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The facilitating effect of gangliosides on the electrogenic (Na^+/K^+) pump and on the resistance of the membrane potential to hypoxia in neuromuscular preparation*

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Abstract. The effects have been investigated of a mixture of gangliosides from beef brain cortex (GM_1, GD_{1a}, GD_{1b} and GT_1) either added to the bathing medium or injected intraperitoneally on muscle fibres and nerve terminals in mouse diaphragm. The electrogenic (Na^+/K^+) pump activity of muscle fibres enriched with sodium was increased by 38% after 2-h pretreatment with gangliosides (5×10^{-8} mol $\cdot l^{-1}$). Muscles from animals treated with gangliosides did not show the substantial depolarization of the resting membrane potential (RMP) in K^+-free solution (6 h) shown by control muscles. Further, treatment with gangliosides slowed the changes in muscle fibre RMP and frequency of the miniature end-plate potentials in oxygen deprived muscles.

Key words: Gangliosides – Mouse diaphragm fibres – Miniature end-plate potentials – Sodium pump – Hypoxia

Introduction

Gangliosides are typical acidic glycolipids of the mammalian plasma membrane and are especially abundant in nerve cells (Svennerholm 1980). Application of these substances to neurons in culture or during nerve regeneration in vivo enhances neurite formation and sprouting (Gorio et al. 1980; 1983; Ferrari et al. 1983). Furthermore, in animals with diabetic neuropathy, treatment with gangliosides leads to an increase in nerve conduction velocity, axonal morphometry and myelin particle density (Norido et al. 1982; 1984; Gorio et al. 1984). These data suggest a broad effect of these agents that cannot easily be explained. It has, however, been reported that gangliosides may regulate the activity of membrane bound enzymes, i.e. (Na^+/K^+)ATPase (Leon et al. 1981). In vitro incubation of rat brain synaptosomal preparations with GM_1 ganglioside in nanomolar concentrations activates the enzyme, whereas higher amounts (100 nmol $\cdot l^{-1}$ or over) are without effect. This observation on membrane fractions, although corroborated by some in vivo data (Aporti et al. 1981), does not imply a priori that similar activation occurs in living cells, where the (Na^+/K^+)ATPase and the corresponding (Na^+/K^+) pump (Skou 1975) may be affected differently. Such a different action was, for example, found in the case of vanadate ions. They are very potent inhibitors of the (Na^+/K^+)ATPase in membrane fractions of many tissues, where the inner part of enzyme moiety with phosphate binding sites is accessible (Cantley et al. 1978; for review see e.g. Ramasarma and Crain 1981). When applied to the intact cells, they are either without effect (Cantley et al. 1978; Karlish et al. 1979) or may even hyperpolarize the cell (Dlouhá et al. 1981).

It was therefore of interest to study the direct effect of gangliosides on the (Na^+/K^+) electrogenic pump. We used the mouse diaphragm, which can be enriched with sodium and on which the electrogenic effect during pump activation can be measured by intracellular microelectrode techniques (Dlouhá et al. 1979). During the course of this work, the effects were also measured of gangliosides on several other properties of muscle fibres such as resting membrane potential (RMP), input resistance (R_i) and action potential (AP). In addition, we studied the influence of gangliosides on hypoxia-induced changes of RMP and frequency of miniature end-plate potentials (m.e.p.p.), which can be considered a monitor of synaptic functional integrity.

Materials and methods

Experiments were performed on diaphragm muscles of female white mice (about 20 g body weight). After isolation, muscles were equilibrated for 4–6 h in normal or K^+-free physiological saline of the following composition (in mmol $\cdot l^{-1}$): Na^+, 149; K^+, 5.0 (or zero); Ca^{2+}, 2.0; Mg^{2+}, 1.0; Cl^-, 160 (or 155); HCO_3, 12.8; H_2PO_4, 1.0; glucose, 11.0. This solution was oxygenated with 95% O_2 + 5% CO_2 and had a pH of 7.2.

Hypoxia was produced either by bubbling the $NaHCO_3$ buffered solution (without phosphate) with N_2 or by covering the muscle bath containing a decreased $NaHCO_3$ concentration (7 mmol $\cdot l^{-1}$; pH = 7.2) with a 5 mm layer of pure paraffin oil to prevent the access of oxygen from the air. The results were essentially similar in both instances. The layer of saline under the oil was about 0.2 mm and muscles of control and ganglioside treated animals were examined simultaneously in the same chamber.

