, 2 mmol/l CaCl_2 , 5 mmol/l Tris-HCl, pH 7.6 (further referred to as control solution) or in conditioned growth media. Then, the cells were transferred to the large (nonperfused) compartment of an experimental chamber (Zubov et al. 1980) where a selected cell was sucked into a plastic pipette until ionic integral currents appeared. Dialysing internal solution (mmol/l 1:140 KF, 10 tetraethylammonium chloride, 5 Tris-HCl, pH 7.6) blocked calcium and potassium channels. At the time of the experiment, the cell was transferred with a pipette to the small (perfused) compartment of the chamber allowing changes of solutions with various additives. During this time, both compartments remained separated from each other.

Fig. 1 shows records of the ionic currents and the experimental dependencies obtained on this basis. These parameters were used to assess the action of serum and its components. A standard set of measurements included: a. measurements of sodium currents (Fig. 1A) to obtain current-voltage relationships (Fig. 1B), and to determine the reversal potential of sodium current (E_{rev}), the potential-dependence of peak sodium conductance $g_p(E)$, and the values of the maximal peak conductance g_{pmax} (Fig. 1D); b. measurements using the pulse protocol (Fig. 1C), to obtain the potential-dependence of steady-state sodium inactivation $h_\infty(E)$; c. measurements of the maximal (at $E = -10$ mV) peak current I_{pmax} . The experimental dependencies $g_p(E)$ and $h_\infty(E)$ for neuroblastoma cells were reported previously (Zubov 1980; Grishchenko et al. 1983) to be satisfactorily described by

$$g_{Na} = \frac{\exp[a_g(E_t - E_g)]}{1 + \exp[a_g(E_t - E_g)]} \quad (1)$$

$$h_\infty = \frac{1}{1 + \exp[a_h(E_c - E_h)]} \quad (2)$$

where E_g is the half-conductance potential, E_h is the half-potential of steady-state inactivation, a_g and a_h (slope factors)—are parameters the values of which were obtained by the least squares method, and E_t and E_c are potentials during the test and conditioning pulse, respectively. The sequence of measurements during an experiment was as follows: a, b, c—before solution exchange, and c, a, b—after solution exchange. Thus the changes of I_{pmax} reflect the fastest components of the sodium system reaction in response to the exchange of the media.

The effects of the substances tested were estimated by the relative changes in I_{pmax} and g_{pmax} expressed as percentages of their initial levels, and by the shifts of E_g and E_h (in millivolts), calculated as the difference between the pre- and post-treatment values. Values of E_{rev} , a_g and a_h are not shown: they remained constant throughout the experiment.

The data presented in Tables as mean values with their standard errors. The numbers of measurements are shown in brackets, $P \leq 0.05$ was chosen as the level of significance.

Since there were no significant differences, clones N18A-1 and Neuro 2a were considered together.

Media tested: The following media were used: 1. bovine blood serum (Kiev Meat Combine); 2. lyophilized fetal calf serum (Serva); 3. fractions of bovine blood serum, molecular weights (kDa): <15; >15; 15-20; 25-50; >50, obtained by dialysis through Visking dialysing tubing with pore diameter of 1.5-2 nm and subsequent ultrafiltration through Centrifo membrane cones (Amicon Grace Company) types CF-25 and CF-50; 4. human blood plasma deficient in PDGF, kindly provided by M. Blagosklonny (Blagosklonny and Kuznetsova 1987); 5. albumin (Reanal) and γ -globulin of human blood serum (Gödöllő, Hungary); 6. modified Eagle growth medium (MGM) with bovine blood serum; 7. conditioned modified growth medium (CMGM) (Eagle medium with bovine blood serum after incubation with cells for 24 hours; 8. serum-free modified growth Eagle medium (SFMGM). The media were modified by the addition of HEPES and CaCl_2 to final concentrations of 16.7 and 2.0 mmol/l, respectively. The modifications were used to support pH and to increase the resistance of plasma membrane to electrical breakdown. Serum and plasma were used after 1:20 dilution by control solution (the so-called "5%" serum or plasma). Serum fractions and solutions containing albumin and γ -globulin were prepared to give final concentrations of the components equal to those in intact 5% serum.

