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THE EFFECTS OF THE CALCIUM IONOPHORE, A23187, ON THE AXOPLASMIC TRANSPORT OF DOPAMINE β-HYDROXYLASE

E. ESQUERRO, A.G. GARCIA & P. SANCHEZ-GARCIA

Departamento de Farmacología y Terapéutica, Facultad de Medicina Autónoma, Arzobispo Morcillo, 1, MADRID-34, Spain

1 The effects of the ionophore, A23187, on the intra-axonal transport of dopamine β-hydroxylase (DBH) were investigated in the cat hypogastric nerve-inferior mesenteric ganglion preparation by monitoring, *in vitro*, the enzyme accumulation above a ligature, 2 to 2.5 cm distal to the ganglion.

2 DBH accumulation in the proximal segment immediately above the ligature (P_1) increased linearly up to 6 h, during incubation in normal Krebs solution at 37°C. The ionophore, A23187, interfered with the enzyme accumulation, but did not modify the previously accumulated DBH activity present in P_1.

3 The blocking effects of A23187 on DBH transport were greatly impaired in the absence of extracellular calcium ions; an excess of calcium in the bathing solution (7.5 mM) itself blocked the enzyme transport by 50%.

4 A23187 did not significantly modify the levels of adenosine triphosphate (ATP) in the segments P_1 and P_2 of the nerve proximal to the ligature.

5 Nerves incubated in an A23187-containing medium showed many mitochondria of normal shape and fine structure; however, typical microtubules or filaments were not seen in these preparations.

6 The results suggest that the ionophore A23187, by considerably raising the axoplasmic ionized calcium levels, interferes with the assembling of microtubules. In this manner, the ionophore would inhibit the transport of adrenergic vesicles and therefore of DBH along the axon. The results also provide additional evidence in favour of the view that for the transport system to work adequately, it is necessary to maintain the intra-axoplasmic ionized calcium concentration between certain critical levels.

Introduction

It is now well established that noradrenaline storage vesicles formed in the perikarya of sympathetic neurones are transported down their axons toward the nerve terminals via a rapid axoplasmic flow system (Banks, Mangnall & Mayor, 1969; Dahlström, 1971; Geffen & Livett, 1971). Since antimitotic agents such as colchicine and vinblastine, which are known to interact with protein subunits of cytoplasmic microtubules (Borisy & Taylor, 1967), inhibit the axoplasmic transport of noradrenaline (Dahlström, 1970; Banks, Mayor, Mitchell & Tomlinson, 1971) it is generally accepted that the axonal microtubular system is probably intimately involved in the process of rapid axoplasmic transport.

Recently it has been shown that the transport of adrenergic vesicles involves an active process which requires energy and is greatly influenced by the extracellular concentrations of sodium and potassium ions (Banks, Mayor & Mraz, 1973; Kirpekar, Prat & Wakade, 1973; García, Kirpekar, Prat & Wakade, 1974). 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 adenosine triphosphate (ATP). In analogy with muscle excitation–contraction coupling, a role for calcium in the above process was postulated. Subsequently, several contradictory reports appeared concerning the role of extracellular calcium on axoplasmic transport *in vitro* (Edström, 1974; Hammerschlag, Dravid & Chiu, 1975; Ochs, Worth & Chan, 1977).

The antibiotic, A23187 (Ely Lilly), acts as a cation carrier by selectively increasing the permeability of artificial and biological membranes to calcium (Reed & Lardy, 1972; Pressman, 1973). In view of the contradictory findings surrounding the role of extracellular calcium on the axoplasmic transport of materials, we studied the effects of the ionophore, A23187, on the axoplasmic transport of DBH, a marker for adrenergic vesicles, in isolated constricted hypogastric

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nerves of the cat. A preliminary report of some of these findings has been published (Esquerro & García, 1977).

Methods

Hypogastric nerve preparation

Cats were anaesthetized with ether followed by chloralose (60 mg/kg, i.v.). The abdomen was opened by a mid-line incision, and the hypogastric nerves were carefully dissected, together with the inferior mesenteric ganglion. The nerve, with the ganglion attached, was then tied with a silk thread about 2 to 3 cm distal to the ganglion. In a group of experiments, cats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and the nerves ligated *in vivo* 2 to 3 cm distal to the inferior mesenteric ganglion; 24 h later the animals were re-anaesthetized with ether plus chloralose and the nerves isolated as described above. The nerve was then placed in an incubation chamber containing 2 ml bathing solution bubbled with 95% O_2: 5% CO_2 at 37°C and incubated for different periods of time. The ionophore was dissolved in ethanol; the final concentration of ethanol in the incubation solution was always less than 0.2%.

