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Orthograde and retrograde axonal transport of calmodulin in a cat noradrenergic neurone

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1 Subcellular distribution studies of calmodulin in cat sympathetic ganglia demonstrated that about 90% of the protein remained in the 27,000 *g* supernatant, suggesting that it is a cytosolic protein. Only 4.5% was recovered in the microsomal fraction pellet.

2 The inferior mesenteric ganglia contained 93.3 ± 3 ng calmodulin per ganglion, and segments of unligated cat hypogastric nerves had 6.53 ± 0.32 ng per 5 mm segment.

3 When the nerve was ligated in the middle and left in the cat for 1–6 days, substantial amounts of calmodulin accumulated in segments of nerve immediately proximal (P_1) and distal (D_1) to the ligature. The amounts found in P_1 amounted to 15.3, 20, 30.4 and 39.4 ng calmodulin per 5 mm segment 1, 2, 3 and 6 days after ligation, respectively. The average rate of transport was 5.5 mm per day, which corresponds to a slow component b of axonal transport (SCb).

4 The accumulation of calmodulin in D_1 was also increased with the time of ligation. After 1, 2, 3 and 6 days, the amounts of the protein found in D_1 were 14.4, 17.7, 19 and 21 ng per 5 mm segment, respectively. The calculated mean rate for the retrograde transport was 3.9 mm per day.

5 Decentralization of the inferior mesenteric ganglia did not affect the rate of accumulation of calmodulin or the basal amounts found in ganglia and nerves. Local injection inhibited the orthograde, but not the retrograde axonal transport of the protein.

6 It is concluded that calmodulin undergoes a process of slow orthograde axonal transport probably incorporated into the axoplasmic matrix of a network of actin microfilaments. The protein is also transported in a retrograde manner.

Introduction

Many of the effects of calcium on living tissues are mediated by calcium-binding regulator proteins. Calmodulin is a major calcium receptor protein which has been well characterized and shown to affect several important enzyme systems (Cheung, 1980; Means *et al.*, 1982). Recently, it also has been implicated as a modulatory protein in neural processes such as neurotransmitter release and axonal transport (De Lorenzo *et al.*, 1979).

It has been suggested that calmodulin initiates synaptic vesicle-axolemmal interactions during the release of noradrenaline from brain synaptosomes through the modulation of the effects of calcium on synaptic protein phosphorylation (De Lorenzo *et al.*, 1979). If this protein plays a similar role in peripheral noradrenergic neurones, it must be synthesized in the cell body and transported down the sympathetic axons to the nerve terminal where the physiological calcium-dependent, exocytotic release of noradrenaline takes place (see Kirpekar, 1975).

Calmodulin has indeed been shown to undergo a process of axoplasmic transport in mammalian nerves. However, there is no agreement on its subcellular distribution and also on the rate of transport of components to which the protein is associated during the process of axonal transport. Iqbal & Ochs (1978) found that $^{45}Ca^{2+}$ was transported at a fast rate of 410 mm per day and is associated with a calcium-binding protein, mol. wt 15,000 daltons, in cat sciatic nerves (later shown to be calmodulin; Iqbal & Ochs, 1980); but Erickson *et al.*, (1980) found that most of

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the calmodulin was transported at a slow rate (2–4 mm per day) in the rabbit visual system. Brady *et al.*, (1982) also concluded that calmodulin was transported down the axons solely as a part of the slow component complex of proteins (the axoplasmic matrix).

The cat hypogastric nerve ligated *in vitro* or *in vivo* has been extensively used in our laboratory to study the axoplasmic transport of noradrenergic vesicles and cholinoceptors (Ceña *et al.*, 1980; Alonso *et al.*, 1982). We have also studied the release of noradrenaline from such ligated nerve trunk segments (neurosomes: Esquerro *et al.*, 1980a,b).

