

EFFECTS OF Ca^{2+} AND Mg^{2+} ON RAPID AXONAL TRANSPORT OF PROTEINS IN VITRO IN FROG SCIATIC NERVES

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INTRODUCTION

Rapid axonal transport is likely to be dependent upon the integrity of microtubular systems (see reviews by Samson, 1971; Ochs, 1972). Actomyosin-like protein has been isolated from the brain (Berl et al., 1973). In a hypothesis for fast axonal transport, Ochs (1972) suggested the existence of axonal transport filaments, which would slide along microtubules by means of cross-bridges activated by ATP. In analogy with muscle contractility the importance of Ca^{2+} was suggested. In these circumstances it seemed of interest to study the effects of Ca^{2+} and Mg^{2+} on fast axonal transport.

MATERIALS AND METHODS

The Transport System

An in vitro system from the frog *Rana temporaria* was used. The preparation, which consists of the dorsal ganglia (nos. 8 and 9), the sciatic nerve, and the gastrocnemius muscle, was placed in frog Ringer solution in an incubation chamber with three compartments (A, B, C). The parts were separated from each other with silicone grease barriers. [^3H]Leucine was added to the ganglionic compartment (compartment A), which made it possible to follow the transport of labeled proteins from the ganglia, along the sciatic nerve (compartment B) towards the muscle (compartment C). Application of the silicone grease barrier between the nerve and the muscle compartments was not necessary for the experiments described in this paper. The preparation and incubation of the system has earlier been described in detail (Edström and Mattsson, 1972). It was demonstrated that proteins synthesized in the ganglia were transported within the axons in anterograde direction at a rate of 127 ± 10 mm/day at 18°C (Edström and Mattsson, 1972; Edström and Hanson, 1973). The two paired preparations from the same animal were used in each experiment. One preparation served as a control and was incubated in a standard Ringer solution while the contralateral preparation was incubated in a modified Ringer's. A ligature was placed at the middle level of the nerve and the preparation was incubated for 17 h at 18°C . After treatment with trichloroacetic acid (TCA) the distribution of TCA-insoluble labeled components in the ganglia and along the nerve was determined as described previously (Edström and Mattsson, 1972; Anderson et

al., 1972). Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) dissolved samples in a 0.55% Permablend III (Packard Instrument Co.) solution in toluene were analyzed for radioactivity with a Packard TriCarb (model 3375) liquid scintillation spectrometer.

Atomic-Absorption Spectrophotometry

Nerves exposed to elevated levels of Ca^{2+} were washed twice, 20 s each time, with standard Ringer's before wet ashing. After wet ashing of nerves in concentrated H_2O_2 , calcium was determined in a LaCl_3 solution by the use of a Perkin-Elmer model 303 atomic-absorption spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Contents were referred to initial wet weights of nerves.

Electron Microscopy

Preparations which had been incubated in modified Ringer's were rinsed twice, 30 s each time, with standard Ringer's immediately before fixation. The fixation solution consisted of purified glutaraldehyde and freshly prepared formaldehyde in a cacodylate buffer (Karnovsky, 1965) containing 0.5% (vol/vol) dimethylsulfoxide. The specimens were postfixed in cacodylate-buffered osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Epon. Sections, $1 \mu\text{m}$ thick, were prepared on an LKB Pyramitome or an LKB Ultratome III ultramicrotome and examined in a light microscope. Thin sections were prepared from selected areas and double stained with uranyl acetate and lead citrate before the examination in a Siemens 1 A electron microscope.

