The Influence of Skeletal Muscle Incubation Medium on Fatigue of Neuromuscular Preparation and on Transmitter Release at Neuromuscular Junctions in the Frog

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Abstract. The effect of frog skeletal muscle incubate on fatigue was studied in frog sciatic nerve, sartorius muscle preparation. Fatigue was produced by prolonged repetitive (1 s^{-1}) stimulation of motor nerve or of curarized muscle.

The incubate partially restored isometric contraction amplitudes of muscle fatigued by nerve stimulation. This effect of partial recovery from fatigue (PRF effect) was exerted mainly by a relatively low-molecular fraction (LMF; < 10 kDa) of the incubate. The incubate and its fractions failed to produce the PRF effect in experiments with directly stimulated muscle.

The action of LMF on synaptic transmission in unfatigued cutaneous-pectoris muscle was examined using binomial analysis of quantal transmitter release. LMF produced an increase in the end-plate potential quantal content (m) at synapses with low initial m values. In contrast, it produced a decrease in m at synapses with higher m values. Both effects were due to respective changes in binomial parameter n.

It is assumed that the stimulatory presynaptic action of the incubate on synapses the effectiveness of which was lowered during fatigue, could account for the PRF effect. A possible contribution of low- and high-molecular components of the incubate is discussed.

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Key words: Fatigue — Neuromuscular synapse — Muscle metabolites — Antidromic regulation

Introduction

Antidromic (retrograde) effects of substances released from muscle fibres on motor nerve terminals have been studied in our laboratory since 1970. These antidromic effects may be common for all excitatory chemical synapses and they play an important role in learning synapses of the brain (Matyushkin 1989).

The antidromic influences of potassium ions accumulated in the microenvironment of active neuromuscular junction have been characterized previously (Matyushkin et al. 1978, 1984, 1993).

The present study was concerned with the search for muscle metabolites with presynaptic action which could be involved in the regulation of neuromuscular transmission during fatigue induced by prolonged low-frequency stimulation.

As early as 1935, a humoral factor of non-protein nature has been described in the perfusate of the vascular supply of frog skeletal muscle, which partially restored contractions of muscle fatigued by indirect stimulation (Kibjakov 1935). Much later we could confirm this result, and have shown that vascular perfusate of frog hind limbs enhanced the transmitter release at neuromuscular synapses (Drabkina et al. 1988; Matyushkin 1989).

Among possible candidates for the role of regulatory factors of antidromic (presynaptic) action, our interest focused on metabolites of muscular dipeptide carnosine (β-alanyl-L-histidine). This dipeptide has been found in large amounts in innervated parts of twitch skeletal muscles (Severin and Vulfson 1963; Crush 1970). A definite physiological role for carnosine has not been established. Speculations concerned its possible relation to the function of cholinergic neuromuscular transmission (Severin and Vulfson 1963; Boldyrev 1977; Matyushkin et al. 1986; Matyushkin 1989).

Exogenous carnosine and its component, histidine (HIS), are capable of restoring the working ability of fatigued neuromuscular preparation (Severin and Vulfson 1963; Boldyrev 1977). Exogenous HIS significantly enhances the evoked transmitter release at neuromuscular synapses with lowered synaptic effectiveness (Shabunova 1977; Matyushkin et al. 1986). Exogenous carnosine also acts as a modulator of presynaptic function at the neuromuscular synapse (Boldyrev 1977; Drabkina et al. 1990).

The sartorius muscle of the frog, fatigued by nerve stimulation, loses about 20% of its carnosine store (Dupin and Stvolinsky 1986; Matyushkin et al. 1986). The fall of the dipeptide content is not accompanied by its release from the muscle or by an increase in the contents of its components in the muscle (Dupin and Stvolinsky 1986), but it is accompanied by an enhanced release of β-alanine (Drabkina et al.

1986) and of unidentified HIS-containing compound(s) found to be neither high-molecular protein (Shabunova 1979) nor free HIS (Dupin and Stvolinsky 1986). Neither of the described effects has been observed with directly stimulated muscle under blockade of neuromuscular transmission (for a review, see Matyushkin et al. 1986).

Based on these data a hypothesis has been proposed stating that activation of muscle via the cholinergic synapse leads to the breakdown of a part of muscular carnosine; HIS-residues formed in this way leave the muscle fibres being incorporated in a low-molecular compound, possibly of oligopeptide nature. This compound has been presumed to play a role in antidromic functional regulation, in particular during fatigue, and to act similarly as exogenous HIS or carnosine (Matyushkin 1989).

In the present study we used the incubation medium of frog sartorius muscles as the source of muscle metabolites in extracellular spaces (which, in the frog, have direct contacts with subcutaneous lymphatic bags). Among these metabolites some of the presumed regulatory factors could be expected to be present. The influence of the incubate and its high- and low-molecular fractions on fatigue development as well as on synaptic quantal release parameters were studied in experiments on isolated neuromuscular preparations of the frog. Attention was paid to possible association of physiological effects with HIS-containing compounds of the incubate.

Materials and Methods

Acquisition and fractionation of muscle incubate

Several isolated sartorius muscles of the frog *Rana temporaria* were incubated for 2 h in normal Ringer solution at room temperature, each muscle being soaked in a separate plastic dish 5 ml in volume. The solution contained (mmol/l): NaCl – 112; KCl – 2; $CaCl_2$ – 1.9; $NaHCO_3$ – 2.9; pH 7.4. Samples obtained from all incubated muscles were then pooled. A half of the total incubate volume obtained was immediately used for physiological experiment. The other half was fractionated by ultrafiltration method (Williams and Wilson 1975) at 5 °C using DIAFLO millipore filter (Amicon Corp.) with the pore size of 10 kDa. Ninety to ninety-five percent of the incubate volume passed through the filter during 3–4 h, thus forming the low-molecular fraction (LMF). The fluid residuum above the filter, i.e. the high-molecular fraction (HMF), was diluted with normal Ringer solution up to an initial volume. LMF and HMF were further used in physiological experiments.