"Serum" molar concentrations of the proteins were based on the albumin (40-50 g/l) and γ -globulin (20-30 g/l) contents in whole serum (Macleod and Drummond 1980; Beriozov and Korovkin 1982; Petrov 1987). Molecular masses of both were assumed to be 70 kDa and 150 kDa, respectively. Albumin and γ -globulin were used also in other concentrations (see below).

Results

Effects of intact serum. With our technique of cell preparation for the experiment (see above), the cells were deprived of serum prior to electrical measurements. The length of serum deprivation was estimated as the interval since the replacement of the growth medium in the cell culture by control solution to the point of the first serum application to the given single cell. Consequently, the time of serum deprivation was rather variable for different cells. The question was raised as to whether there is some correlation between the length of serum deprivation and the cell responses to serum application. A special analysis in this respect in one of the preliminary experimental series showed no such correlation, at least for the time range from 16 to 450 min in 50 experiments, with the correlation coefficient r being 0.29. Therefore, the transition of the sodium electro-excitabile system to a serum-reactive state after previous serum deprivation was apparently fast in our experimental conditions.

Typical results concerning serum effects are shown in Figs. 1 and 2. An increase in the sodium currents was observed immediately after the addition of 5% serum into the superfused compartment of the experimental chamber. The initial fast rise in the peak current was followed, as a rule, by a slower one, until a plateau level was reached and kept as long as the serum was present (usually 10 min). In addition, a shift in the sodium peak conductance curve towards more negative potentials occurred in the presence of the serum (Fig. 1D). Physiologically, the shift is equivalent to lowering the excitability threshold. A change in the linear part of the current-voltage relationship seen in Fig. 1 indicates that the increase in peak current values is due to the rise of the maximal peak conductance, probably at the expense of the mobilization of some additional quantities of nonfunctional (silent) sodium channels present in the membrane before the serum application. Obviously, this mobilization cannot be explained by the observed shift of inactivation towards more positive potentials (Fig. 1D) because the fraction of noninactivated channels h_{∞} effectively is 1 both before and after the serum application under our holding potential of -130 mV. It has not been excluded that a part of the current increase may result from the above mentioned potential-dependent inactivation shift under physiological conditions of the cell, i.e. under a more positive membrane potential.

Figs. 1 and 2 illustrate two basic serum effects which together result in a higher level of activity of the sodium excitable system: 1) a stimulating effect expressed