Chemicals

Aqueous solutions of L-[4,5- ^3H]leucine (36–50 Ci/mmol, 1 mCi/ml) were purchased from The Radiochemical Centre, Amersham, England. The standard Ringer solution had the following millimolar composition: NaCl, 111.2; KCl, 1.9; MgCl_2 , 1.6; CaCl_2 , 1.1; NaHCO_3 , 2.4; and glucose, 5.5. Ca^{2+} -high and Mg^{2+} -high Ringer's were obtained by isotonic substitution of 10–30 mM CaCl_2 and of 20 mM MgCl_2 for NaCl ($78.2 \text{ mM } \text{CaCl}_2 = 111.2 \text{ mM NaCl}$, $78.7 \text{ mM } \text{MgCl}_2 = 111.2 \text{ mM NaCl}$). The pH of the various Ringer solutions was adjusted to 7.4 by adding small amounts of HCl. The solutions were gassed with O_2 before use. Ethylene glycol-bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Transport in Ca²⁺- and Mg²⁺-Free Ringer's

The preparations were incubated for 17 h at 18°C with [³H]leucine present in the ganglionic compartment (compartment A). At the beginning of the incubation a ligature was placed on the nerve (compartment B), 30 mm from the ganglia. Standard Ringer solution was used in the ganglionic compartment. The distribution of protein-incorporated radioactivity in control nerves, perfused with standard Ringer solution, was compared with that in paired nerves perfused with Ca²⁺-free Ringer's containing 1 mM EGTA. The ganglionic part of the test preparation was incubated in standard Ringer solution, in order not to affect the protein synthesis in the neuronal perikarya in the ganglia. The high radioactivity recovered in front of the ligature (Fig. 1) is due to a damming-up of

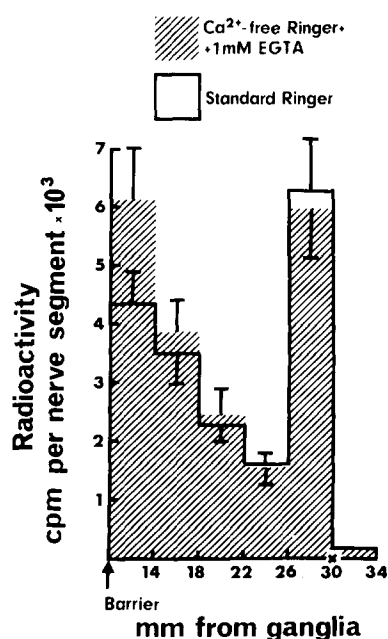


FIGURE 1 The effect of Ca²⁺-free Ringer's, containing 1 mM EGTA, on the transport of [³H]leucine-labeled TCA-insoluble material in the sciatic nerve. The preparations were incubated for 17 h at 18°C. The two preparations from the same animal were used, one serving as a control and the other as a test preparation. Compartment A contained 10 μ Ci [³H]leucine (38 Ci/mmol) in 1 ml standard Ringer's. Compartments B and C of the control were perfused with standard Ringer's and of the test preparation with Ca²⁺-free Ringer's. The Ca²⁺-free Ringer's contained 1 mM EGTA. The position of the ligature is shown (X). Each bar represents the mean of 17 experiments. The vertical lines indicate the SEM.

proteins transported within the axons towards the muscle at a rate of 127 mm/day (Edström and Mattsson, 1972; Edström and Hanson, 1973). As can be seen from Fig. 1 the transport function was not impaired after perfusion of the nerve for 17 h with Ca²⁺-free Ringer's containing 1 mM EGTA. During these conditions the total content of Ca⁺⁺ in the nerves dropped to about 19% of that of control nerves perfused with standard Ringer's (Table I). Raising the EGTA concentration to 5 mM in four other experiments (not shown) did not affect the transport.

To test if the presence of Mg²⁺ in the Ringer's maintained the transport function, nerves were perfused with Ringer solution containing 1 mM EGTA but deprived of both Ca²⁺ and Mg²⁺. Three such experiments were performed. There was in no case more than a slight decrease in the amount of accumulated radioactivity in the test as compared with the control preparation of the same animal.

Transport in Ca²⁺- and Mg²⁺-High Ringer's

The ganglia were exposed to standard Ringer's and the nerve to a Ringer solution containing 20 or 30 mM Ca²⁺ (Fig. 2). In other respects the preparations were treated as described earlier. While 20 mM Ca²⁺ had no effect on the transport, as judged by the accumulation of TCA-insoluble radioactivity in front of the ligature, the presence of 30 mM Ca²⁺ inhibited the amount of accumulated radioactivity by about 40.0% ($P < 0.005$, $n = 14$, P values in this and following experiments obtained by the use of a paired-sample two-tailed t test).