Incubation media

The normal incubation medium was Krebs-bicarbonate solution with the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11. This solution was equilibrated with 95% O_2: 5% CO_2, and the final pH was 7.4 to 7.5. Calcium-free or high-calcium, and magnesium-free solutions were made by removal or addition of $CaCl_2$ or $MgSO_4 \cdot 7H_2O$; no osmotic adjustments were made.

Assay of dopamine β-hydroxylase

At the end of the incubation period the nerves were laid against a ruler; sufficient tension was applied to straighten the nerve, which was then cut in 5 mm segments, which starting just proximal to the ligature and going toward the ganglion, were designated P_1 and P_2.

Each individual segment was homogenized in 0.5 ml Tris (5 mM) buffer, pH 6.8 containing 0.2% Triton X-100 and 0.25% bovine serum albumin. The homogenate was centrifuged at 27,000 *g* for 10 min, and DBH was assayed in 0.2 ml of the supernatant according to the method of Goldstein, Freedman & Bonnay (1971) with some modifications (García *et al.*, 1974). In order to allow for any variation in the concentration of endogenous inhibitors of DBH in the nerve homogenate, a sample of purified bovine adrenal DBH (Aunis & Miras-Portugal, 1976) of known activity was always added to one aliquot of homogenate of each nerve in every experiment. The recovery of partially purified DBH added to the various samples was very reproducible and varied between 90 and 100%. Data were not corrected for recovery. DBH activity is expressed as nmol of octopamine formed h^{-1} 5 mm^{-1} segment.

Assay of neuronal adenosine triphosphate

The hypogastric nerves with ganglia attached were incubated at 37°C for a 6 h period in oxygenated Krebs solution or in the presence of A23187, 33 μM. At the end of the incubation period the 1 cm segment ($P_1 + P_2$) immediately proximal to the ligature was homogenized in cold 2 N perchloric acid. The homogenate was centrifuged at 4000 rev/min and aliquots of the supernatants were used to estimate their ATP content by the luciferin-luciferase method described by Stanley & Williams (1969). Results are expressed as nmol of ATP per cm of nerve.

Electron microscopy

The hypogastric nerves were incubated for a 6 h period, at 37°C in normal Krebs or in Krebs solution containing A23187 33 μM. At the end of the incubation period the nerves were fixed at 4°C in Karnowsky solution (1965) for 3 h, washed with collidine buffer 0.2 M, pH 7.4 for 1 h and fixed for 2 h in 2% osmium tetroxide solution in collidine buffer containing 5% $CaCl_2$. After dehydration in ethanol and acetone, the tissues were embedded in Epon 812, and they were cross sectioned at approx. 1 mm proximal to the ligature in ultrathin sections (600 to 800 Å). The sections were stained with uranil acetate and lead citrate (Venable & Coggeshall, 1965) and examined in a Philips 301 electron microscope.

Results

Accumulation of dopamine β-hydroxylase in a section of a nerve immediately proximal to constriction (P_1)

DBH activity was distributed uniformly all along the hypogastric nerve. In 20 nerves the mean DBH activity was 1.50 ± 0.33 nmol h^{-1} 5 mm^{-1}. When the hypogastric nerve with its inferior mesenteric ganglion was incubated at 37°C in normal Krebs solution the accumulation of DBH in the segment immediately proximal to the ligature (P_1) was linear during the 6 h incubation period; no accumulation over basal levels was seen in P_2. DBH accumulated only in P_1. No accumulation was observed in the more proximal seg-