The electrogenic (Na^+/K^+) pump activity (Kernan 1962) was studied electrophysiologically in muscles enriched with sodium for 4–6 h in K^+-free medium at a room temperature of 20° C. According to our previous studies (Dlouhá et al. 1981; Zemková et al. 1982), diaphragm muscle fibres treated in K^+-free medium for 4–6 h lose about 30 mmol $\cdot l^{-1}$ K^+ and gain a corresponding amount of Na^+. This is accompanied by depolarization of about 10 mV from the resting membrane potential (RMP) −72 to −74 mV.

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RMP was recorded from superficial muscle fibres using an intracellular glass microelectrode (filled with 3 mmol · l^{-1} KCl, 15–30 MΩ) before and after addition of 5 mmol · l^{-1} K^+ to the bath. The difference between these two values was considered as a measure of the activity of the electrogenic (Na^+/K^+) pump (Kernan 1962; Dlouhá et al. 1979). The effective input resistance of the muscle fibre membrane was calculated in the conventional way from the passive response of the membrane to a subthreshold rectangular pulse of more than 10 ms of duration passed through a second intracellular microelectrode.

A transmembrane current was also used to initiate action potentials in individual muscle fibres. During the period when the measurements were made, a small constant anodal current was applied to hyperpolarize the muscle fibre membrane to about −95 mV (Redfern and Thesleff 1971). Passive responses, AP and m.e.p.p. were photographed from the screen of a Tektronix storage oscilloscope from which the frequency of m.e.p.p. was also counted. As a rule, the mean values of each parameter were pooled for at least ten (usually 15–20) fibres in individual muscles and the final values reported are expressed as mean ± S.E.M. from 3–5 muscles, unless otherwise stated. In the figures, ± S.E.M. is indicated by bars.

Beef brain cortex gangliosides (FIDIA, Abano Terme, Italy) were purified by high pressure liquid chromatography to a high level of purity (Gorio et al. 1983). The final composition of the mixture was $GM_1 = 21\%$; $GD_{1a} = 39.7\%$; $GD_{1b} = 16\%$ and $GT_1 = 19\%$. The remaining components of other gangliosides such as GQ occurred in trace quantities.

For treatment in vitro, the 10^{-6} mol · l^{-1} stock solution was prepared by dissolving the mixture powder in distilled water and bubbling with N_2 2 h and this was stored at + 4° C for a maximum of 14 days.

A group of mice was given daily intraperitoneal injections of 10 or 1 mg/kg of ganglioside mixture (GS) of the same composition as used in vitro (Cronassial, FIDIA). The control group was treated with saline only. The muscles of both groups of animals were used simultaneously according to the experimental protocol.

Results

The effect of ganglioside treatment on the frequency of miniature end-plate potentials and the parameters of the resting membrane of diaphragm fibres

The frequency of miniature end-plate potentials (m.e.p.p.) in mouse diaphragm in oxygenated physiological saline was unchanged by exogenous GS. Before adding GS to the muscle bath, the frequency was 0.78 ± 0.21 per s and after 6 h incubation with 5×10^{-8} mol · l^{-1} GS, the mean value was 0.85 ± 0.60. Also chronic treatment of the animals with GS did not influence spontaneous quantal release. The m.e.p.p. frequency was 0.75 ± 0.22 and 0.80 ± 0.25 per s respectively in control muscles and in muscles from mice treated with GS (10 mg per kg) for 10 days.

RMP did not change after 6 h GS treatment in vitro either; it was −75.6 ± 0.5 mV before and −76.4 ± 0.8 after GS application. In chronic experiments, the RMP of controls was −73.4 ± 0.9 mV and −74.1 ± 0.8 mV and −75.1 ± 0.8 mV in muscles treated with 10 mg/kg GS for 3 and 10 days respectively.