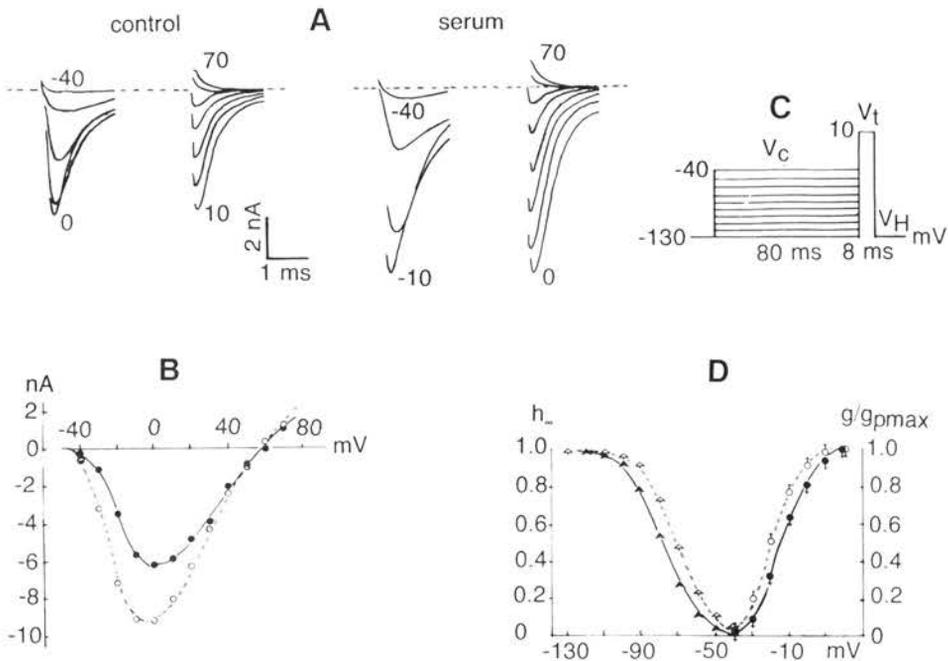


Figure 1. Evaluation of serum action on sodium channels in neuroblastoma cells. *A:* Currents through sodium channels induced by short (8 ms) displacement of the membrane potential fixed at a holding level of -130 mV to values ranging from -40 to $+70$ mV in 10 mV steps. The initial and final values of membrane potential (mV) are shown for each family of current curves. *B:* Current-voltage dependencies obtained from data in *A* in control solution (filled circles) and in the presence of 5% serum (open circles). *C:* Pulse protocol used to obtain the inactivation curves ($h_\infty - E$) of sodium channels. E_c - potential during conditioned pulse, E_t - potential during test pulse, E_H - holding potential (mV). *D:* Experimental relaxations between normalized maximal peak conductance (circles), fraction of noninactivated channels (triangles) and membrane potential; (solid and dotted lines were drawn by eye). Filled symbols: measurements in control solution, open symbols: the same in medium containing 5% serum. The relations were obtained from data in *A* and *B* and from measurements made according to the pulse protocol *C*. Cell 7-83, clone Neuro 2a.

through the increase of I_{pmax} and g_{pmax} values and presumably explained by the mobilization of some additional quantities of functional channels, and 2) a modulating effect in the form of counter-directional shifts in activation and inactivation curves.

It is difficult to interpret a counter-directional shift of the conductance and inactivation curves in terms of macroscopic changes in membrane surface charge.

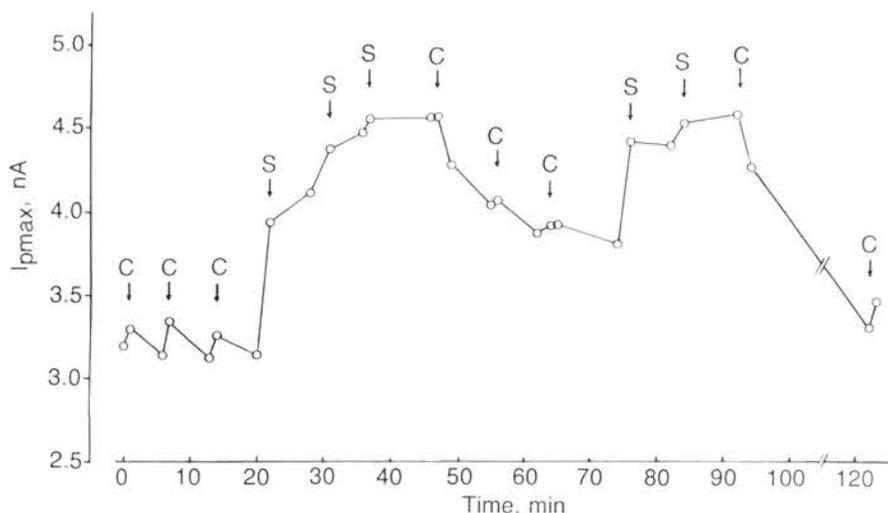


Figure 2. Time course of changes in maximal peak current values measured at -10 mV during application and washout (arrows) of 5% serum. C indicates perfusion with control solution, S - the same with a solution containing 5% serum. The interval of each perfusion was approx. 1 min. Cell 17-84, clone N18A-1.

This question, however lies beyond the scope of this paper.

Both effects mentioned above were highly reproducible in different experimental series and with different serum batches, and differed from one another only quantitatively. The increase in current values with the serum application, as shown in Fig. 2, occurred more rapidly than the reverse change after serum washout. For example, with foetal calf serum the changes in I_{pmax} were: 16.6 ± 2.8 (8), $p < 0.05$; -1.91 ± 1.0 (7), $p > 0.05$; and -8.52 ± 2.98 (7), $p < 0.05$, corresponding to serum application, immediately after serum washout, and 15-25 min later, respectively. Similar results were obtained in all other experimental series. The tables list changes in the current parameters observed immediately after serum washout. For this reason the figures relating to washout are, as a rule, smaller in their absolute values than the respective figures for serum application, and the reversibility of the serum effects is underestimated. The same is true for the results of experiments with serum fractions and purified serum proteins (see below).