If both the ganglia and the nerve were exposed to elevated levels of Ca²⁺ the transport inhibitory effect was more pronounced (Fig. 3). Whereas a 10 mM concentration of Ca²⁺ had no significant effect, the transport was inhibited by 27.2% by 15 mM Ca²⁺ ($0.02 < P < 0.05$, $n = 8$). The protein synthesis in the ganglia was not affected by 15 mM Ca²⁺ (101.9% of controls, P , NS, $n = 8$). At 20 mM Ca²⁺, which had no effect when it was present exclusively in the nerve compartment, the amount of accumulated TCA-insoluble radioactivity was reduced by 68.0% ($P < 0.005$, $n = 15$). Since, however, 20 mM Ca²⁺ inhibited leucine incorporation into ganglionic protein by 35.0% ($P < 0.005$, $n = 15$) the real transport inhibition was about 33%.

In contrast to the effects with elevated Ca²⁺ concentrations, the presence of 20 mM Mg²⁺ in both the ganglionic and the nerve compartments did not influence the transport (Fig. 4). Nor did the

TABLE I
Calcium Content of Sciatic Nerves Exposed to Different Concentrations of Ca^{2+}

Incubation conditions	Calcium content $\mu\text{mol/g}$ wet weight nerve
Nonincubated nerves, analyzed after isolation	2.10, 2.19
Standard Ringer's (1.1 mM Ca^{2+})	$1.72 \pm 0.05^*$
Ca^{2+} -free Ringer's + 1 mM EGTA	$0.35 \pm 0.03^*$
20 mM Ca^{2+} Ringer's	$13.92 \pm 0.18^*$
20 mM Ca^{2+} + 20 mM Mg^{2+} Ringer's	$15.47 \pm 0.27^*$

Nerves were perfused with different Ringer solutions for 17 h at 18°C and subsequently analyzed as described in the text.

* The values are means \pm SEM of four experiments.

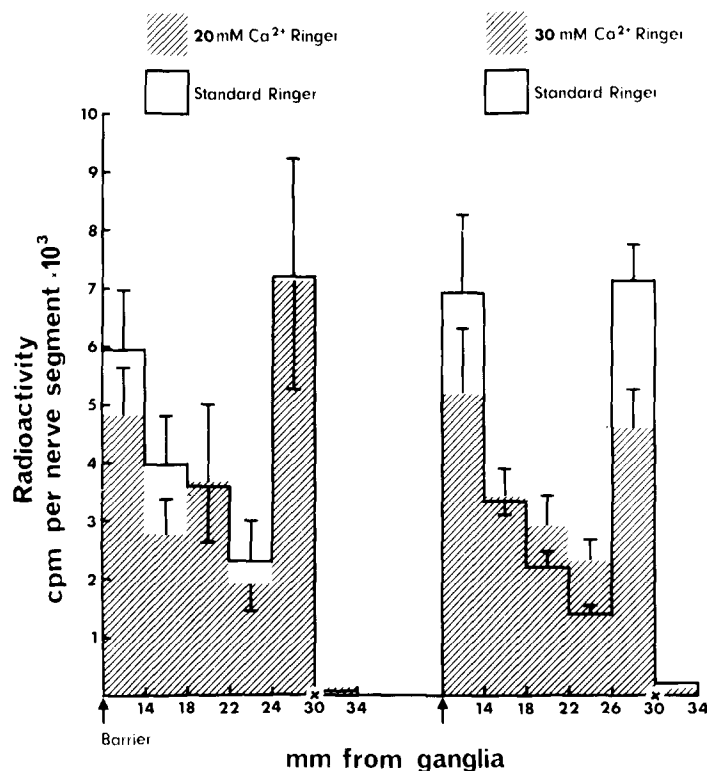


FIGURE 2 The effect of Ca^{2+} -high Ringer's on the transport of $[^3\text{H}]$ leucine-labeled TCA-insoluble material in the sciatic nerve. The nerve, but not the ganglia, was exposed to Ca^{2+} -high Ringer's. The preparations were incubated for 17 h at 18°C . The two preparations from the same animal were used, one serving as a control and the other as a test preparation. Compartment A contained $10 \mu\text{Ci}$ $[^3\text{H}]$ leucine (38 Ci/mmole) in 1 ml standard Ringer's. Compartments B and C of the control contained standard Ringer's and of the test preparation Ca^{2+} -high Ringer's. The position of the ligature is shown (X). Each bar represents the mean of six (20 mM Ca^{2+}) and of 14 (30 mM Ca^{2+}) experiments. The vertical lines indicate the SEM.