The ultrafiltration procedure was performed also with normal Ringer solution used to obtain the incubate, and "pseudo-fractions" obtained were used either as control samples in spectrophotometric measurements or as control solutions in physiological experiments.

Spectrophotometry was used to measure the contents of protein and HIS-containing substances in the incubate and its fractions. The total protein content was measured by the Lowry method (Lowry et al. 1951), the content of HIS-containing substances by the Pauly diazoreaction method (Darbre and Clamp 1986) and using reaction with a HIS-specific reagent, diethylpyrocarbonate (Ovadi et al. 1967).

Fatigue experiments

An isolated sartorius neuromuscular preparation was mounted in a Perspex chamber with constant perfusion of Ringer solution at room temperature.

In a first series of experiments fatigue was produced by prolonged indirect repetitive stimulation at 1 s^{-1}. The nerve was stimulated through a suction electrode with supramaximal pulses of 0.3 ms duration. Normal Ringer solution or its respective "pseudofraction" were used as control solutions in studying the effects of incubate or its fractions.

In a second series of experiments fatigue was produced by prolonged direct repetitive stimulation at 1 s^{-1}. The neuromuscular transmission was blocked by 6.4×10^{-6} mol/l d-tubocurarine (Sigma) added to the control and test solutions. The muscle was stimulated via superficial silver electrodes with supramaximal pulses of several ms duration.

The isometric contractions of the muscle were recorded using a mechanotron.

The experimental protocol was as follows. The preparation was stimulated for 1 h in control solution. Then, the control solution was replaced by incubate or one of its fractions. After 1 h stimulation in the tested medium the preparation was washed with the control solution for 1 h. Stimulation continued throughout the experiment (3 h).

In a separate control series of experiments with indirect or direct stimulation (1 s^{-1}) the preparations were fatigued for 3 h in Ringer solution without or with d-tubocurarine. To simulate experimental conditions with the use of incubate (see above), "mock solution exchange" was performed after 1 h of stimulation by replacing Ringer solution with the same solution from another container.

Mean amplitudes of isometric contractions during 1 min (an average of 60 contractions) were calculated at different intervals during the fatigue development, and were expressed as percents of the mean amplitude of the first 10 contractions.

Experiments on neuromuscular junctions

Spontaneous miniature end-plate potentials (MEPP) and neurally evoked end-plate potentials (EPP) were recorded intracellularly from junctions of the frog cutaneous-pectoris muscle using standard microelectrode technique. The nerve was stimulated through a suction electrode at 0.5 s^{-1} with suprathreshold pulses of 0.3 ms duration. Muscle contractions were abolished by adding 10–15 mmol/l $MgCl_2$, equally to control and experimental solutions.

After equilibration of the preparation with the control solution (for 40–60 min) control MEPP and EPP were recorded. Then the solution was replaced by incubate. MEPP and EPP were recorded from the same synapse 15, 30, 45 and 60 min after the solution replacement. Subsequently, the preparation was washed out with control solution. The nerve was not stimulated in intervals between recordings.

For analysis, mean amplitudes of 100–150 MEPP and 100 EPP were calculated. Mean EPP quantal content (m) was calculated as a ratio of mean EPP amplitude to mean MEPP amplitude. Binomial parameters p (quantum release probability) and n (number of release sites or size of available transmitter store) were calculated using formulae: $p = 1-(\sigma^2_{epp}/(A_{epp} \times A_{mepp}))$; $n = m/p$, where σ^2_{epp} is variance of EPP amplitudes; A_{epp} and A_{mepp} are the mean amplitudes of EPP and MEPP, respectively (Miyamoto 1975). Values of the parameters were related to those recorded from the same synapse in control solution. Student's t-test was used to determine statistical significance. Values are presented as means ± S.E.M. throughout.

Table 1. Concentrations of protein and HIS-containing compounds in muscle incubate and its fractions ($\times 10^{-9}$ mol/l)

Substance	Incubate	Low-molecular fraction (< 10 kDa)	High-molecular fraction (> 10 kDa)
Proteins*	671.1 ± 48.0 (37)	87.3 ± 1.2 (37)	489.8 ± 3.0 (37)
HIS-containing compounds**	761.88 ± 62.6 (37)	626.2 ± 104.9 (35)	93.9 ± 26.1 (35)

The number of experiments is shown in parentheses
* – Bovine serum albumine (Sigma) was used for calibration
** – Histidine chloride (Reanal) was used for calibration

Results

Contents of some incubate components

The concentrations of protein and of HIS-containing substances detected in the incubates and their fractions are shown in Table 1. HMF included 73% of protein contained in the whole incubate and 12% of its HIS-containing substances. LMF included 13% of protein and 84% of HIS-containing substances of the whole incubate. Thus, the latter compounds are, most probably, of non-protein nature and of a relatively low molecular mass (less than 10 kDa). This distribution of HIS-containing substances is similar to that observed previously in vascular perfusate of frog hind limb muscles (Drabkina et al. 1988).

Influence of incubate and its fractions on fatigue process

The time courses of fatigue at indirect and direct stimulation of the sartorius muscle in a control series of experiments (with Ringer solution only) are shown in Fig. 1. Two main phases are seen: 1) An initial rapid fall of contractions during the first 10–15 min (down to 10.1 ± 2.1% and 6.9 ± 1.6% at indirect and direct stimulation, respectively); and 2) a very slow further decline down to 3.9 ± 0.8% and 1.4 ± 0.6% by the end of experiment. The second phase can be designated a plateau phase.

As it is seen in Fig. 1, the deepness of fatigue at the plateau phase was more profound at direct than at indirect stimulation.