In view of the contradictory findings on the rate of transport of calmodulin, and of its possible implications in the axonal transport of noradrenergic vesicles and receptors, as well as the release of noradrenaline from sympathetic nerves, we initiated this study to determine first the subcellular distribution of calmodulin within peripheral sympathetic neurones, and then to demonstrate the accumulation of this protein proximal and distal to a constriction made on the cat hypogastric nerve trunk.

Methods

Subcellular distribution of calmodulin

Superior cervical ganglia and the thoracic sympathetic chains were isolated by a careful dissection procedure from 3 adult cats anaesthetized with ether. The tissues were finely minced and homogenized with a ground glass to teflon homogenizer in 10 ml of ice-cold 0.32 M sucrose containing 10 mM Tris, pH 7.3. All subsequent procedures were performed at 2°C.

The homogenate was first centrifuged at 800 *g* and the pellet resuspended in 5 ml of 0.32 M sucrose Tris buffer. An aliquot of the supernatant was saved and the rest spun down at 27,000 *g* for 20 min (Sorvall SS-34 rotor). The supernatant was saved and the pellet resuspended in 4 ml of sucrose-Tris buffer.

To estimate the amount of calmodulin present 200 µl of each fraction were diluted to 1 ml with 10 mM Tris buffer containing 0.1 mM ethyleneglycol bis β-amino ethyl ether, N_1N'tetraacetic acid (EGTA), and 0.1 mM mercaptoethanol. Calmodulin was determined as described below.

Ligation of the nerves

Cats of either sex weighing 2.5–4 kg were anaesthetized with ether, the abdomen was opened by a midline incision under aseptic conditions, and the hypogastric nerves were carefully tied with a silk thread at two different points separated by 3 mm (Alonso *et al.*, 1982).

Calmodulin accumulation after the ligation

One to six days after ligation, the animals were again anaesthetized, the abdomen reopened, and the nerves, together with the inferior mesenteric ganglia, gently dissected out proximally and distally to the double ligature. The nerve was then cut into 5 mm segments. The segments, starting just proximal to the proximal ligature and going toward the ganglion, are designated as P_1, P_2 and P_3; the segments distal to the ligation are called D_1, D_2 and D_3 (see lower part of Figure 1).

In a group of experiments, the inferior mesenteric ganglion was decentralized by cutting all preganglionic fibres to it; the nerves were then ligated as above. In other experiments, colchicine (1 mM solution) was applied locally to the inferior mesenteric ganglion and the nerves ligated as usual.

Assay of calmodulin

Each individual segment or the inferior mesenteric ganglion was homogenized in all-glass homogenizers in 1 ml of 10 mM Tris buffer, pH 7.7, containing 0.1 mM EGTA and 0.1 mM mercaptoethanol. The homogenates were centrifuged at 27,000 *g* for 10 min; the supernatants and aliquots from different fractions resulting from the subcellular fractionation procedure were heated at 90°C for 5 min. Calmodulin was assayed in 100 µl aliquots of the supernatants diluted appropriately and in 100 µl of the 27,000 *g* supernatant from tissue homogenates, using a competitive-inhibition type calmodulin radioimmunoassay system (New England Nuclear, NEK-018). The amount of calmodulin in the subcellular fractions was expressed as ng per fraction or as ng mg^{-1} protein. Calmodulin present in the inferior mesenteric ganglia or nerve segments was expressed as ng per ganglion or ng per 5 mm segment of nerve, respectively.

The statistical significance of the differences between means was determined using Student's *t* test for group data.

Results

Subcellular distribution of calmodulin in peripheral sympathetic neurones

Since proteins seem to move in axons as a part of cytologically identifiable structures (Black & Lasek, 1980) and these structures have different transport kinetics, it is convenient, before attempting to study the axonal transport properties of calmodulin, to know its subcellular distribution. The subcellular distribution of calmodulin could not be studied in

Table 1 Subcellular distribution of calmodulin in peripheral sympathetic neurones

		Homogenate	*800g supernatant*	*800g pellet*	*27,000g supernatant*	*27,000g pellet*
Experiment 1	Calmodulin (ng per fraction)	2687	1187	1500	1056	62
Experiment 2	Calmodulin (ng per fraction)	3500	1543	2000	1348	140

Superior cervical ganglia and the thoracic sympathetic chains from 2 cats were pooled, homogenized, and subjected to the differential centrifugation procedure described in Methods. Calmodulin is expressed as total ng per fraction.

peripheral sympathetic axons because of the small amount of tissue, but it was studied in the neuronal cell bodies of the sympathetic ganglia.