admixture of 20 mM Mg²⁺ in six other experiments (not shown) potentiate the transport inhibition caused by 20 mM Ca²⁺.

Electron Microscopy

The structure of ganglia and sciatic nerves incubated under standard conditions has previously been described (Edström et al., 1973). Swelling of profiles of endoplasmic reticulum, of Golgi complex, and of mitochondria could occasionally be observed, but the number of 250-Å microtubules and of 100-Å filaments remained remarkably constant after incubation for 17 h. In the present experiments ultrastructural changes were observed in perikarya of test preparations incubated for 17 h with 20 mM Ca²⁺. Microtubules, which were rather numerous in cell bodies of controls, were reduced in number in most cells exposed to 20 mM Ca²⁺. In the latter an increased number of 100-Å

filaments, often arranged in dense strands or bundles, were observed. After Ca²⁺ treatment the Golgi complex in several cell bodies showed more profiles and the granular endoplasmic reticulum, often split into short tubulosaccular fragments, a more even distribution. The nerve axons, on the other hand, showed less conspicuous ultrastructural changes after exposure to 20 mM Ca²⁺. In the latter a moderate reduction in the number of microtubules and possibly an increase in the number of 100-Å filaments were observed in scattered axons. However, more definite information of the possible effects of Ca²⁺ on axonal structures must await further and extended electron microscope studies.

DISCUSSION

Ca²⁺ is likely to play a key role in maintaining membrane structure, and to be the coupling factor