In experiments with nerve stimulation the incubate and both its fractions reversibly slowed down the fatigue development, or even caused a partial restoration of contractions comparing to those at the plateau phase before solution exchange (Fig. 2). A large variability of the effects was observed in individual experiments,

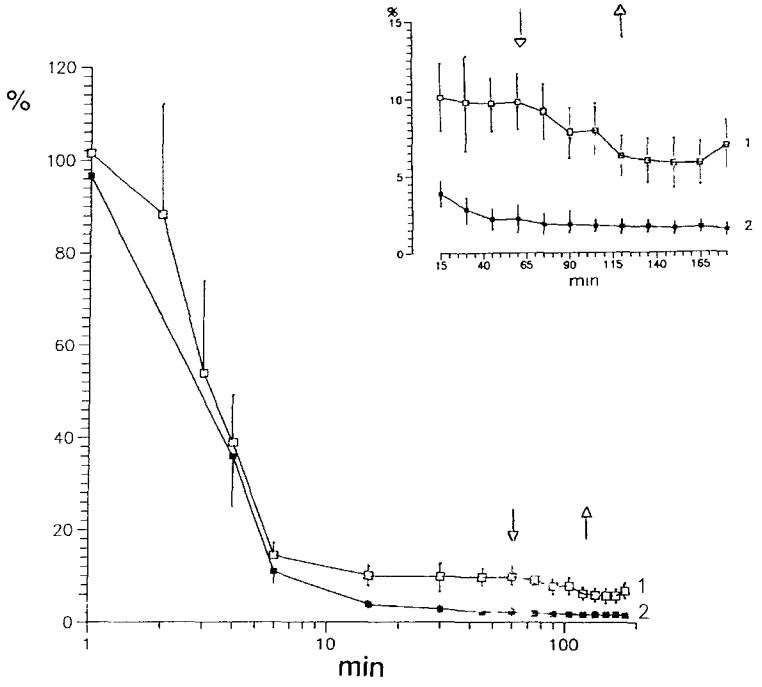

Figure 1. Fatigue development in frog sartorius muscle during prolonged repetitive stimulation at 1 s^{-1}. 1 – Motor nerve stimulation; 2 – Direct stimulation of curarized muscle. Abscissa (logarithmic scale) : Time from onset of stimulation, minutes. Ordinate: Amplitude of isometric contractions, percents of mean amplitude of the first 10 contractions. Each point represents mean (± S.E.M.) of 7 (in 1) or 4 (in 2) experiments. Arrows indicate "mock solution exchange" (see Materials and Methods).
Insert: Time course of fatigue at the plateau phase shown on an enlarged, linear scale.

especially with HMF. The most prominent effect was produced by LMF (curve 3 in Fig. 2).

Fig. 3 illustrates the obvious LMF-produced change in the time course of fatigue. The slope of the regression line of the contraction amplitude-versus-time plot for LMF (curve 2 in Fig. 3) differred significantly ($P < 0.05$) from that obtained for control experiments (curve 1 in Fig. 3).

Thus, the incubate, especially its LMF, contains factors capable of producing a partial recovery from fatigue (PRF effect) in neurally activated muscle.

In experiments with direct stimulation of the muscle neither incubate nor its fractions caused any PRF effect (Fig. 4). On the contrary, whole incubate and LMF both induced a slight, statistically insignificant ($P > 0.05$) acceleration of fatigue (curves 2 and 3 in Fig. 4), whereas HMF produced a profound, highly significant

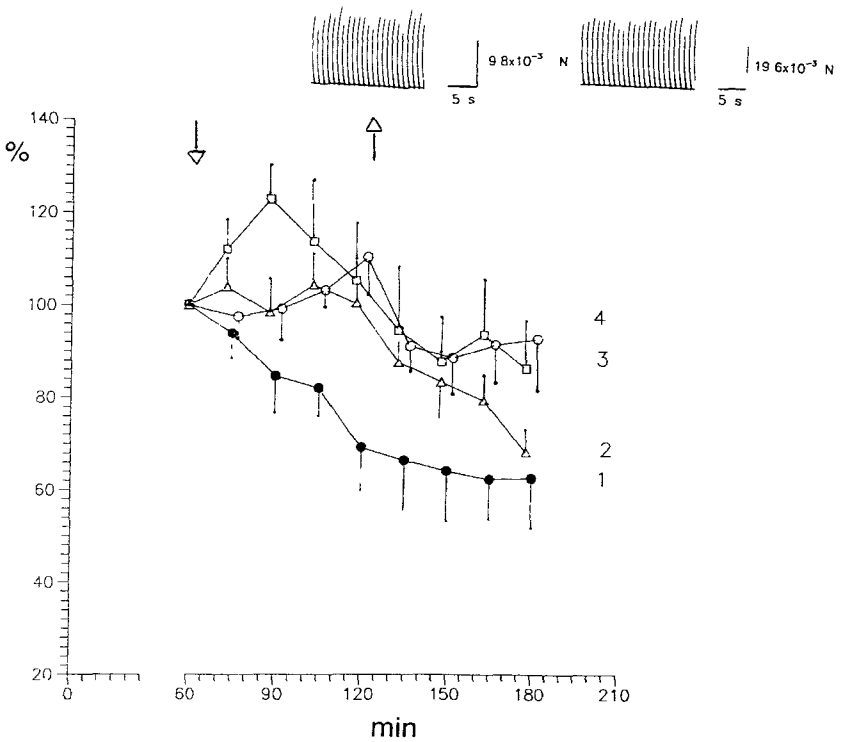

Figure 2. Effects of incubate and its fractions on frog sartorius muscle fatigued by motor nerve stimulation (1 s^{-1}). 1 – Control; 2 – Incubate; 3 – Low-molecular fraction, LMF; 4 – High-molecular fraction, HMF. Abscissa: Time from onset of stimulation, minutes. Ordinate: Amplitude of contractions, percents of amplitude recorded 60 min after onset of stimulation. (The values of these amplitudes related to the initial 10 responses in 1, 2, 3 and 4 were 9.8 ± 1.8%, 13.4 ± 3.6%, 11.7 ± 3.1% and 11.2 ± 2.4%, respectively). Each point represents the mean amplitude for 7–8 experiments. The downward arrow indicates onset of perfusion with the tested solution, the upward arrow indicates wash-out.
Insert: Records of isometric contractions after 1 h stimulation in control solution (left) and after 45 min LMF action (right). Note the difference in amplitude calibration: left 9.8×10^{-3} N; right 19.6×10^{-3} N. Time calibration 5 s.