Table 1 shows that after homogenization and low speed centrifugation of several sympathetic ganglia, a large fraction of the total calmodulin present in the original homogenate remains in the 800 *g* pellet, probably associated with unbroken cells and large particles. Of the 44% of calmodulin remaining in the 800 *g* supernatant, as much as 88% remained in the 27,000 *g* supernatant, suggesting that the majority of the protein is soluble in the cytosol. Only 4.5% of calmodulin was found in the 27,000 *g* pellet.

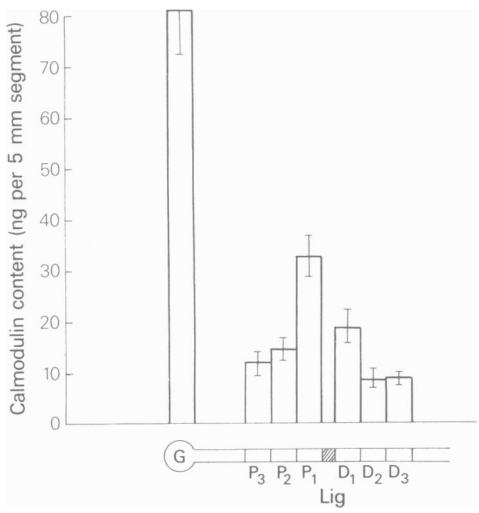

Figure 1 Distribution of calmodulin in the hypogastric nerve ligated for 72 h *in vivo*. Three days after ligation *in vivo*, the nerves were dissected out, cut into 5 mm segments proximal and distal to the ligation (Lig), homogenized and analysed for their calmodulin content as described in Methods; inferior mesenteric ganglia (G) were also dissected out to estimate their calmodulin content. Data are means ± s.e. (vertical bars) of 8 nerves.

Distribution of calmodulin in the inferior mesenteric ganglion-hypogastric nerve preparation

In 40 inferior mesenteric ganglia the mean calmodulin content was 93.3 ± 6.2 ng per ganglion. The protein distributed uniformly along different segments of unligated hypogastric nerves. In 16 segments from unligated nerves, the amount of calmodulin found was 6.53 ± 0.32 ng per 5 mm segment (range 4–8.6).

Accumulation of calmodulin in segments of nerves immediately proximal and distal to constriction

Three days after ligation of the nerves, calmodulin clearly accumulated in P_1 as well as in D_1 segments (Figure 1). The amounts of calmodulin found in P_1 (30.4 ± 2.4 ng per 5 mm segment) were 2.5 and 3.5 times the quantities found in P_2 and P_3, respectively. Maximal accumulation in P_1 occurs at about 3 days and there is only a small increase over this value after 6 days of ligation (Figure 2). In addition, it is worth noting that P_2 and P_3 segments contained greater amounts of calmodulin when compared to unligated nerves; apparently, due to the slow rate of transport, the newly synthesized calmodulin remains distributed along the nerves (Figure 2). This is in contrast to the distribution of dopamine-β-hydroxylase activity or noradrenaline in nerves ligated *in vivo*; because these materials are associated with noradrenergic vesicles and therefore transported at a very fast rate (more than 100 mm per day), they accumulate quickly only in the segments of nerves most proximal to the ligation (P_1 and D_1; García *et al.*, 1974; Alonso *et al.*, 1982). It also should be pointed out that the calmodulin contents of ganglia did not vary after ligation of their nerves for 1, 2, 3 or 6 days.