Moolenaar et al. (1981) showed that serum depleted of growth factors fails to induce any of the electric and ionic events caused by intact serum. In order to estimate the stability of our active factor(s), a special series of experiments was performed using modified growth media (see Materials and Methods). A cell culture grown for a period of 1 day without media substitution was suspended in conditioned media (CMGM) and used for the experiment. Our initial assumption

Table 1. Effects of 5% blood serum on activity parameters of sodium channels in neuroblastoma cells.

	Parameter			
	$I_{pmax},\%$	$g_{pmax},\%$	E_g, mV	E_h, mV
Application	26.9±2.2(47)	16.0±1.6(37)	-4.11±0.50(28)	5.78±0.86(29)
Washout	-5.57±1.12(45)	-5.42±1.89(33)	-0.10±0.52(27)	-3.76±0.87(27)

The asterisk indicates insignificant change ($p > 0.05$).

Table 2. Changes of activity parameters of sodium channels in neuroblastoma cells after media replacement. Conditioned (CMGM), serum-free (SFMGM), and initial (MGM) medium. Arrows show direction of media replacement.

Parameter	CMGM	CMGM	MGM
	↓ SFMGM	↑ SFMGM	↑ SFMGM
	1	2	3
$\Delta I_{pmax},\%$	-4.62±0.81(11)	12.7±1.8(6)	12.1±1.3(4)
$\Delta g_{pmax},\%$	-6.81±1.09(11)	10.1±3.6(6)	12.8±2.4(3)
$\Delta E_g, mV$	-0.86±1.03(11)*	-1.40±0.65(6)	-2.68±1.23(4)*
$\Delta E_h, mV$	-5.72±1.30(11)	6.49±2.20(6)	7.33±0.94(4)

The asterisk indicates insignificant change ($p > 0.05$).

was that if any serum activity remained in CMGM and the cells in turn did not lose their sensitivity to active serum factors, sodium current parameters would continue to change relative to their values in serum-free media. Then, a transition from CMGM to SFMGM (serum-free medium) should give an effect similar to that of serum washout. Data in Table 2 (first column) show that this is indeed the case (compare with Table 1 and Fig. 2), the delayed negative changes of I_{pmax} and g_{pmax} being increased after several minutes in SFMGM to -16.9 ± 1.4 (10) and -18.7 ± 2.0 (11), respectively.

Changes in sodium current parameters as a result of returning the cell from SFMGM to CMGM, or to initial (nonconditioned) media, were actually of the same order (compare columns 2 and 3, Tab.2). It can be concluded from this that the serum activity in the growth media with regard to the sodium potential-dependent system remains essentially unchanged during at least 1 day of media conditioning.

Identification of the active serum components. The above data on serum conditioned media show that the changes of the sodium current parameters studied are caused by a metabolically stable serum constituent(s). Biologically active substances operative in regulation systems, such as neurotransmitters or some growth

Table 3. Changes in values of sodium channel activity parameters induced by 5% blood plasma or serum fractions differing in molecular masses of their constituents; Neuroblastoma cells.

Preparation	$\Delta I_{pmax},\%$	$\Delta g_{pmax},\%$	$\Delta E_g, mV$	$\Delta E_h, mV$
Plasma without PDGF:				
23.7 \pm 3.2(16)	8.01 \pm 2.12(15)	-5.17 \pm 0.65(14)	3.79 \pm 1.01(14)	
Serum:				
12.4 \pm 2.4(13)	4.36 \pm 1.61(12)	-2.44 \pm 0.55(12)	3.16 \pm 0.57(12)	
Serum fractions:				
> 15	18.6 \pm 2.1(27)	11.4 \pm 1.8(27)	-3.06 \pm 0.56(28)	6.33 \pm 0.67(28)
< 15	1.66 \pm 1.11(38)*	-1.44 \pm 1.23(36)*	-1.71 \pm 0.42(36)	-3.56 \pm 0.41(36)
> 25	11.5 \pm 1.8(5)	9.71 \pm 1.24(5)	-3.38 \pm 0.73(5)	5.30 \pm 0.97(5)
15-25	0.34 \pm 1.25(8)*	-3.06 \pm 3.23(8)*	2.30 \pm 1.08(7)*	-3.05 \pm 0.35(8)
> 50	14.4 \pm 3.7(18)	10.4 \pm 2.7(15)	-3.49 \pm 0.96(15)	8.40 \pm 0.82(15)
25-50	-9.25 \pm 2.61(16)	-9.97 \pm 2.85(16)	2.19 \pm 1.87(14)*	-0.66 \pm 0.63(14)*