Table 1. Action potential parameters and input resistance (R_i) of mouse muscle fibres in control diaphragms (C) and in diaphragms treated for 6 h with 5×10^{-8} gangliosides (GS)

Parameter and unit	C	GS
MRR (V/s)	284 ± 18	274 ± 15
MRF (V/s)	194 ± 25	180 ± 30
E_{crit} (mV)	57 ± 3	55 ± 3
OS (ms)	25 ± 3	26 ± 3
Dur (ms)	0.77 ± 0.01	0.75 ± 0.03
R_i (MΩ)	0.73 ± 0.02	0.80 ± 0.02

MRR, maximum rate of rise of action potential, MRF, maximum rate of fall of action potential, E_{crit}, critical level for action potential generation (threshold), OS, amplitude of overshoot of action potential, Dur, duration, or time-course of action potential at zero potential. The difference between control values and values after application of gangliosides are not statistically significant.
Temperature = 18–20° C

The data on input resistance and AP parameters from muscles treated for 6 h with 5×10^{-8} GS are summarized in Table 1. Examples of AP and passive responses, used for calculation of R_i, are given on Fig. 1 (two upper records). No significant difference for any parameter was found between the two groups of muscles indicating the absence of a shortlasting ganglioside effect on the main passive and active ionic channels in diaphragm fibres. Furthermore, no difference was observed after the animals had been treated with GS for 10 days (data not given).

The effect of ganglioside treatment on the electrogenic (Na^+/K^+) pump

The RMP of diaphragm muscles bathed several hours in K^+-free solution depolarized from −73.6 ± 0.4 mV to −61.3 ± 0.5 mV (10 muscles, 188 fibres). When 5×10^{-8} mol · l^{-1} GS was added 2 h before measurements were made, the RMP depolarized to −63.6 ± 0.6 mV (12 muscles, 205 fibres). The readmission of 5 mmol · l^{-1} K^1 (Fig. 2, arrow) to the control muscles hyperpolarized the RMP temporarily, the maximum level −80.1 ± 0.6 mV) occuring between approximately 7 and 15 min after potassium application. The RMP then depolarized slowly to −71.6 ± 0.7 mV within 30 min. The hyperpolarization of RMP, which is abolished by ouabain (Dlouhá et al. 1981), apparently represents the activation of (Na^+/K^+)ATPase dependent, active transport of Na^+ and K^+ ions across the membrane (Akaike 1975). The electrochemically imbalanced transport gives rise to a net flow of charge, so affecting the RMP.

If the maximum hyperpolarization of the RMP in the absence of GS is taken as 100%, then the 2-h pretreatment with GS increased the maximum electrogenic effect by 38% (Fig. 2, squares). The peak RMP value was −90.4 ± 0.2 mV, obtained 15 min after the addition of K^+. The time course of the RMP changes was similar to that in the control (Fig. 2, circles). However, the RMP remained significantly more hyperpolarized at the end of the "electrogenic period" (−78.2 ± 1.0 mV and −71.2 ± 0.7 mV respectively) 30 and 60 min after K^+ application in GS treated muscle as compared with control ones (−71.6 ± 0.7 mV and −65.3 ± 1.4 mV respectively).