($P < 0.01$) reversible deepening of fatigue (curve 4 in Fig. 4).

Thus, the PRF effect is related to a function of motor nerve endings since it was absent in directly stimulated muscle (cf. Fig. 2 and 4).

In a few experiments we tried to estimate the concentration range of active anti-fatigue factors of the incubate. For this purpose, effects of the incubate were studied either after its dilution with normal Ringer solution or with the concentrations of extracted components raised. "Concentrated" incubates were obtained by

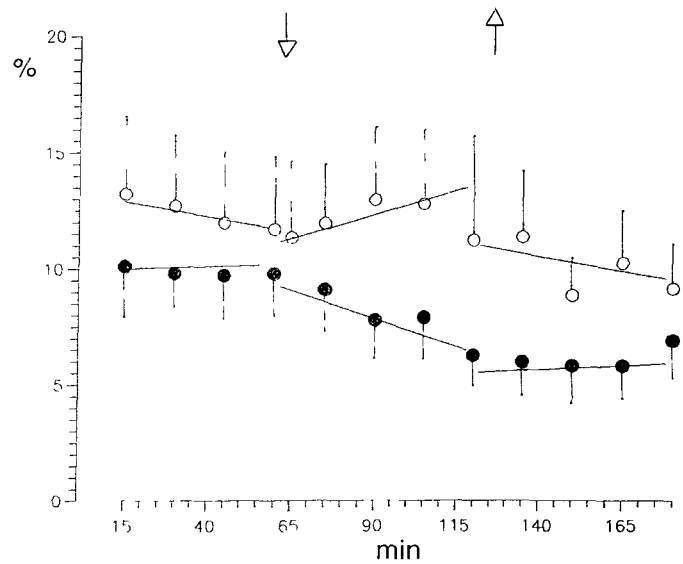

Figure 3. Effect of the incubate low-molecular fraction (LMF) on frog sartorius muscle fatigued by motor nerve stimulation. Only the plateau phase is shown. 1 – Control; 2 – LMF. The same experiments as in Fig. 2. Abscissa: Time from onset of stimulation, minutes. Ordinate: Contraction amplitude, percents of mean amplitude of the first 10 contractions. Linear regression lines (amplitude versus time) are shown for intervals before, during and after "mock solution exchange" (in 1) and LMF action (in 2). For other designations see legend to Fig. 2.

increasing the numbers of muscles incubated in the same total volume of Ringer solution (as in experiments shown in Fig. 2) and by prolongating the exposure time by 1 h. The resulting concentrations of protein and HIS-containing substances in the "concentrated" incubates appeared to be 3 times those in the non-concentrated ones, the distribution of the components between LMF and HMF being similar to that shown in Table 1. The incubates had pH values similar to those of control solutions and the non-concentrated incubates.

The activities of the diluted or "concentrated" incubates were studied with indirectly stimulated muscles only.

A 5-fold dilution of non-concentrated incubates as well as of their LMF or HMF completely abolished the PRF effect (results of experiments with 3 incubates and their fractions; not shown).

Two "concentrated" incubates and their fractions were tested. These incubates as well as their LMF and HMF markedly reduced the contractions of neurally fatigued muscle (by 53%, 55% and 69%, respectively; not shown) compared to

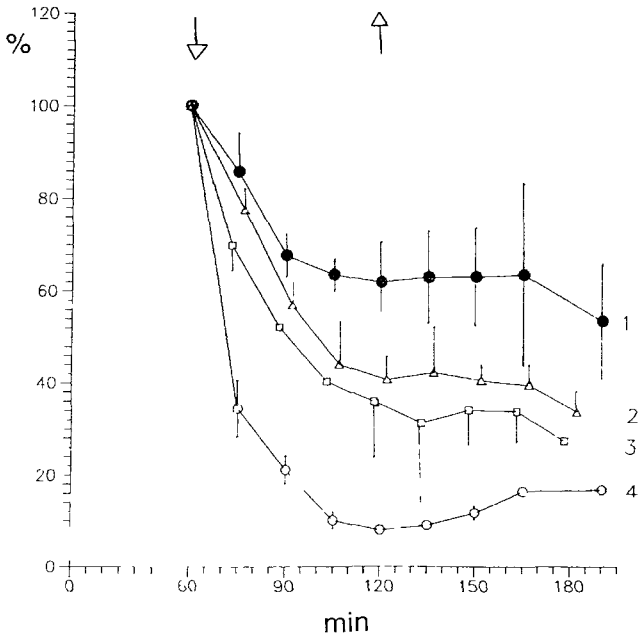

Figure 4. Effects of the incubate and its fractions on frog sartorius muscle fatigued by direct stimulation ($1s^{-1}$) in the presence of d-tubocurarine (6.4×10^{-6} mol/l). 1 – Control; 2 – Incubate; 3 – LMF; 4 – HMF. For legend see Fig. 2. The contraction amplitudes recorded 60 min after onset of stimulation (100%) related to the initial 10 responses in 1, 2, 3 and 4 were $2.2 \pm 0.9\%$, $3.9 \pm 1.6\%$, $3.8 \pm 2.5\%$ and $4.1 \pm 0.9\%$, respectively. Each point represents the mean for 3–4 experiments.

those recorded after 1 h stimulation in control solutions. (Interestingly, the pattern was similar to that observed during HMF action on directly stimulated muscle, see Fig. 4). These effects developed rapidly and were reversible.

Influence of LMF on quantal release parameters at neuromuscular junctions

A decrease in the EPP quantal content at synapses of neurally fatigued isolated frog sartorius muscle was assumed to be the main cause of the muscle performance failure at stimulation frequency as used in our fatigue experiments (Nikolsky and Poletaev 1977). This suggests that the mechanism underlying the observed PRF effect might be stimulation of presynaptic function.

To test this possibility, we studied the influence of LMF (a fraction producing the most obvious PRF effect) on quantal secretion parameters at synapses of unfatigued cutaneous-pectoris muscle with the neuromuscular transmission blocked by Mg^{2+}. These conditions may represent a more or less adequate model of presynaptic function inhibited during fatigue.