In the portions of nerve distal to ligation, the protein accumulated in D_1 when compared to the amounts found in D_2 and D_3. The distribution of calmodulin in distal segments after different periods of ligation is shown in Figure 2. It can be appreciated that as the time of ligation increased, the amounts of calmodulin found in all three distal segments also increased.

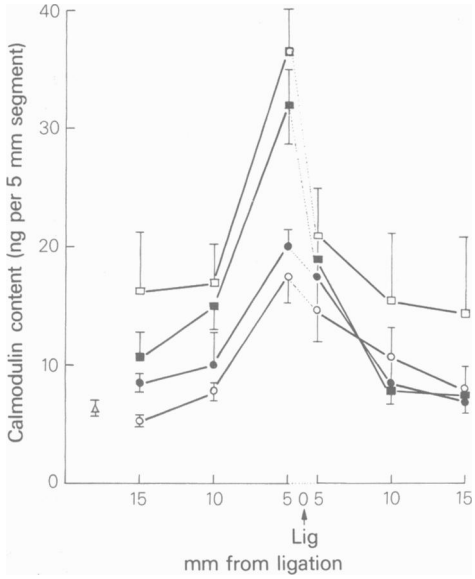

Figure 2 Distribution of calmodulin along the proximal and distal portions of nerves ligated for 0(△), 1(○), 2(●), 3(■) and 6(□) days. Abscissa scale shows the distance from ligation of the nerve segment in mm (P, proximal to ligation; D, distal to ligation). Data are means ± s.e. of 4–6 experiments.

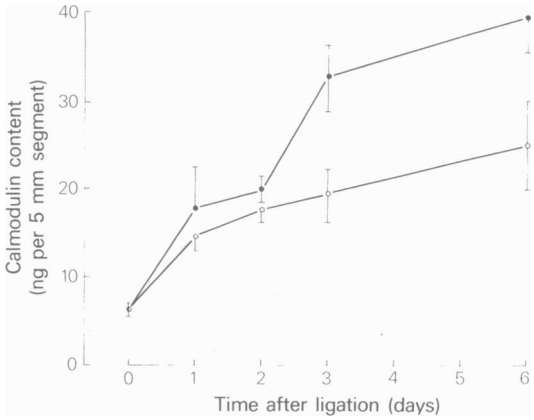

Figure 3 Accumulation of calmodulin in segments of nerves immediately proximal (P_1) (●–●) and distal (D_1) (○–○) to a ligature on the cat hypogastric nerves placed 2 cm distally to the inferior mesenteric ganglion 1–6 days before removal of the nerves for assay. Data are means ± s.e. of 4–6 nerves.

The rate of transport of calmodulin

The rate of transport of calmodulin can be calculated approximately from the slope of the lines representing the protein accumulation in P_1 and D_1 segments against time. Figure 3 shows that the accumulation of calmodulin in P_1 was not linear; but after 24 h, for example, the amount of protein found in P_1 was three times that found in the more proximal segment, P_3; this would indicate that the amount of calmodulin found in 10 mm of nerve above P_1 must have moved into P_1 within 24 h, and indicates a rate for the orthograde axonal transport of about 10 mm per day. Taking the amount present in non-ligated nerves

Table 2 Estimation of the rates of orthograde axonal transport of calmodulin in ligated cat hypogastric nerves

	Basal	*1 day*	*2 days*	*3 days*	*6 days*
Calmodulin in P_1 after ligation (ng per 5 mm segment)	6.53 ± 0.32 (16)	15.3 ± 2.6 (4)	19.8 ± 1.3 (6)	30.4 ± 2.4 (6)	39.4 ± 5.1 (4)
Accumulated net calmodulin in P_1 (P_1 basal, ng per 5 mm segment)	—	8.8	13.3	23.9	32.9
Factor = accumulated net calmodulin in P_1/basal content)	—	1.35	2.04	3.66	5.04
Estimated rate of transport (mm per day = factor × 5/number of days)	—	6.75	5.1	6.1	4.2

Mean rate of orthograde axonal transport: 5.54 mm per day.