The asterisk indicates insignificant change ($p > 0.05$).

factors (for example insulin), usually are chemically unstable. It seems reasonable to suppose that the serum effects observed are not related to substances of this type. Earlier this was investigated by us in several experiments with identified serum components of known physiological action on neuroblastoma cells (Zubov and Salnikov 1986). Epidermal growth factor, insulin, transferrin, ATP, serotonin, and dexamethasone were ineffective or decreased slightly I_{pmax} both separately and in various combinations with each other.

Taking into account the complicated and so far insufficiently investigated serum composition, the examination of serum fractions differing in their molecular weight was recognized to be a more reasonable approach to ascertain the nature of modulating factor(s) than the sorting out of accessible identified serum components. The present data as well as those obtained earlier on serum fractions and PDGF-free blood plasma effects (Zubov et al. 1988) are summarized in Table 3. It can be seen that serum fractions containing components with a molecular weight above 50 kDa, as well as blood plasma deficient in PDGF, affect the sodium current in the same manner as does intact serum. On the contrary, all of the low-molecular weight fractions (below 50 kDa) were either ineffective or produced an opposite effect. Such a behavior was noted for all parameters studied except E_g , which did not discriminate between high or low molecular weight fractions. The changes of E_g and E_h in the low molecular weight media were in the same direction (to the negative), however, they were of opposite direction (to the negative and to the positive, respectively) in high-molecular weight serum fractions and in PDGF-free plasma.

Table 4. Effects of serum, γ -globulin and albumin on the activity parameters of sodium channels in neuroblastoma cells.

Applications	Parameter			
	$\Delta I_{pmax}, \%$	$\Delta g_{pmax}, \%$	$\Delta E_g, mV$	$\Delta E_h, mV$
γ -Globulin, 4 μ mol/l	6.0 \pm 3.6(25)*	-2.3 \pm 5.8(14)*	-2.1 \pm 0.9(14)	-0.3 \pm 0.6(18)*
γ -Globulin, 22 μ mol/l	4.2 \pm 3.7(6)*	4.1 \pm 3.7(6)*	-2.4 \pm 0.6(6)	0.3 \pm 1.0(5)*
Albumin, 4 μ mol/l	23.5 \pm 4.2(13)	8.0 \pm 6.5(10)*	-4.5 \pm 1.9(10)	0.2 \pm 1.1(10)*
Albumin, 8.7 μ mol/l	39.4 \pm 8.0(10)	14.0 \pm 14.3(12)*	-5.6 \pm 1.6(12)	2.4 \pm 1.0(11)
Albumin, 22 μ mol/l	42.0 \pm 7.2(15)	24.3 \pm 8.2(8)	-6.8 \pm 0.9(8)	4.6 \pm 1.7(6)
Washout of albumin, 22 μ mol/l	-12.5 \pm 2.6(7)	-4.6 \pm 3.3(6)*	-2.5 \pm 0.5(6)	-2.7 \pm 0.8(5)
γ -Globulin after albumin (4 μ mol/l both)	-7.2 \pm 2.3(8)	3.7 \pm 7.7(8)*	-1.8 \pm 1.3(8)*	-1.6 \pm 0.6(6)
Albumin after γ -globulin (4 μ mol/l both)	32.1 \pm 3.2(9)	-2.9 \pm 8.5(5)*	-4.7 \pm 1.4(5)*	3.4 \pm 1.1(5)
Albumin 8.7 after γ -globulin 4 (μ mol/l)	32.2 \pm 6.6(11)	3.2 \pm 8.0(11)*	-2.9 \pm 1.6(11)*	2.8 \pm 1.1(11)
Albumin 22 after γ -globulin 4 or 22 (μ mol/l)	44.5 \pm 8.6(9)	24.0 \pm 5.8(6)	1.6 \pm 2.2(6)*	4.9 \pm 1.2(5)

The asterisk indicates insignificant change ($p > 0.05$).