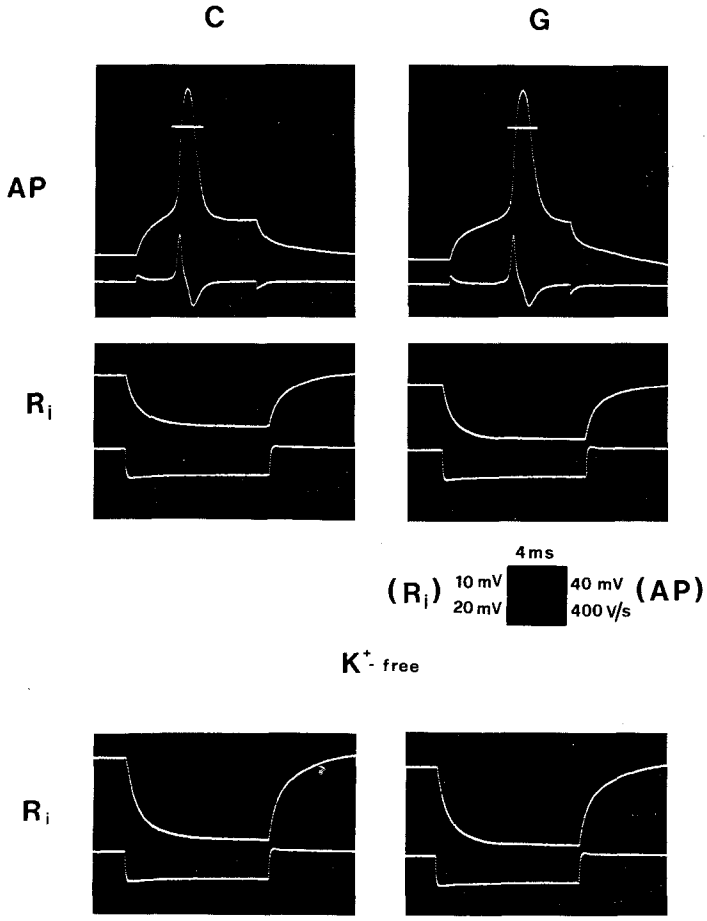

Fig. 1. Illustration of the absence of effect of 6 h ganglioside (5×10^{-8} mol $\cdot$ l^{-1}) treatment of diaphragm muscle fibres on action potential (*AP*) and input resistance (R_i). AP and hyperpolarizing pulses were generated in muscle fibres of diaphragms bathed either in normal physiological solution (control, *C*) for 6 h or in saline with gangliosides (*G*). Upper beam on the oscilloscope records – membrane potential, lower beam – either first derivation of the membrane response from which the rise and decay rates of AP were estimated or rectangular current pulse applied intracellularly with second glass microelectrode inserted into the muscle cell near (10–20 μm) to recording electrode. K^+-free = demonstration of the increase in passive membrane response in the fibres bathed in K^+-free saline for 6 h

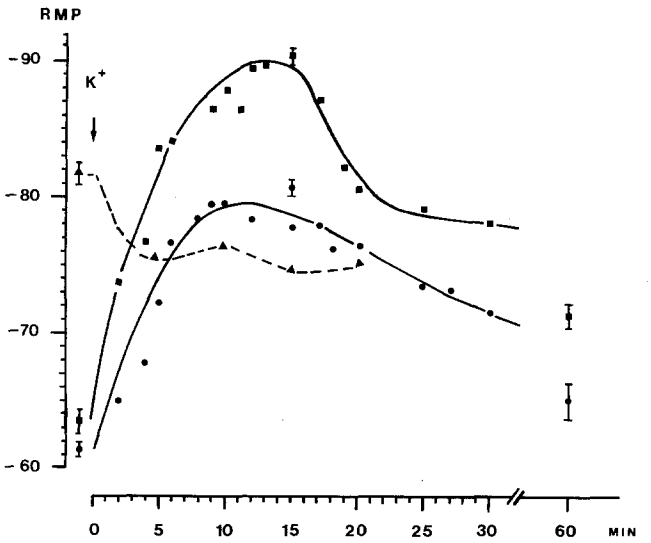

Fig. 2. Times course of hyperpolarization after addition of 5 mmol $\cdot$ l^{-1} K^+ to Na^+-loaded muscles at time zero. *Full circles*, control muscles, *squares*, muscle treated for 2 h with gangliosides 5×10^{-8} mol $\cdot$ l^{-1} immediately before potassium was applied. *Triangles*, muscles bathed for 6 h in K^+-free solution plus 5×10^{-8} mol $\cdot$ l^{-1} gangliosides. The resting membrane potentials (RMP in mV) are the means of at least 40 readings from 4 muscles. The S.E.M.'s were about 1 mV or less for most of the readings and are indicated only in several points

The amplitudes of the passive membrane responses to similar rectangular pulses increased during the 6 h period of loading the muscles with Na^+ in K^+-free medium (Fig. 1, bottom records) and the membrane R_i increased from 0.90 ± 0.04 MΩ to 1.40 ± 0.06 MΩ (two muscles, 20 fibres for each value). A similar increase was also observed in two muscles treated with GS for 6 h; the values of R_i were 0.93 ± 0.05 MΩ before and 1.48 ± 0.04 MΩ after Na^+-loading.