The initial values of quantal secretion parameters m, p and n (m_0, p_0 and n_0, respectively) recorded in control solutions at different Mg^{2+} concentrations (10–15 mmol/l) varied considerably in different preparations, the least variable being p_0 (Table 2).

Table 2. Initial (control) values of quantal release parameters (m_0, p_0 and n_0) at synapses of frog cutaneous-pectoris muscle in experiments with the low-molecular fraction (LMF) of incubate

Parameter	Populations of synapses				
	I Whole population	II "Low-quantal" synapses ($m_0 < 15$)	III "High-quantal" synapses ($m_0 > 15$)	IV Synapses with $n_0 < 60$	V Synapses with $n_0 > 60$
m_0	19.2 ± 4.0	27.1 ± 1.7	30.4 ± 5.5	10.4 ± 2.6	33.1 ± 8.1
	(19)	(9)	(10)	(8)	(7)
*CV_{m_0}	0.907	0.729	0.576	0.717	0.646
		$P_{I-II} < 0.05$		$P_{IV-V} < 0.05$	
p_0	0.35 ± 0.05	0.27 ± 0.15	0.41 ± 0.08	0.35 ± 0.05	0.34 ± 0.10
	(15)	(7)	(8)	(8)	(7)
CV_{p_0}	0.597	0.642	0.555	0.425	0.799
		$P_{I-II} > 0.05$		$P_{IV-V} > 0.05$	
n_0	85.0 ± 29.7	38.3 ± 16.9	125.8 ± 50.8	27.0 ± 6.1	151.2 ± 54.6
	(15)	(7)	(8)	(8)	(7)
CV_{n_0}	1.353	1.130	1.142	0.643	0.955
		$P_{I-II} > 0.05$		$P_{IV-V} < 0.05$	

*CV – coefficient of variation
Figures in parentheses show numbers of experiments

In experiments with LMF, the quantal release parameters averaged for the whole nonuniform population of synapses studied did not differ significantly from control values. However, in some individual experiments more or less pronounced increases or decreases in the EPP quantal content were seen. In these experiments values of m_0 were the lowest or the highest, respectively, from the whole population.

It is well known that presynaptic effects of many substances depend on the initial levels of quantal secretion. The same should be true for the production of the assumed endogenous regulatory factors expected to be similar to those contained in the external incubate (see Introduction).

Taking this into account, we analyzed the LMF action on subpopulations of synapses showing different m_0 values.

The whole population of the studied synapses was divided into two groups, about equal in size, with a low m_0 value (less than 15) and with a higher m_0 value (exceeding 15) (Table 2).

With "low-quantal" synapses the LMF appeared to increase the EPP quantal content, whereas a decrease in m was observed with the LMF-treated "high-quantal" synapses (Fig. 5). Although the magnitudes of the effects of both kinds were less at later periods of LMF action than at the earlier ones (see Fig. 5), the differences between them were statistically significant for the whole period of LMF treatment (see legend to Fig. 5).

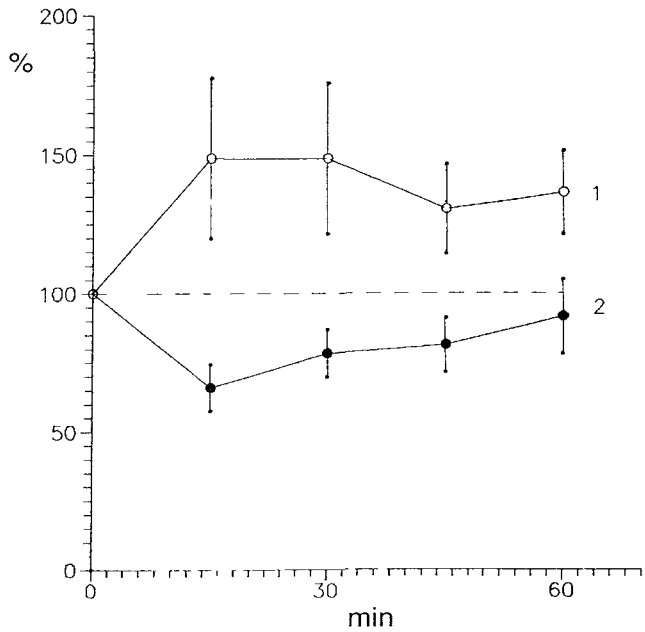

Figure 5. Effects of the incubate low-molecular fraction (LMF) on EPP quantal content (m) at synapses of unfatigued frog cutaneous-pectoris muscle. 1 – "Low-quantal" synapses; 2 – "High-quantal" synapses (see Table 2). Abscissa: Time of LMF action, minutes. Ordinate: EPP quantal content, percents of control. Each point represents the mean for 9 (in 1) or 10 (in 2) experiments. Statistical significance of differences between 1 and 2: at 15 and 30 min: $P < 0.01$; at 45 and 60 min: $P < 0.05$.

These results suggest an asymmetry in LMF action on synapses with low and high m_0. The same conclusion could be drawn from the observed negative correlation between m_0 values and relative changes in m (percents of control) induced by LMF ($r = -0.48$; 19 synapses; $P < 0.05$).

In addition, LMF seemed to change the binomial parameter n, increasing it for "low-quantal" synapses and slightly decreasing it for "high-quantal" synapses.

For a more detailed analysis of the LMF action on parameter n, we divided the whole population of synapses into two groups according to n_0 values (the groups with different m_0 and n_0 values were partially overlapping but did not coincide: see Table 2).

With synapses with a relatively low n_0 LMF increased n up to $152.0 \pm 19.9\%$ ($P < 0.05$) whereas with synapses with a higher n_0 it caused a slight decrease in n (down to $86.1 \pm 19.0\%$, $P > 0.05$). A negative correlation was observed between n_0 values and relative changes in n produced by LMF ($r = -0.57$: 15 synapses; $P < 0.05$).

