Is attenuation of tRNA charging responsible for the inhibition of protein labelling accompanying depolarisation of the cerebral cortical cells?

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Summary. Topical application of concentrated solutions of KCl resulted in reduction of charging of tRNA 7 min after s.c. injection of L-(U-^{14}C) leucine. Neither radioactivity nor pool size of the total amino acids changed during this period.

Inhibition of protein labelling in the cerebral cortex by depolarizing agents, particularly potassium ions, has been repeatedly demonstrated by in vivo[1-3] and in vitro[4-7] experiments. The effect is evenly distributed among the proteins of the subcellular[3] and submitochondrial[8] fractions and between the bodies of neurons and glial cells[9] suggesting a general, nonspecific effect. Depolarisation is accompanied by decrease of the high energy phosphates and by stimulation of aerobic glycolysis and oxidative metabolism[10-12]. Inhibition of protein labelling may come from a preferential utilisation of high energy phosphates for the restoration of membrane polarity at the expense of other energy requiring processes like protein synthesis.

The aim of the present study was to estimate the incorporation of a labelled amino acid into the tRNA fraction of the cerebral cortex in vivo under the influence of topically applied potassium ions. Since the size of tRNA pool is very small and its turnover high, short-time (7 min) interval after injection of (^{14}C) leucine was used.

Experiments were performed on the male adult hooded rats (Druckrey strain). Under pentobarbital anaesthesia (40 mg/kg, i.p.), trepan openings 4 mm in diameter were made above the parietooccipital regions of both hemispheres. To the experimental animals, a piece of filter paper (3 × 3 mm) soaked with 3.2 M KCl was applied into the trepan opening of both hemicortices 20 min before killing. Saline was applied to the control rats in the same way. 7 min before decapitation of rats, 0.5 ml saline containing 15 or 25 μCi L-(U-^{14}C) leucine (s.a. 125 mCi/mmole, from the Institute for Research, Production and Application of Radioisotopes, Prague) was administered s.c. In a cold room the brains were quickly dissected out and hemicortices prepared on an ice-chilled petri dish were stored in liquid nitrogen. 2 hemicortices were pooled for each type of biochemical analysis, primarily because of low radioactivity in tRNA. Transfer RNA fraction was isolated from the frozen pulverized samples by a cold phenol method by a slightly modified procedure[13]. Briefly, after homogenisation in a mixture of phenol followed by treatment with amylalcohol in chloroform, RNA was precipitated with ethanol. 1 M NaCl in acetate buffer, pH 5.0 was used for extraction of low molecular RNA, largely tRNA. Absorption at 260 nm and radioactivity[14] were measured in separate aliquots. Specific radioactivity of proteins[15,16] and of the total amino acids[14,17] were measured in the contralateral hemicortices.

In the control experiments[18], efficiency of 1 M NaCl extraction of tRNA was verified. Protein contamination of tRNA fraction was checked by addition of radioactive brain proteins to the nonradioactive frozen pulverized tissue in the phenolacetate buffer mixture before homogenisation. No radioactivity was detected in the final solution of tRNA. A small variability of specific radioactivity of the charged tRNA related to that of the total free amino acids reveals very small variations inspite of different amounts of the labelled amino acid used, suggesting that tRNA is in equilibrium with free amino acids (table).

In the potassium-treated cortex, the ratio of radioactivity bound to tRNA to that of the free amino acids is 24% lower than in the control cortex (table). Transport of amino acids is obviously not affected as shown by their unchanged specific radioactivity. Neither is their pool size altered (23.5±0.9 μmoles/mg protein in the K^+-treated cortex, 23.9±1.3 in controls). Protein labelling is inhibited to a similar extent as reported earlier[3]. It appears that aminoacylation of tRNA is inhibited. This can be demonstrated only for short labelling pulses, when tRNA saturation by the radioactive amino acid is not yet completed. At 15-min interval, no difference between tRNA bound radioactivities can be detected. Protein labelling remained decreased to the same extent at at 7 min, providing further evidence of absence of protein contamination in the tRNA fraction[18]. Both percentage of uncharged tRNA in vivo and aminoacylation capacity measured in vitro are unaffected by potassium ions applied under conditions described in the present communication[18]. Thus the present results support the hypothesis that tRNA charging is attenuated. It is probably due to a shortage of ATP. Involvement of other energy-requiring steps of the protein synthesis cannot be excluded, however.

The notion about the mechanism of inhibition of protein synthesis induced by depolarisation as presented here, implies a kind of regulatory mechanism by which a cell is able to produce energy preferentially for 1 compartment and/or to redistribute utilisation of existing energy reserves in favour of maintaining 'more essential' function at the expense of a 'less important' one (s).

Effect of potassium ions on the labelling of tRNA, total amino acids, and proteins after injection of (^{14}C)leucine

	Control	K^+	Difference (%)	p
tRNA $\left(\frac{\text{dpm}/\mu\text{g}}{\text{dpm}/\mu\text{mole am.a.}}\right)$	39.1±0.7 (n=6)	29.8±1.4 (n=6)	−24	<0.003
Total amino acids (dpm/μmole)	615±42 (n=12)	608±47 (n=12)	−1	n.s.
Proteins (dpm/mg)	58.9±6.1 (n=12)	41.4±4.4 (n=12)	−30	<0.05

For the statistical evaluation Student's t-test was used.