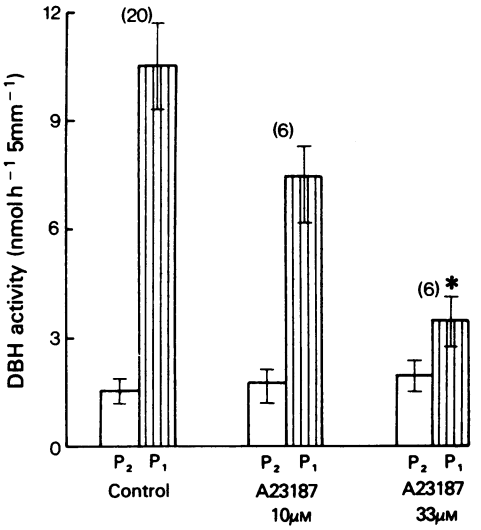

Figure 1 Accumulation of dopamine β-hydroxylase (DBH) activity in P_1 (lined column) during a 6 h incubation period in Krebs-bicarbonate solution (control) and in two concentrations of A23187. Vertical lines represent s.e. means. The number of experiments is given in parentheses. * $P < 0.01$, compared to control.

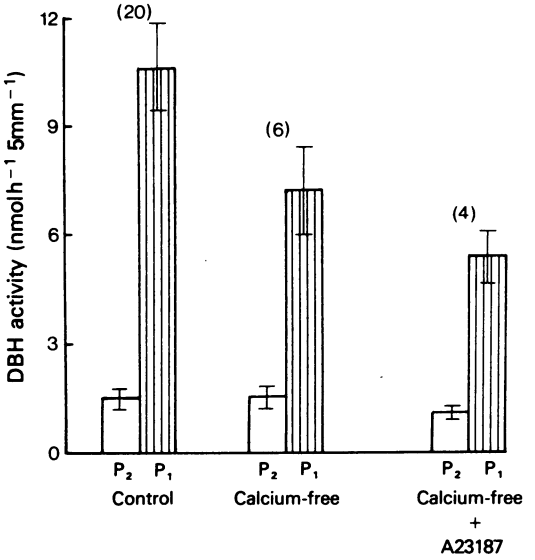

Figure 2 Effect of calcium removal and A23187 on the accumulation of dopamine β-hydroxylase (DBH) activity in P_1 (lined column). Incubation of the nerves was carried out for a 6 h period in normal (control) or calcium-free solution with or without ionophore (33 μM). Vertical lines represent s.e. means. The number of experiments is given in parentheses.

ments in which the DBH values were similar to the basal levels found in non-incubated nerves (García *et al.*, 1974). Therefore, in the experiments described in this paper we analyzed DBH content only in the P_1 and P_2 segments proximal to the ligature. The addition of ethanol up to 0.2% did not affect the rate of accumulation of DBH. Figure 1 shows that DBH activity in P_1 after 6 h incubation was 10.57 ± 1.2 nmol h^{-1} 5 mm^{-1}, a value about seven fold greater than that found in P_2. These values compare favourably with those previously found by us (García *et al.*, 1974).

Influence of A23187 on dopamine β-hydroxylase accumulation in P_1

In a group of nerves we investigated the effects of A23187 on the accumulation of DBH activity in P_1. At concentrations lower than 10 μM, this ionophore had no effect on DBH accumulation. However, incubation of the nerves for a 6 h period in the presence of 10 and 33 μM of A23187 inhibited the enzyme accumulation by about 30% and 70% ($P < 0.01$), respectively (Figure 1).

That the ionophore does not directly inhibit the nerve enzyme activity is proved by the fact that duplicate samples of homogenate added to a purified bovine adrenal medulla DBH preparation gave recoveries of the activity of the purified enzyme which varied from 90 to 100%. In addition, when nerves ligated *in vivo* 24 h before the experiment were incubated *in vitro* in the presence of the ionophore, the previously accumulated large enzyme activity was not modified. These experiments strongly suggest that A23187 specifically interferes with DBH transport and accumulation and not with its activity.

Effects of extracellular calcium and A23187 on dopamine β-hydroxylase accumulation in P_1

Figure 2 shows that incubation of the nerves during 6 h in a calcium-free Krebs solution caused a 30% inhibition of accumulation of DBH in P_1. Addition of A23187 33 μM to calcium-free medium blocked the enzyme accumulation by an additional 20% (Figure 2). It is interesting to note that this inhibition of DBH accumulation by A23187 in calcium-free solution is significantly lower ($P < 0.05$) than that evoked by the drug in the presence of 2.5 mM extracellular calcium.