The asymmetry of presynaptic action could be convincingly confirmed in two experiments where we had an opportunity to examine the influence of the same LMF on two synapses with considerably different quantal release parameters. The values of m_0 and n_0 were 2.8 and 8.5, respectively, for one synapse; and 16.0 and 66.0, respectively, for the other one. In the first synapse LMF increased the EPP quantal content and parameter n by 146% and 110%, respectively, while in the second one it decreased the EPP quantal content by 30% without substantial change in n.

In some experiments, namely in those using synapses with low m_0 values, the EPP quantal content was not altered significantly by the LMF action, but it increased dramatically (by 100–200%) in response to wash-out of LMF. Such a kind of presynaptic after-effect has been described previously for exogenous carnosine (Drabkina et al. 1990).

For the whole nonuniform population of synapses, the LMF-induced relative changes in the EPP quantal content (both increases and decreases) were due to respective changes in binomial parameter n: the coefficient of correlation was +0.59 (15 synapses; $P < 0.05$).

In experiments with LMF the relative changes in EPP quantal content appeared the larger, the higher the concentration of HIS-containing substances in this fraction. For the whole population of synapses studied, the coefficient of correlation was +0.50 (19 synapses; $P < 0.05$). An even higher correlation between relative changes in m and the concentration of HIS-containing compounds was obtained for the group of synapses with low n_0 values ($r = +0.87$; 7 synapses; $P < 0.05$). It was exactly in these synapses that LMF produced significant increase in n (see above).

No correlation was found between changes in m and protein concentration.

No statistically significant change in binomial parameter p was observed, when analyzing LMF action either on the whole population of synapses or on their above described subpopulations. We were unable to separate any representative subpopulations of synapses with different p_0 values because of the relatively low variability

of this parameter in our experiments (see Table 2).

In experiments with LMF no substantial changes were observed in muscle fibre resting potential or in amplitude or time course of MEPP. This suggests that LMF does not significantly modify electrogenesis in the muscle cell membrane or the characteristics of the acetylcholine-receptive postsynaptic membrane.

Discussion

Fatigue of isolated skeletal muscle or neuromuscular preparation is a very complicated process which remains not completely understood. Different cellular mechanisms can be involved in the reversible reduction of muscle performance in fatigued preparation depending on the stimulation pattern or the mode of muscle activation (Nikolsky and Poletaev 1977; Westerblad et al. 1991; Westerblad and Allen 1992; Edman and Lou 1992).

In a comprehensive study of fatigue mechanisms in isolated, directly stimulated muscle fibres, Edman and Lou (1992) have shown that the force reduction during an initial period of fatiguing stimulation at 1 s^{-1} is caused by an impairment on the level of contractile elements. This functional disorder (termed "true myofibrillar fatigue"), is generally assumed to be a consequence of metabolic shifts in the myoplasm during repetitive contractions (Renaud and Mainwood 1985; Westerblad et al. 1991; Nagesser et al. 1992).

More prolonged stimulation at the above frequency causes a failure of the contractile apparatus activation due to a failure of action potential propagation along transverse tubules (Westerblad et al. 1991; Edman and Lou 1992).

It should be noted that such a blockade of excitation-contraction coupling prevents the myofibrillar apparatus from further exhaustion (see Edman and Lou 1992). Probably, this is manifested in the slow phase of contraction decline (plateau) seen in our experiments with directly stimulated muscles (Fig. 1, curve 2).

In isolated frog sartorius muscle fatigued by nerve stimulation at 1 s^{-1}, a block develops firstly at the presynaptic level (Nikolsky and Poletaev 1977). It occurs due to a decrease in EPP quantal content below a threshold for muscle action potential generation. However, in this case the presynaptic block is not a result of "fatigue" of the presynaptic function per se; rather, it is a consequence of inhibitory antidromic actions of metabolites released from contracting muscle fibres and accumulated in extracellular clefts (Nikolsky and Poletaev 1977).

Although direct evidence is lacking, one can suggest the involvement of metabolites such as K^+, H^+, lactate, adenosine, etc. These substances are expected to be released from muscle fibres, and are known to inhibit the transmitter release (Lucier and Mainwood 1972; Hník et al. 1976; Matyushkin et al. 1978, 1984; Silinsky 1980; Gaudri-Tallarmain 1986; Matyushkin 1989; Smith 1991).

As a result of the presynaptic blockade, a periodic failure of activity can occur in a fraction of motor units in a randomized manner. Such randomized block operating as negative feed-back could protect the subsequent elements (excitation-contraction coupling, myofibrills) from overload. We suppose that this is reflected in less profound fatigue during the plateau phase at indirect compared to direct stimulation of muscle (Fig. 1).

At the same time, there are many reasons to assume that, besides the described negative feed-back, some positive feed-backs operate in fatigued preparation tuning the synapses with reduced quantal secretion to more effective transmission (a latent "training" effect). These processes might be mediated by factors released from synaptically activated muscle fibres (see Introduction, and a review by Matyushkin 1989), and their effects would be revealed when the muscle fibres are "silent" during partial presynaptic blockade and do not produce "inhibitory" substances. The nature of these factors remains to be determined. Our study represents one initial step on this way.

Our results brought evidence for the incubation medium of resting skeletal muscles containing chemical factors capable of restoring contractions in fatigued muscle (PRF effect, Figs. 2 and 3). A target of their action is the transmission of signals from nerve to muscle, which is evidenced by the lack of PRF effect in experiments with direct muscle stimulation (cf. Figs. 2 and 4). The most prominent PRF effect was seen on exposure to LMF (Fig. 2, curve 3, and Fig. 3).

We have shown that the incubate, namely its LMF, contains factors modulating the evoked transmitter release at Mg^{2+}-blocked neuromuscular junctions of unfatigued muscle. This modulation was asymmetric depending on the initial level of quantal secretion: the LMF effect on EPP quantal content at "low-quantal" synapses was mainly stimulatory, whereas it was mainly inhibitory at relatively "high-quantal" synapses (Fig. 5).

Quite probably, the stimulatory presynaptic action of the incubate on synapses the effectiveness of which was lowered during fatigue, could account for the PRF effect.