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Metal binding to myosin and to myosin DTNB-light chain

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Summary. The effects of various divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+} on the intrinsic fluorescence of heavy meromyosin (HMM) and myosin 5,5′-dithio-bis-(2-nitrobenzoate) DTNB-light chain of rabbit striated muscle, are compared. At pH 6.4, the fluorescence change induced by the metal ions is present only in the isolated light chain and disappears in HMM, thus indicating an interaction between the heavy and light chains with respect to the binding of the metal ions. Whereas Mg^{2+} binds more strongly than Ca^{2+} to myosin, this order is reversed in the case of the DTNB-light chain.

In vertebrate striated muscle, it is believed that Ca^{2+}-regulation is controlled by thin filaments components, troponin and tropomyosin[2]. Myosin-linked regulation has been described in molluscan muscle[3] and in vertebrate smooth muscle[4], and in some cases a dual system of regulation, involving both myosin and the thin filaments components, has been found[5]. Some evidence for the existence of myosin-linked regulation in vertebrate striated muscle has also previously been presented[6-9]. This hypothesis is supported by the finding that striated muscle myosin and its fragment, heavy meromyosin (HMM), bind Mg^{2+} [10,11] and Ca^{2+} [12,11] with dissociation constants in the micromolar range. Because tight binding sites of Ca^{2+} are also present on the light chains of myosin which can be released by 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB-light chains), it has been suggested[13] that they might constitute part of the regulatory system of striated muscle. Evidence in favor of this assumption came from studies[14] in which it was shown that the DTNB-light chain from rabbit striated muscle could replace a scallop myosin light chain, which is responsible for Ca^{2+} regulation in this molluscan muscle, and restore the Ca^{2+} sensitivity of the system.

It has previously been speculated[13] that the metal-binding DTNB-light chain might be located at the hinge between subfragment-1 and subfragment-2 of myosin. This hypothesis is supported by the fact that both with trypsin[15,16] and chymotrypsin[17] either subfragment-1 or heavy meromyosin is produced, depending on whether digestion is carried out in the presence of EDTA or divalent cations. Recent work[18,19] has established that saturation of the DTNB-light chain site on myosin with divalent ions protects this subunit against proteolysis, and this in turn inhibits the cleavage of the heavy chain at the subfragment-1–subfragment-2 hinge. The present work describes the effect of Ca, Mn and Mg divalent ions on the intrinsic fluorescence of rabbit myosin DTNB-light chain and of HMM. The binding constants of these metal ions to myosin, HMM and the DTNB-light chain are compared in an attempt to evaluate the role of the light chain in metal-binding and regulation in the thick filaments.

Table 1. Effect of metal ions or metal substrate complexes on the intrinsic fluorescence of HMM[a]

Metal ion or metal-substrate complex	$-\Delta I/I$[b] pH 7.4[c] (%)	pH 6.4[d] (%)
Mg^{2+}	6.2	0.5
Ca^{2+}	6.7	0.3
Mn^{2+}	5.7	-
Mg-ATP	−17.0	−17.0
Mn-ATP	−14.3	−17.0

[a]HMM concentration 134 μg/ml; [b]decrease of tryptophan fluorescence at maximum of emission spectrum, $\lambda = 350$ nm (uncorrected spectrum); [c]in 50 mM Hepes (N-hydroxyethylpiperazine-N′-ethane sulfonic acid) buffer, final pH 7.41; [d]in 50 mM cacodylate buffer, final pH 6.35.

Materials and methods. Myosin was extracted from the back muscles of white rabbits. Myosin and HMM were prepared as previously described[20]. DTNB-light chains were purified by preparative gel electrophoresis[21] from a crude light chain extract obtained by urea treatment of myosin[22]. All reagents were of analytical grade. Double distilled water was used throughout.

Fluorescence measurements were performed at 25 °C as previously described[13]. The excitation wavelength used was 295 nm, so as to ensure that only tryptophan residues were excited[20]. The difference spectra induced by metal ions were similar to those previously published, with no shift in wavelength of emission in the case of the DTNB-light chain[13] and with a small blue shift in the case of HMM[11]. The fluorescence effects were expressed as $\Delta I/I$ values (in %), the ratio of the changes in fluorescence intensity at the peak of the spectrum, induced by the metal ions, ΔI, to the intensity measured in their absence, I. The correction for dilution effects (< 5%) was estimated by the addition of aliquots of a stock solution of metal ion to a tryptophan solution. The observed fluorescence intensities were compared to that of a tryptophan solution used as an external standard to correct for source intensity fluctuations. The concentration of HMM was 134 μg/ml ($A_{295} < 0.03$) and that of the DTNB-light chain-120 μg/ml ($A_{295} < 0.02$).

Results and discussion. The effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on the intrinsic fluorescence of HMM is shown in table 1. At pH 7.4, all 3 metal ions induce a quenching of about 6% of the fluorescence, whereas the effect is negligible at pH 6.4. However, as also shown in table 1, there is no decrease in the magnitude of the fluorescence enhancement due to ATP, by lowering the pH from 7.4 to 6.4 with either Mg^{2+} or Mn^{2+}. This is evidence that the pH lowering did