The data obtained allow us to exclude a number of low-molecular weight substances such as hormones, growth factors (PDGF), neurotransmitters, oligopeptides, free amino acids, and a number of others, as candidates for the role of modulators of sodium channel. The noted trend of reversal in the sign of the response with low-molecular weight fractions (lack of increase or even inhibition of sodium current and conductance, shift of E_h to the left instead of to the right) agrees with the effects mentioned above (Zubov and Salnikov 1986) for growth factors and some other agents in the same range of molecular weights. Therefore, the idea seems more probable that the modulation of sodium channels in the presence of serum is due to its high-molecular weight component(s) 50 kDa. These include albumins and globulins as the major portion, and among globulins the γ -globulins are of great interest because of their high biological activity. It was natural to begin with investigating both.

The results of these experiments are summarized in Table 4. It was shown that albumin in a concentration of 22 μ mol/l, which roughly is its concentration in intact 5% serum, activates the sodium channel current, reproducing both qualita-

tively and quantitatively all effects of intact serum and its high-molecular weight fractions (see Tables 1-3). The application of γ -globulin, also in a concentration equal to that in serum ($4 \mu\text{mol/l}$), only resulted in a negative shift of E_g . Also, it can be seen that the effects of serum can be qualitatively reproduced with a lower concentration of albumin as well, while γ -globulin remains inactive even in a concentration equimolar to albumin.

It is necessary to make the qualification that the masses and concentrations of proteins used for the calculations are quite approximate because of wide deviations of the references (Macleod and Drummond 1980; Beriozov and Korovkin 1982; Petrov 1987). However, the wide range of the concentrations used overlaps with this deviation allowing to make conclusions as to the specificity of the albumin action.

This conclusion is supported by data concerning the action of albumin and γ -globulin in concert. Namely, the addition of γ -globulin after albumin had the same effect on the cells as did albumin deprivation. On the contrary, albumin applied after γ -globulin produced the same effect as when applied immediately after the control medium (Table 4).

The restoration of the initial levels of parameters I_{pmax} and g_{pmax} after both albumin and intact serum deprivation is a rather delayed process: it has the form of a partial reversibility of the albumin and serum additions. This fact also supports the similarity between the effects of albumin and serum.

This similarity seems especially evident from the behaviour of parameter E_h , which is strictly timed to the addition or removal of the agents tested; this not the case for E_g .

Discussion

The modulations of sodium channel behavior described above differ from those usually related to the triggering of cell proliferation. First of all, they concern electro-excitable sodium channels. Secondly, the responses to the presence of serum are not reactions to mitogenic components of the latter such as EGF, insulin, PDGF, etc. Thirdly, the modulations are relatively independent of the cellular metabolism and of the concentration of the calcium ions inside the cell, since they could be detected in conditions of broken cells interior (Pogorelaya et al. 1980) in the presence of high intracellular fluoride concentrations.

Also the differences as compared with the previously described changes of membrane potential in neuroblastoma cells (Moolenaar et al. 1979; 1981) should be noted: the former changes were induced by growth factors but not albumin.

A comparison of the effects of albumin, plasma, serum and its fractions allows us to suggest that albumin is the essential, if not the only, factor determining the action of serum on the electro-excitable system of plasma membrane. However,

this suggestion is to be supported by further more detailed investigations of high-molecular serum fractions with respect to their protein composition and current-modulating activity.

It is well known that albumin is able to transfer lipids, hormones, some mineral substances and other plasma components along the blood stream (Maurer 1987). So the question may arise as to whether the modulations described are due to albumin alone or to biologically active substances carried by it. The latter suggestion seems to us of low probability as the same parameter changes were observed both with serum or its high-molecular weight components and with purified albumin, i.e., under conditions where any serum components other than albumin are limited or even eliminated.

The concentrations of albumin in the blood stream are rather stable so the modulation of sodium channels caused by this protein seems hardly to have any regulatory significance. Probably, the role of albumin consists in supporting a definite physiological state of the sodium electro-excitabile system. Nevertheless, the effects described may be of special interest since they reflect the mode of interactions of the channels with their natural environment; this has not completely been investigated so far.

Acknowledgements. The authors are indebted to G. I. Ivanova, E. D. Nosyreva, G. A. Savokhina for cell cultivation; to M. E. Blagosklonny for the PDGF deficient plasma samples; to V. A. Pospelov for consultation on serum ultrafiltration; and to G. N. Mozhayeva and T. V. Pospelova for discussions.

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