Presynaptic actions of the incubate (both stimulatory and inhibitory) were predominantly due to respective modulation of the size of the store of quanta immediately available for release (binomial parameter n), with no change in quantum release probability, i.e. in binomial parameter p (see Results). An alteration of parameter n can be expected to reflect a modification of processes of quanta mobilization (Elmqvist and Quastel 1965) or vesicle exocytosis and recycling (Heuser and Reese 1973). Thus, a positive feed-back regulation of these processes could provide stabilization of presynaptic function, saving resources or setting the function to more effective performance at fatigue conditions.

Similar feed-backs mediated by antidromic factors operate also in unfatigued preparations including Mg^{2+}-blocked ones (Matyushkin et al. 1978, 1984; Ma-

tyushkin 1989). One and the same endogenous factor can act as a presynaptic stimulator or inhibitor (mediating positive or negative feed-back, respectively) depending on its concentration, which, in turn, depends on the level of synaptic activity (EPP quantal content). Such a mechanism has been well documented, for example, for endogenous K^+ (Matyushkin et al. 1978, 1984). Many endogenous peptides may produce opposing physiological effects depending on their concentrations (e.g. Schotman et al. 1985).

If the muscle incubate, used as an external medium, contains factors similar to those present in the tested preparation, the "exogenous" factors of the incubate can be expected to sum up with the endogenous ones. The resulting effect of the incubate would then depend on the final concentration of these factors in extracellular spaces and the synaptic microenvironment, which may vary consistently in individual preparations. This might account for the observed asymmetry of the incubate action on synapses with different m_0 and n_0 (Fig. 5) as well as for the variability of the effects observed in the fatigue experiments (see Results).

The observed stimulatory presynaptic action of LMF correlated with the concentration of HIS-containing substances which were mainly of non-protein, low-molecular nature (see Table 1).

It should be mentioned that the mode of LMF action, namely increasing of EPP quantal content due to an increase in binomial parameter n, is characteristic of exogenous HIS (Shabunova 1977). This fact taken together with the correlation mentioned above could bring forward an attractive presumption that HIS-containing substances mediate both the stimulatory presynaptic-, and the anti-fatiguing actions of the incubate. However, we cannot exclude that some other low-molecular components play a role in mediating these effects. In this case the observed correlation could reflect a relationship between the effect and the concentration of some low-molecular factor(s) which may vary in different incubates in parallel to HIS-containing substances.

Factors mediating the PRF effect appeared to act within a rather narrow concentration range: they lost their activity as soon as after 5-fold dilution of the incubate. On the other hand, raising the concentrations of the incubate constituents (by ca. 3 times, see Results) resulted in a reverse effect, namely in a deepening of fatigue.

It can be suggested that both opposite effects are induced by the same factor(s) acting in an opposite manner at different concentrations (see above).

Alternatively, the incubates may contain some other components which would not affect synaptic transmission at low concentrations but would exert a depressant effect at higher concentrations, acting on a non-synaptic target(s). We have no direct data to support synaptic or non-synaptic action of the "concentrated" incubate. However, we could demonstrate that the non-concentrated incubate, especially its HMF contained substances (most probably proteins) exerted inhibitory

action on excitation-contraction coupling or contractile apparatus of muscle fatigued by direct stimulation (Fig. 4).

The question arises, why the last mentioned action was lacking (or markedly reduced) in experiments with neurally stimulated muscles, even those treated with HMF, the fraction causing profound contraction decrease in muscles stimulated directly (cf. Fig. 2 and 4). One plausible explanation is as follows.

As noted above, in experiments with direct muscle stimulation the contraction decline at the plateau phase was mainly due to excitation-contraction uncoupling in a large fraction of muscle fibres (Edman and Lou 1992). In the remaining fibres, still active, the safety factor of action potential generation in T-tubular membranes can be expected to be reduced due to K^+ accumulation in the tubular spaces (Hník et al. 1976; Juel 1986; Renaud and Light 1992). It can be suggested that some proteins of the external incubate (especially when HMF is used) might be come adsorbed on the mouths or walls of T-tubules. As a result, a slight increase in external resistance can occur, which would be enough for complete blockade of action potential propagation from surface to tubular membrane, if the safety factor of the last membrane was lowered to just suprathreshold levels.

On the contrary, in the case of neurally fatigued preparation the muscle fibres in a large part of motor units are kept unfatigued (with intact electrical properties) due to partial presynaptic blockade. Thus, the above described uncoupling mechanism can be expected to become inoperative in this situation. Whatever the mechanism, the same reasoning seems to be true if the inhibitory action of the incubate on muscle elements requires a fatigue-induced alteration in the properties of these elements.

In conclusion, the results of the present study have shown a complex influence of muscle metabolites, present in the incubation medium of a resting muscle on presynaptic function as well as on muscular elements of motor units. Such an influence could play a role in the regulation of the motor units functioning and of muscle performance in fatiguing conditions. For the determination of the nature of the active substances and of the cellular mechanisms of their actions, experimental conditions are to be created in which the observed stimulatory and inhibitory effects are reliably separated. Efforts in this direction are under way.

Acknowledgements. This work was supported in part by Grant No. 94-04-12118a from Russian Foundation for Basic Research.

References

Boldyrev A. A. (1977): Biochemical Aspects of Excitation-Contraction Coupling. Moscow University Publishing, Moscow (in Russian)

Crush K. G. (1970): Carnosine and related substances in animal tissues. Comp. Biochem. Physiol. **34**, 3—30

Darbre A., Clamp J. (1986): Analytical Methods. In: Practical Protein Chemistry (Ed. A. Darbre), 243—363, J. Wiley & Sons Ltd. Chichester- N.-Y.-Brisbane- Toronto-Singapore

Drabkina T. M., Shabunova I. A., Matyushkin D. P., Gankina E. S., Efimova I. I. (1986): β-Alanine release from frog muscle determined by thin-layer chromatography. Byull. Eksper. Biol. Med. **101,** 38—41

Drabkina T. M., Kubasov I. V., Matyushkin D. P., Romanovsky D. Yu., Yurkyanets E. A. (1988): Some biochemical characteristics of muscular perfusate and its influence on transmitter release in neuromuscular synapse. Byull. Eksper. Biol. Med. **105,** 460—462