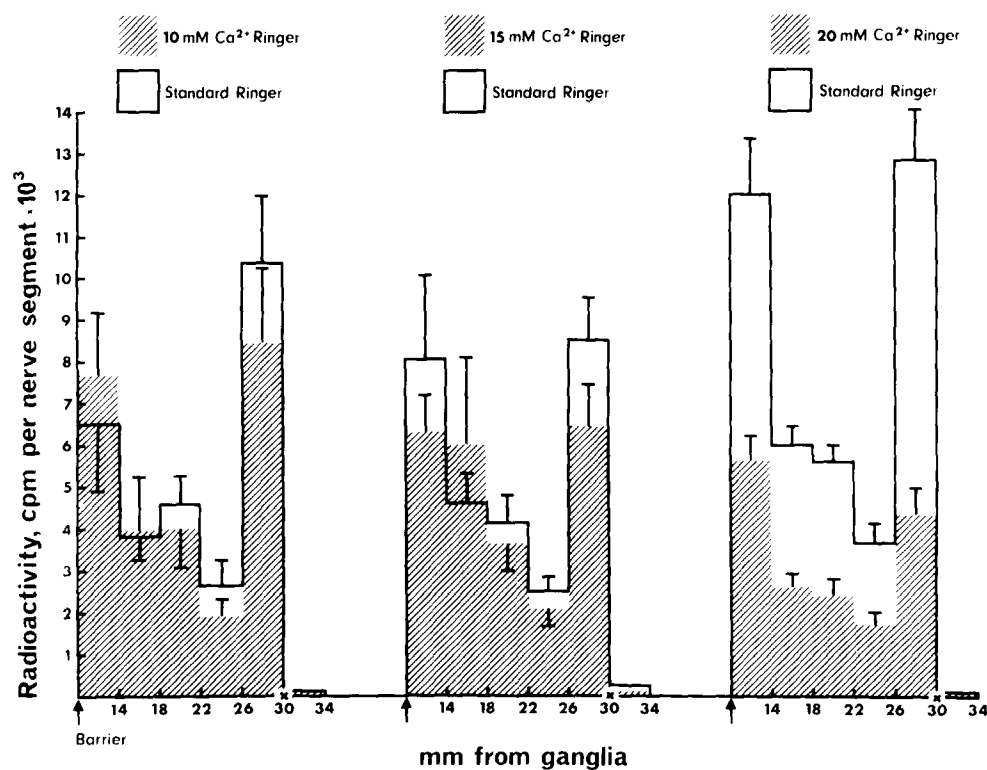


FIGURE 3 The effect of Ca²⁺-high Ringer's on the transport of [³H]leucine-labeled TCA-insoluble material in the sciatic nerve. The nerve and also the ganglia were exposed to Ca²⁺-high Ringer's. The preparations were incubated for 17 h at 18°C. One preparation was incubated with standard Ringer's and the other with Ca²⁺-high Ringer's in the three compartments (A, B, C). Compartment A contained 10 μCi [³H]leucine (50 Ci/mmol) in 1 ml standard Ringer's or Ca²⁺-high Ringer's. Each bar represents the mean of six (10 mM Ca²⁺), eight (15 mM Ca²⁺), and 15 (20 mM Ca²⁺) experiments. The vertical lines indicate the SEM.

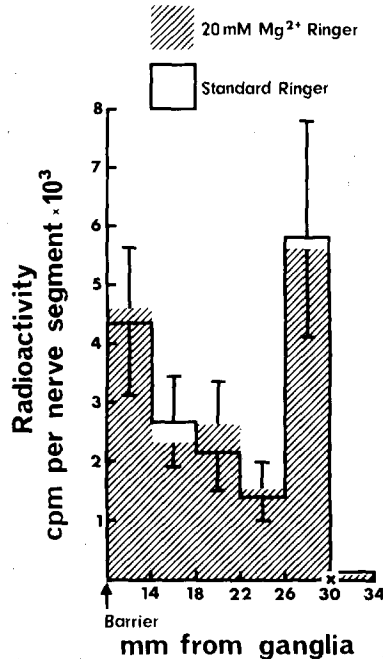


FIGURE 4 The effect of Mg²⁺-high Ringer's on the transport of [³H]leucine-labeled TCA-insoluble material in the sciatic nerve. Conditions as described in Fig. 3, except that Mg²⁺-high Ringer's instead of Ca²⁺-high Ringer's was used. The position of the ligature is shown (X). Each bar represents the mean of four experiments. The vertical lines indicate the SEM.

between excitation and contraction in muscle and also between excitation and secretion in many types of secretory cells (for review see, Cuthbert, 1970). There is a growing body of evidence that the rapid axonal transport is dependent upon the integrity of the microtubular system (see reviews by Samson, 1971; Ochs, 1972). Considering the importance of Ca²⁺ in many cellular mechanisms, some of which may also involve microtubules (Perris and Whitfield, 1967, Goldman and Rebhun, 1969; Rasmussen, 1970), the present results, which show a lack of dependence of rapid axonal transport on low external Ca²⁺ and Mg²⁺, may seem surprising. In spite of perfusion of the nerves for 17 h with a Ca²⁺-free solution containing a strong Ca²⁺ chelator (EGTA), there was no effect on axonal transport. Even if both divalent ions, Ca²⁺ and Mg²⁺, were excluded from the perfusion medium, still containing EGTA, there was only a slight, if any, inhibitory effect. If there was some effect, it is most likely to be an indirect result of the general importance of divalent ions for cell

function and can not be linked to the transport mechanisms as such. The present results are interesting to compare with those of Kirkland and Burton (1972), who recently reported that extracellular Ca²⁺ is probably not involved in the stabilization of neurites and microtubules of neuroblastoma cells. The possible importance of intracellular Ca²⁺ in the mechanism was emphasized. If the present results show that external Ca²⁺ is not a requirement for rapid axonal transport, the next question, whether it depends on intracellular Ca²⁺ or not, may be more difficult to answer. After perfusion for 17 h in a Ca²⁺-free solution containing EGTA the content of total Ca²⁺ of the nerves dropped by about 81%. Considering the long time of perfusion, the remaining Ca²⁺ would be expected to be strongly bound and possibly to exist in chelated form. The latter is uncertain and a possible role of remaining Ca²⁺, not mobilized during the experimental conditions, can not yet be excluded.