The effect of GS on depolarization of the RMP in K^+-free solution was followed in several experiments. When the muscles were bathed with 5×10^{-8} mol $\cdot$ l^{-1} GS in K^+-free solution from the very beginning, the RMP was in most cases well maintained at about -80 mV. In paired hemidiaphragms the mean RMP of the half in K^+-free solution only was -63.7 ± 1.8 mV and that in K^+-free + GS was -81.8 ± 0.7 mV. This indicated that GS gave protection against the loss of intracellular K^+. When 5 mmol $\cdot$ l^{-1} K^+ was readmitted, the RMP of the latter muscles depolarized after 5 min and remained at about -75 mV during the whole period of the experiment (Fig. 2, triangles). Similar results were obtained in muscles dissected from mice treated for 3 or 10 days with 10 mg/kg GS. The RMP of these muscles, when bathed in K^+-free solution for 4–6 h, was -82.3 ± 1.7 mV. In parallel controls, from non-treated animals, the RMP depolarized to -63.7 ± 1.2 mV (Fig. 3, squares). After restoration of potassium, the RMP in treated muscles depolarized to about -73 mV whereas normal hyperpolarization developed in controls (Fig. 3, circles). Also the muscles from two animals treated for 3 days with only 1 mg/kg of GS were resistant to the drop of membrane polarization induced by the K^+-free solution and the RMP of these muscles was -87.2 ± 1.2 mV after 6 h in the absence of potassium.

The effect of gangliosides on hypoxia-induced changes of RMP and m.e.p.p. frequency

In these experiments, the effects of oxygen deprivation on muscle fibre RMP and m.e.p.p. frequency were monitored. In the absence of oxygen, the diaphragm fibres progressively depolarized and after 1 h the RMP changed from -74.6 ± 0.8 mV to -63.2 ± 0.9 mV (N_2-saturated medium, two muscles). When muscles were pretreated with 5×10^{-8} mol $\cdot$ l^{-1} GS for 2 h in oxygenated solution, the change of the RMP in N_2-saturated medium was reduced from -76.1 ± 0.9 to -68.3 ± 0.7 mV. Washing the muscles with oxygenated solution led to a restoration of the muscle RMP; within 15 min the values were -72.2 ± 0.9 mV and -74.0 ± 1.1 mV in the control and treated muscles re-

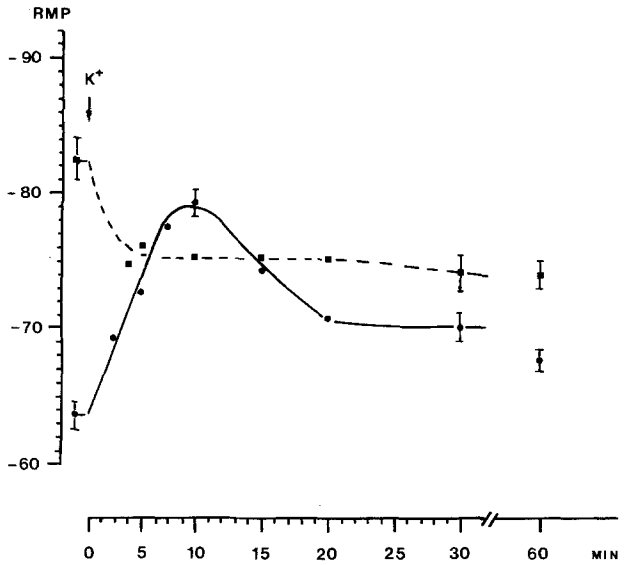

Fig. 3. RMP of diaphragm muscle fibres after 6 h in K^+-free solution. *Circles* indicate saline-treated animals, *squares*, animals treated for 3 and 10 days with 10 mg/kg gangliosides. $K^+ = 5$ mmol · l^{-1}

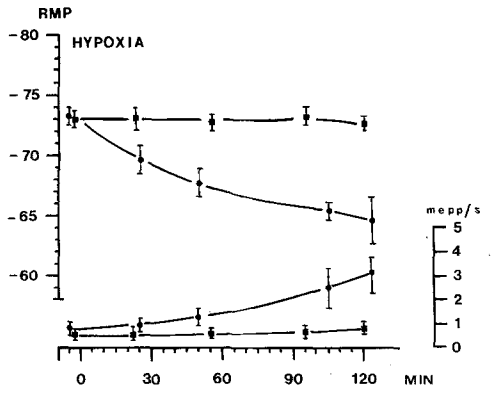

Fig. 4. Hypoxia-induced changes of the resting membrane potential (RMP, left scale in mV) and miniature end-plate potential frequency (m.e.p.p.s, right scale) in control (*full circles*) and ganglioside (GS)-treated mouse diaphragm (*squares*). N^2-saturated solution was applied at time zero to the control two muscles. Two other hemidiaphragms were first preincubated for 120 min with 5×10^{-8} mol · l^{-1} GS in oxygenated solution and then kept in N_2-saturated 5×10^{-8} mol · l^{-1} GS medium for 120 min. Temperature = 20° C