Drabkina T. M., Radzjukevich V. K., Shtanko S. A. (1990): Investigation of carnosine effect on synaptic transmission in the frog neuromuscular junction. Fiziol. Zh. SSSR **76,** 18—25 (in Russian)

Dupin A. M., Stvolinsky S. L. (1986): The change of carnosine content in muscles working at different patterns of activity. Biokhimiya **51,** 160—163 (in Russian)

Edman K. A. P., Lou F. (1992): Myofibrillar fatigue versus failure of activation during repetitive stimulation of frog muscle fibres. J. Physiol. (London) **457,** 655—673

Elmqvist D., Quastel D. M. J. (1965): A quantitative study of end-plate potentials in isolated human muscle. J. Physiol. (London) **178,** 505—529

Gaudri-Tallarmain I. M. (1986): The effect of lactate on acetycholine release evoked by various stimuli from Torpedo synaptosomes. Europ. J. Pharmacol. **129,** 135–143

Heuser J. E., Reese T. S. (1973): Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell. Biol. **57,** 315—344

Hník P., Holas M., Krekule I., Kříž N., Mejsnar J., Smieško V., Ujec E., Vyskočil F. (1976): Work-induced potassium changes in skeletal muscle and effluent venous blood assessed by liquid ion-exchanger microelectrodes. Pflügers Arch. **362,** 85—94

Juel C. (1986): Potassium and sodium shifts during *in vitro* isometric muscle contraction and the time course of the ion-gradient recovery. Pflügers Arch. **406,** 458—463

Kibjakov A. V. (1935): On a humoral factor in the interrelation between nerve and muscle. Proc. Tatar Institute Theor. Clin. Med. **2,** 141—148 (in Russian)

Lowry O. N., Rosenbrough M. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265—275

Lucier G. E., Mainwood G. W. (1972): The measurement of potassium efflux in superfused frog sartorius muscle. Can. J. Physiol. Pharmacol. **50,** 123—131

Matyushkin D. P. (1989): Feed-Backs in the Synapse. "Nauka" Publishing, Leningrad (in Russian)

Matyushkin D. P., Shabunova I. A., Sharovarova G. M., Vinogradova I. M. (1978): On potassium functional feedback in neuromuscular junction. J. Neurosci. Res. **3,** 441—450

Matyushkin D. P., Krivoi I. I., Shabunova I. A. (1984): Reversal potential of end-plate currents and potassium feedback at the neuromuscular synapse. J. Neurosci. Res. **11,** 281—292

Matyushkin D. P., Drabkina T. M., Shabunova I. I. (1986): On the question of the contribution of some muscle metabolites to antidromic regulation of transmitter release from motor nerve terminal. Vestnik Leningradskogo Universiteta Ser. 3, 76—84 (in Russian)

Matyushkin D. P., Krivoi I. I., Drabkina T. M. (1993): The positive action of potassium ions outflow on the neuromuscular transmission. XXXII Congress of the Intern. Union of Physiol. Sciences. August 1st-6th. Glasgow. Abstracts. 244.64/P

Miyamoto M. D. (1975): Binomial analysis of quantal transmitter release at glycerol-treated frog neuromuscular junctions. J. Physiol. (London) **250,** 121—142

Nagesser A. S., van der Laarse W. J., Elzinga G. (1992): Metabolic changes with fatigue in different types of single muscle fibres of *Xenopus laevis*. J. Physiol. (London) **448,** 511—523

Nikolsky E. E., Poletaev G. I. (1977): Study of mechanism of blockade of transmission from nerve to skeletal muscle at low-frequency neuronal stimulation. Neirofiziologiya **9,** 78—85 (in Russian)

Ovadi J., Libor S., Elodi P. (1967): Spectrophotometric determination of histidine in proteins with diethylpyrocarbonate. Acta Biochim. Biophys. Hung. **2,** 455—458

Renaud J. M., Mainwood G. W. (1985): The effects of pH on the kinetics of fatigue and recovery in frog sartorius muscle. Can. J. Physiol. Pharmacol. **63,** 1435—1443

Renaud J. M., Light P. (1992): Effects of K^+ on the twitch and tetanic contraction in the sartorius muscle of the frog, *Rana pipiens*. Implication for fatigue *in vitro*. Can. J. Physiol. Pharmacol. **70,** 1236—1246

Schotman P., Schrama L. H., Edwards Ph. M. (1985): Peptidergic system. In: Handbook of Neurochemistry (Ed. A. Lojtha) **8,** pp. 243—279, Plenum Press, N.-Y.-London

Severin S. E., Vulfson P. L. (1963): The role of carnosine in neurotrophic relations. In: The Effect of Use and Disuse of Neuromuscular Function. (Eds. Gutman E. and Hník P.) pp. 53—62. Elsevier, Amsterdam

Shabunova I. A. (1977): On the presynaptic action of histidine at neuromuscular junction. Fiziol. Zh. SSSR **63,** 964—969 (in Russian)

Shabunova I. A. (1979): The histidine release from the frog skeletal muscles at rest and in rhythmic activity. Fiziol. Zh. SSSR **65,** 1371—1373 (in Russian)

Silinsky E. M. (1980): Evidence for specific adenosine receptors at cholinergic nerve endings. Brit. J. Pharmacol. **71,** 191—194

Smith D. O. (1991): Sources of adenosine released during neuromuscular transmission in the rat. J. Physiol. (London) **432,** 343—354

Westerblad H., Allen D. G. (1992): Changes of intracellular pH due to repetitive stimulation of single fibres from mouse skeletal muscle. J. Physiol. (London) **449,** 49—71

Westerblad H., Lee J. A., Lannergren J., Allen D. G. (1991): Cellular mechanisms of fatigue in skeletal muscle. Amer. J. Physiol. **261,** C195—209

Williams B., Wilson K. (1975): Methods of Chromatography. In: Principles and Technique of Practical Biochemistry (Ed. B. Williams and K. Wilson) pp. 123—134, Edward Arnold, London

Final version accepted April 6, 1995