The absence of effects in Ca²⁺-free medium contrasted with the inhibitory effects by elevated levels of external Ca²⁺. A significant transport inhibitory effect was caused by 15–20 mM Ca²⁺ in the medium surrounding the ganglia and the nerve. First, by raising the Ca²⁺ concentration to about 30 mM a corresponding effect was observed when only the nerve was exposed to Ca²⁺-high Ringer's. The differential sensitivity needs an explanation. The difference could be due to different effective penetration barriers to the movement of Ca²⁺ into the cell compartments of the nerve and of the ganglia. Against this argues the high content of Ca²⁺ recovered in nerves exposed to Ca²⁺-high Ringer's. The concentration of Ca²⁺ of the nerves (microgram/gram wet weight) then attained a value of about 75% of that in the incubation medium (microgram/milliliter). Nervous tissue normally tends to maintain a low level of free Ca²⁺ (Stahl and Swanson, 1971). The considerable rise of Ca²⁺ could reflect a partial breakdown of the barriers or an accumulation of Ca²⁺ in mitochondria or other structures. The difference in transport inhibition by Ca²⁺ in the medium surrounding the ganglia and the nerve could alternatively be due to the presence of microtubules of different stability to Ca²⁺ in the two tissue compartments. In the light of the finding of inhibited microtubule polymerization in cell-free solutions by Ca²⁺ but not by Mg²⁺ (Weisenberg, 1972), it is tempting to mention another possibility. The transport inhibitory effect caused by Ca²⁺ could be a consequence

of its interaction with the assembling of microtubules which mainly could take place in the nerve cell bodies. The morphological effects caused by Ca^{2+} are not inconsistent with this possibility. Electron microscopy showed a much larger decrease in the number of 250-Å microtubules in the perikarya than in the nerve axons after exposure to Ca^{2+} -high Ringer's.

Schlaepfer (1971) reported drastic morphological changes of rat sciatic nerves exposed to various concentrations of Ca^{2+} . With the addition of 2 or more mM of CaCl_2 to the incubation media there was a complete granular transformation of neurofilaments and microtubules in all myelinated fibers. Fixation of the nerves took place in a medium containing elevated levels of Ca^{2+} . In the present experiments the preparations were rinsed in standard Ringer's immediately before fixation in a Ca^{2+} -free medium. It seems more likely that the discrepancies in morphological effects caused by Ca^{2+} in the two investigations reflect differences in methodology, i.e., presence and absence of Ca^{2+} during fixation, rather than a true difference between the two species studied. It is interesting to note that high Ca^{2+} in the present study produced effects which remind one of those caused *in vivo* by colchicine in several systems, i.e. loss of microtubules and a proliferation of neurofilaments (see review by Wuerker and Kirkpatrick, 1972).

SUMMARY

Effects of Ca^{2+} and Mg^{2+} on rapid axonal transport of proteins were studied *in vitro* in frog sciatic nerves. The transport of [^3H]leucine-labeled proteins was followed from the dorsal ganglia, along the sciatic nerve towards the muscle. Perfusion of the nerve for 17 h with Ca^{2+} -free Ringer's containing 1 mM EGTA reduced the Ca^{2+} level to 19% of that of control nerves but did not affect the rapid axonal transport. Nor was the transport influenced by removal of both Ca^{2+} and Mg^{2+} from the medium. In contrast the transport was partially inhibited by elevated levels of Ca^{2+} but not by Mg^{2+} . The transport inhibitory effect by Ca^{2+} was more pronounced if the ganglia, than if exclusively the nerve axons, were exposed to Ca^{2+} -high Ringer's.

Electron microscopy of nerves treated with elevated levels of Ca^{2+} showed a reduced number of 250-Å microtubules and an increased number of 100-Å filaments. The changes were more pronounced in the perikarya than in the nerve axons.

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