Effects of high calcium solutions and magnesium removal on dopamine β-hydroxylase accumulation in P_1

Since A23187 seemed to block DBH transport by an extracellular calcium-dependent mechanism, it was of

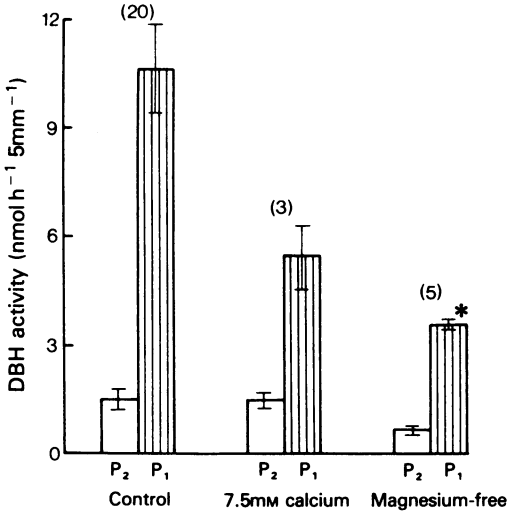

Figure 3 Effect of high-calcium solution and magnesium removal on the accumulation of dopamine β-hydroxylase (DBH) activity in P_1 (lined column). Incubation of the nerves was carried out for a 6-h period in normal (control), high-calcium (7.5 mM) or magnesium-free Krebs solutions. Vertical lines represent s.e. means. The number of experiments is given in parentheses. * $P < 0.01$, compared to control.

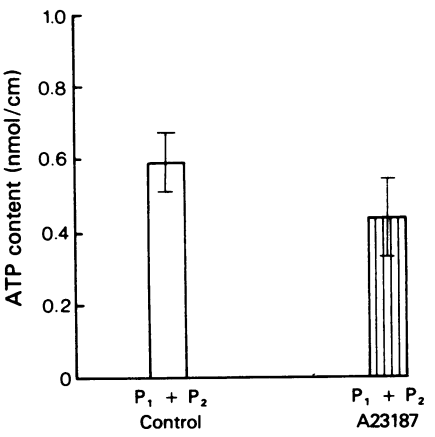

Figure 4 Adenosine triphosphate (ATP) content of segments $P_1 + P_2$ of the hypogastric nerve immediately proximal to the constriction after a 6-h incubation period in Krebs-bicarbonate solution (control) or Krebs containing A23187 (33 μM). Vertical lines represent s.e. means. The number of paired experiments was 4.

interest to test whether an excess of calcium would, itself, modify the rate of transport and accumulation of the enzyme. Figure 3 shows that incubation of the nerves in excess calcium (7.5 mM) did indeed produce a 50% inhibition of DBH accumulation in P_1.

It is well known that both divalent cations, calcium and magnesium, behave antagonistically in neural secretory processes (Baker, 1975). Therefore, we felt that the effects of incubating the nerves in magnesium-free Krebs solution on the axonal transport of DBH should be tested. Figure 3 shows that incubation of the nerves in the absence of magnesium caused a 60% inhibition of DBH accumulation in P_1 ($P < 0.01$).

Effects of A23187 on the ATP content of the nerve

Since the transport of DBH involves an active process which requires energy (García *et al.*, 1974) it was of interest to measure the total neural concentration of ATP after incubation of the nerves for 6 h in normal Krebs or in Krebs solution containing A23187 (33 μM). Figure 4 shows that A23187 did not significantly affect the levels of ATP in the 1 cm nerve proximal to the ligature ($P_1 + P_2$).

Electron microscope observations

In these studies nerves were incubated for 6 h at 37°C in Krebs-bicarbonate solution (control nerves) or in Krebs containing 33 μM of A23187.

Control Nerves. In control nerves the majority of both myelinated and unmyelinated fibres above the ligation exhibited normal fine structure. The incubation did not apparently affect their microtubular and filamentous components as well as the number and shape of mitochondria (Figure 5). However, evidence of damage was found in some unmyelinated fibres. These showed an increased population of granulated vesicles. Schwann cells had a normal appearance.

A23187-treated nerves. The nerves incubated in A23187 (33 μM)-containing medium showed a striking swelling of axons and a considerable retraction of Schwann cell envelopes. This is illustrated in Figure 5b. Typical microtubules or filaments were not seen in these preparations. Nerve fibres were crowded by a flocculent material. On the other hand, both, axons and Schwann cells showed many mitochondria of normal shape and fine structure (inset, Figure 5b).