The rationale for such estimation is based on the assumption that the amount of calmodulin found in 5 mm segments of unligated nerves (basal content) moved into the segments of nerves immediately proximal (P_1 for the orthograde transport) or distal (D_1 for the retrograde transport) to the ligation. Since there was no strict linearity between the accumulation of calmodulin in P_1 or D_1 and the time of ligation, it was difficult to calculate the exact rate of transport from the slope of the lines in figure 3. Therefore, an approximation has been made by calculating the individual rates of transport at different ligation times and averaging them. The numbers in parentheses represent the number of nerves.

Table 3 Estimation of the rates of retrograde axonal transport of calmodulin in cat hypogastric nerves

	Basal	*1 day*	*2 days*	*3 days*	*6 days*
Calmodulin in D_1 after ligation (ng per 5 mm segment)	6.53 ± 0.32 (16)	14.5 ± 5.7 (4)	17.7 ± 2.8 (6)	19.11 ± 1.9 (4)	21.2 ± 8.5 (4)
Accumulated net calmodulin in D_1 (D_1 basal, ng per 5 mm segment)	—	8	11.2	12.5	14.5
Factor = accumulated net calmodulin in D_1/basal content)	—	1.23	1.72	1.92	2.23
Estimated rate of retrograde axonal transport (mm per day = factor × 5/number of days)	—	6.15	4.29	3.19	1.85

Mean rate of retrograde transport = 3.91 mm per day
Calculations are similar to those presented in Table 2

(6.53 ng) as basal calmodulin content of 'control' nerves, the rate of transport should be about 8 mm per day. Table 2 shows the relative rates of transport calculated from the accumulation of calmodulin in P_1 at different time periods after ligation. On the average, it can be assumed that the overall rate of orthograde transport of calmodulin in the hypogastric nerve is about 5.5 mm per day.

The retrograde transport was calculated in a similar way. The mean rate of transport calculated in nerves ligated 1–6 days was approximately 3.9 mm per day (Table 3). Since more distal segments (D_2 and D_3) contain lower amounts of calmodulin than those found in non-ligated segments, it is obvious that if these segments were taken as the basal protein contents, the actual rate of transport should be higher.

Effects of decentralization on the axonal transport of calmodulin

Decentralization of the inferior mesenteric ganglia by cutting the preganglionic fibres did not affect, 3 days later, the amounts of calmodulin found in the ganglia (76 ng per 5 mm segment; $n = 2$) or in unligated nerve segments (5.04 ± 0.61 ng per 5 mm segment; $n = 8$). Six days after the ligation of nerves from decentralized ganglia, the amounts of calmodulin found in P_1 were 25 ng per 5 mm segment ($n = 2$), amounts slightly lower than those found in nerves from normal, innervated ganglia.

Effects of colchicine on the orthograde and retrograde axonal transport of calmodulin

Even though calmodulin seems to be transported by a slow-transport system, it was interesting to see whether colchicine inhibited its transport as it does with the fast axoplasmic transport of noradrenergic vesicles (Dahlström, 1970). Colchicine was initially applied locally to the hypogastric nerve with a cotton pellet in mM concentrations, but the animals died. Therefore, 20 µl of a 1 mM colchicine solution was injected with a Hamilton syringe directly into the inferior mesenteric ganglion on one side; the contralateral ganglion was injected with an equal volume of saline and both nerves ligated at the moment of the injection. Three days later, nerves and ganglia were removed and analysed for their content of calmodulin.

Colchicine inhibited completely the accumulation of calmodulin in P_1, as compared to P_2 and P_3 segments; however, all 3 segments had greater amounts of calmodulin than that present in unligated nerve segments. So, while unligated segments contained 6.5 ± 0.3 ng calmodulin per 5 mm segment, P_1, P_2 and P_3 nerve segments of colchicine-treated ganglia had 13.3 ± 2.2, 13.4 ± 3.3, and 13.9 ± 2.8 ng per 5 mm segment, respectively ($n = 4$, $P < 0.05$ when compared to controls).