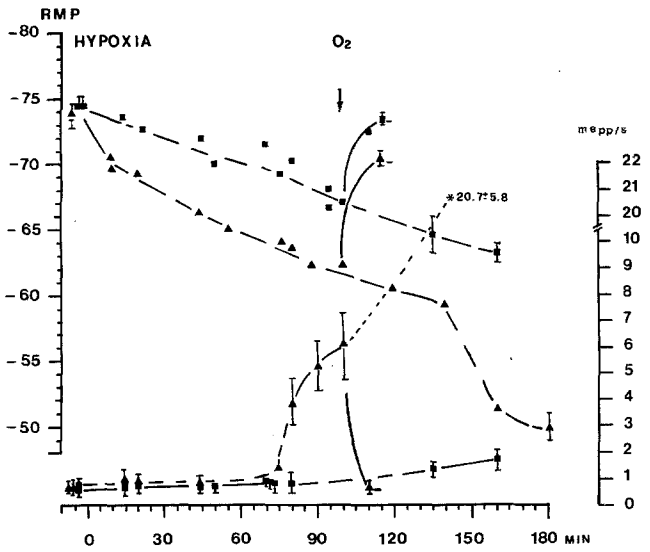

Fig. 5. Hypoxia-induced changes of RMP (*left scale*) and m.e.p.p. frequency (per s, right scale) in control muscles (*triangles*) and muscles from 3 day treated animals with 10 mg/kg gangliosides. During the experiment, no gangliosides were present in the bath, which was covered with oil

spectively. The change of the RMP was small, as compared with controls, when the muscles were pretreated with GS and bathed in O_2-deprived solution, in the presence of GS (2 h, Fig. 4).

The effect of hypoxia on the RMP in muscles from control and GS injected mice (10 mg/kg, 3 days) are shown in Fig. 5. The results are essentially similar to those already described, showing the slower rate of hypoxia-induced depolarization in GS-treated diaphragms.

The effect of hypoxia on the m.e.p.p. frequency was also recorded. Oxygen deprivation increased the m.e.p.p. frequency significantly in control muscles (from 0.8 ± 0.3 to 3.0 ± 0.7 per s) but had no effect in muscles bathed with GS. Interestingly, the m.e.p.p. frequency did not change substantially during the first 60 min of hypoxia when the RMP of muscle fibres had depolarized. Subsequently, the mean frequency increased and the number of fibres with a high frequency of m.e.p.p. (more than 10 per s) increased in control muscles, but not in the experimental ones. After about 100 min in O_2-deprived solution, the addition of oxygen led to restoration of both parameters: the RMP and m.e.p.p. frequency of control and experimental muscles returned to their original values. If the muscles were maintained under the oil for more than 2 h, the frequency of m.e.p.p.s increased in most fibres from the control muscles, but subsequently a lot of fibres (33% after 130–140 min) became completely silent in the end-plate zone. The mean m.e.p.p. frequency in GS-treated muscles increased more slowly and the final value at 160 min was 1.7 ± 0.4 per s (Fig. 5). The effect of hypoxia observed in the oil covered preparation was somewhat stronger (m.e.p.p. frequency higher) probably due to a greater O_2 deprivation.

Discussion

The reported results show that gangliosides, added to the bathing medium or injected intraperitoneally, affect both muscle fibres and nerve terminals by increasing their ability to withstand hypoxia and the absence of K^+.

Membrane polarization is the necessary prerequisite for excitable cells to generate action potentials and to transmit signals along their membrane. The treatment of muscle fibres in vitro with K^+-free solution for several hours or by lowering temperature below 10° C causes the loss of intracellular K^+ and muscles become enriched with Na^+ (Kernan 1962; Sato et al. 1967; Tanai and Kagiyama 1968; Taylor et al. 1970; Dlouhá et al. 1981). On re-admission of potassium or re-warming, the (Na^+/K^+) pump is activated, which results in transient hyperpolarization of muscle fibres because the number of positively charged sodium ions transported out of cell exceeds the number of potassium ions transported in (Thomas 1969; Brinley and Mullins 1968; Den Hertog 1973; Rang and Ritchie 1968).