Discussion

Our data show that the ionophore, A23187, blocked the transport and accumulation of DBH in P_1 and that this effect was greatly diminished when calcium ions were removed from the extracellular medium. The reduction of accumulation of enzyme activity in

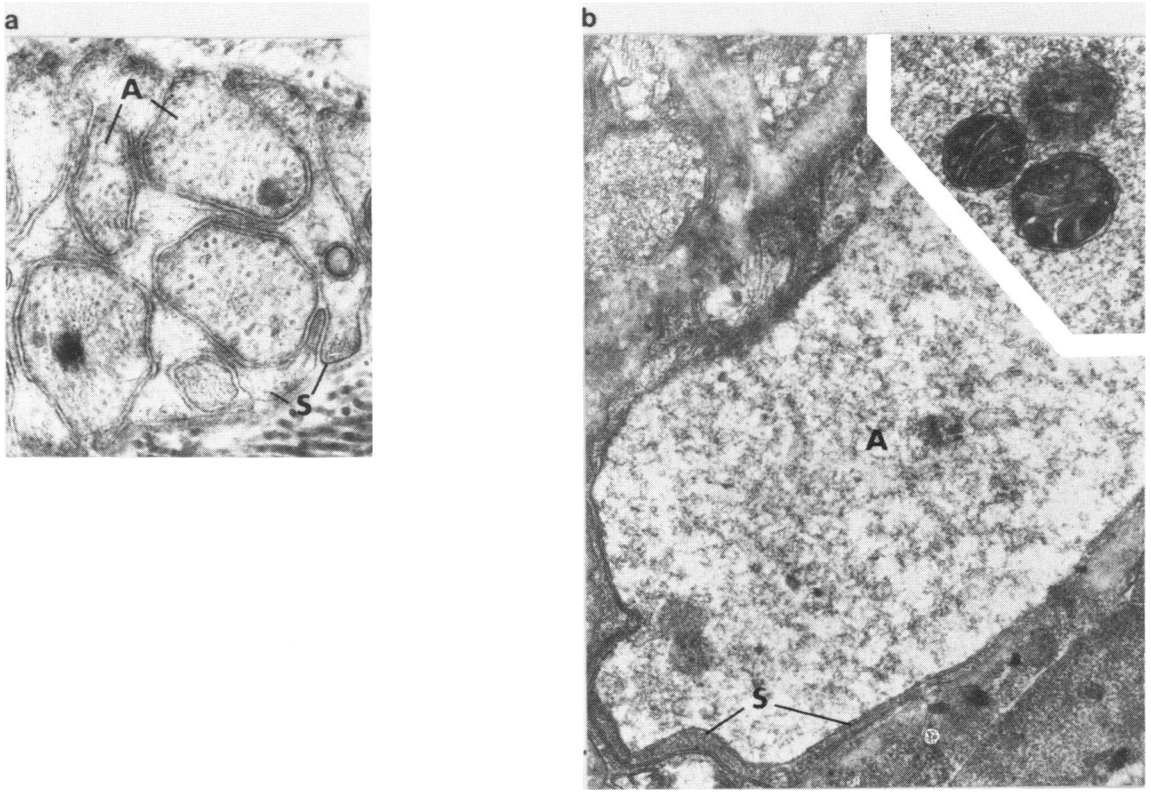

Figure 5 Electron microscopic plates of hypogastric nerves incubated for a 6 h period in normal Krebs (control) or Krebs solution containing A23187 (33 μM). (a) Control nerve. A: axons. S: Schwann cells. Magnification ×24,000. (b) Nerve incubated in A23187 illustrating a swollen axon profile (A) and retracted Schwann cell envelopes (S). Mitochondria in the inset look normal. Magnification ×24,000.

P_1 evoked by A23187 was not the result of non-specific damage of the storage vesicles, since no loss of DBH activity from P_1 occurred in nerves which were first allowed to accumulate the enzyme *in vivo* for 24 h, and were then incubated *in vitro* with the ionophore. In addition, A23187 did not interfere with the different steps involved in DBH assay. Since the rate of DBH transport and accumulation of DBH is not affected in a nerve separated from its ganglion, it follows that the effects of A23187 are exerted locally on the nerve transport system.

The ionophore A23187 specifically increases the permeability of membranes to calcium ions (Reed & Lardy, 1972) and, therefore, its blocking effects could be related either to the transport of calcium from the outside to the inside of the axon or to a mobilization of intracellularly stored calcium, resulting in an increase of cytoplasmic free calciim. The first possibility seems likely since the blocking effects of the ionophore are decreased when external calcium is removed.

Our results seem to be explained best by the assumption that the ionophore A23187 increases the intracellular ionized calcium concentration and that this increase in free calcium ultimately leads to the blockade of the axoplasmic transport system by a not yet clearly understood mechanism. If this is indeed the case, then procedures which lead to a rise in intracellular ionized calcium should also block the axoplasmic transport of DBH. Some of these known procedures are (Baker, 1975):

(1) A rise in external calcium. It was observed that a three fold increase in extracellular calcium concentration inhibited the accumulation of DBH by 50% (Figure 3). Also, preincubation of the nerve in a high-calcium Krebs solution at 4°C, and then reincubation at 37°C, completely prevented the enzyme accumulation (García *et al.*, 1974). Edström (1974) and Ochs *et al.* (1977) have also shown that fast axonal transport of proteins is blocked by raising the extracellular calcium concentration. DBH transport was also inhibited when the nerves were incubated in magnesium-free Krebs, a situation in which calcium uptake is likely to be increased.

(2) A reduction in external sodium. We have previously shown that removal of sodium from Krebs solution greatly inhibited the transport of DBH (García *et al.*, 1974).

(3) A rise in internal sodium. Potassium-free solution, ouabain, N-ethylmaleimide and oligomycin, by slowing the sodium pump increase the internal sodium concentration and the intracellular concentration of ionized calcium. All these procedures have been shown to inhibit effectively the axonal transport of DBH (García *et al.*, 1974).

(4) A reduction of the effectiveness of the intracellular buffers, for instance, inhibitor of mitochondrial calcium uptake or any procedure mobilizing bound calcium. In isolated mitochondria of rat and rabbit brain the presence of A23187 reduced calcium uptake while calcium efflux was increased (Nordmann & Currell, 1975; Fairhurst, Julian & Whittacker, 1975). On the other hand, it is well known that a large part of the mitochondrial calcium is discharged by uncoupling agents and that mitochondria have an energy-dependent transport of calcium which is inhibited by 2,4-dinitrophenol (DNP) and oligomycin (Carafoli & Rossi, 1971; Vickers & Dowdall, 1976). We have also previously shown that DNP and oligomycin reduced the accumulation of DBH in P_1 (García *et al.*, 1974).

Rapid axonal transport of adrenergic vesicles is dependent upon the integrity of the microtubular system (Dählström, 1970; Banks *et al.*, 1971). The ability of crude or partially purified rat brain tubulin to polymerize was carefully studied by Weisenberg (1972). He reported that the formation of microtubules in the presence of a nucleotide triphosphate and $MgSO_4$ was strongly inhibited by calcium ions at concentrations of free calcium of only 6 μM. This concentration is about 20 times greater than the estimated concentration (0.3 μM) of ionized calcium inside resting nerves (Baker, 1975). The ionophore, by considerably raising the intracellular ionized calcium levels will interfere with the assembling of microtubules and in this manner will inhibit the transport of DBH and therefore, of adrenergic vesicles along the axon, preventing the accumulation of enzyme activity in P_1.

The electron microscopic studies showed that in the nerves incubated in A23187-containing Krebs, typical microtubules or filaments disappeared. Shilwa (1976) has also shown in specimens of *Actinosphaerium eichhorni* that A23187 in the presence of calcium produces a degradation of the axonal microtubular array, suggesting that microtubule formation *in vivo* is influenced by micromolar concentrations of calcium. These morphological findings agree well with the interpretation given above concerning the role of calcium in the assembling of microtubules, since the ionophore A23187 is very selective for calcium ions (Reed & Lardy, 1972).

It is interesting to note that A23187 did not affect the neural ATP content. These biochemical data agree well with the morphological data, since the nerve incubated with A23187 showed many mitochondria of normal shape and structure, indicating that the ionophore blocking effects of axoplasmic transport are not mediated by energy interference, but by disruption of the microtubular system. Although microtubules are present continuously in many cell types, they also may be formed or broken down as needed by the cell. Our experiments are consistent with the idea that these microtubular changes are

controlled by the intracellular levels of ionized calcium according to the requirements of the adrenergic neurone for transport of materials along its axon.

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