In the mouse diaphragm in K^+-free solution (Dlouhá et al. 1981) the gain of sodium is accompanied by a reduction of intracellular K^+ (Zemková et al. 1982) which is the main reason for the drop of RMP after several hours of bathing in K^+-free solution. There was no significant difference between the RMPs in control and 2 h ganglioside-pretreated diaphragm fibres; it seems that this indicates the same loss of K^+ and gain of Na^+ in both cases.

Pretreatment of muscle cells for the whole period of Na^+-loading or injection of gangliosides in vivo led to an effective prevention of the depolarization of the muscle cells in K^+-free solution. This depolarization is due to a leakage of K^+ from the muscle fibres; the absence of this depolarization in ganglioside pretreated muscles therefore suggests that ganglioside treatment reduced the K^+ leakage due to the lack of external K^+. Ganglioside-induced potentiation of the pump (see below) could be the basis for such an effect. Indeed, in some preliminary experiments on diaphragms from 14 day treated mice (10 and 1 mg/kg gangliosides per day), when the pump was inhibited by cooling for 15 h (Vizi and Vyskočil 1979) the decrease of RMP was approximately the same as in control muscles (i.e. treated and untreated muscles were equally loaded). After re-warming, however, the electrogenic effect in the treated muscles was higher than in controls confirming again the positive effect of gangliosides on the pumping process. This effect may also underlie the better survival of diaphragms in hypoxic conditions. The mouse diaphragm fibres (about 20 μm) are apparently more sensitive to hypoxia than either thicker muscles or the muscles of the rat, as indicated in control experiments by the relatively quick change of the RMP. In our experiments, we observed that if the RMP depolarized to about 55 mV (Fig. 4, triangles) the muscle fibres were no longer directly excited by current passing through the electrode. The depolarization of the nerve terminal RMP can be estimated from the frequency of m.e.p.p.s. Both parameters, the RMP of muscle fibres and m.e.p.p. frequency changed in ganglioside-treated muscles more slowly than in controls. The better functioning of the pump probably enabled both muscle fibres and nerve terminals to survive the lack of oxygen for longer period of time.

An increase of effective membrane input resistance could be one of the reasons for the observed higher electrogenic effect of the pump (see e.g. Thomas 1972). However, there was no effect of gangliosides on the R_i and thus this membrane parameter is apparently not responsible for the GS-induced potentiation of the pump.

The most probable explanation for the potentiating effect of the gangliosides is a direct influence on the membrane (Na^+/K^+)ATPase. It has been shown in biochemical experiments that GM_1 and other gangliosides, when inserted into the membrane, increase the ouabain-sensitive part of the enzyme activity (Leon et al. 1981). The potentiation of the electrogenic effect was very similar (38%). These observations may explain at least in part the beneficial influence of gangliosides on many cell functions, such as differentiation, growth and regeneration (for details see Rapport and Gorio 1981) which are undoubtedly dependent on the cell polarization.

The stable insertion of the gangliosides, present in the medium in concentrations equal or lower than 5×10^{-8} mol $\cdot$ l^{-1}, has been shown to have a positive effect on the (Na^+/K^+)ATPase activity (Leon et al. 1981). This is in good agreement with our results. The biochemical experiments also showed that ganglioside-activated enzyme had an increases V_{max}, i.e. the velocity of the ATP splitting was higher. In electrophysiological experiments, the increase in the reaction rate may well correspond to the higher electrogenic effect of the pump; however it is not quite clear why the increased rate of ATP splitting should support the survival of muscle fibres in anaerobic conditions. The available amount of ATP would probably be exhausted more quickly and the ganglioside-treated muscle should, thus, be more sensitive to hypoxia. Because the results obtained just were the opposite, one can speculate about the possible influences of gangliosides on energy metabolism. It would be of interest to check whether gangliosides are able to shift metabolic pattern of the excitable cells to the anaerobic production of ATP, as is indicated by the hypoxia experiments.

We have previously shown that gangliosides affect neuronal plasticity; however, such an action is dependent upon the presence of growth factors (Gorio et al. 1980; Ferrari et al. 1983). On the other hand, we showed that gangliosides improve the neurological deficits in mouse diabetic neuropathy only in the later phase when insulin is ineffective. Earlier, during first several weeks of the life, they are without effect on these same parameters (Norido et al. 1984; Gorio et al. 1984).

There must be a general action of gangliosides after incorporation into an excitable or neurally derived cell. As the present results show, this enzymatic or metabolic change may make the neuron either more ready to respond to the stimulatory action of growth factors or improve its excitability.

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