Concerning the portions of nerves distal to ligation, it is curious to see that the accumulation of calmodulin in D_1, well above D_2 or D_3, was not affected in colchicine-treated nerves. It is also not clear whether colchicine reaches the distal portions of the ligated nerve following its injection into the ganglion. The amounts of calmodulin in control and colchicine-treated ganglia were similar.

Discussion

We have shown in this paper that calmodulin, an ubiquitous calcium receptor protein (Cheung, 1980;

Mean *et al.*, 1982), is present in the cell bodies and axons of peripheral sympathetic neurones. The protein accumulates in the portions of nerve segments proximal and distal to a ligation placed in the middle of the cat hypogastric nerve; the accumulation is more prominent in segments P_1 (immediately proximal) and D_1 (immediately distal to the ligation), suggesting that calmodulin moves along sympathetic axons via processes of anterograde and retrograde axonal transport. While decentralization does not seem to affect these processes, colchicine inhibits the anterograde transport system.

Subcellular distribution of calmodulin

An important question for understanding the functional significance and the rate of transport of calmodulin is its distribution between membrane and cytosol compartments. Discrepancies have arisen from different laboratories; some authors claim that brain calmodulin is mostly released into soluble fractions (Kakiuchi *et al.*, 1978), but others found that a significant portion (about half) of calmodulin from various fractions of brain is membrane-bound (Costa *et al.*, 1977).

In peripheral noradrenergic neurones, we found that only 4% of calmodulin is associated with a subcellular fraction containing mitochondria, noradrenergic vesicles, and other microsomal membranes; most of the calmodulin was found in the supernatant fraction, and thus it seems to be freely soluble in the cytosol. Since soluble proteins are usually slowly transported, it is likely that calmodulin is indeed transported by a slow axoplasmic flow system.

The rates of transport of calmodulin

The fact that calmodulin is a soluble protein, not associated with any particle precipitable with the microsomal fraction (at least in its major part), accords with its slow rate of anterograde axonal transport, about 5.54 mm per day. This rate of transport is closely related to the rate found for the slow component b (SCb) by Black & Lasek (1980) in retinal ganglion cell axons, and suggests that calmodulin may be transported down the axon with the axoplasmic matrix, a network of actin microfilaments to which calmodulin and other proteins are complexed as they are transported down the axons. However, the complex of calmodulin with this matrix must be weak, as during tissue homogenization calmodulin is released into the soluble supernatant.

These results accord with those found by Erikson *et al.*, (1980) in the rabbit visual system; these authors analysed [^{3}H]-leucine incorporated into calmodulin, revealing that most of it was axonally transported at a slow rate (2–4 mm per day). Brady *et al.*, (1982) also concluded that calmodulin was transported down the axons as a part of the slow component of proteins (the axoplasmic matrix). However, our results are dissimilar to those of Iqbal & Ochs (1978), who found that calmodulin and ^{45}calcium were transported at the fast rate of 410 mm per day in cat sciatic nerves.

One of the reasons for these discrepancies may be the different technical approaches used to quantitate calmodulin and monitor the rates of its axonal transport. Iqbal *et al.*, (1978) and Erikson *et al.*, (1980) measured [^{3}H]-leucine incorporation into proteins, and these authors purified calmodulin by a two-step polyacrylamide gel electrophoresis. We are directly measuring endogenous calmodulin by using a sensitive and specific radio-immunoassay (Chafouleas *et al.*, 1979; Wallace & Cheung, 1979). A second reason might be that only a minor fraction of the 15,000 daltons mol. wt. protein labelled by [^{3}H]-leucine in the experiments of Iqbal & Ochs (1978) really accounts for calmodulin.

Calmodulin might also suffer a process of retrograde axonal transport as demonstrated by its considerable accumulation in the segment of nerve immediately distal to ligation (D_1). The rate of transport was 3.91 mm per day, slightly lower than the rate of anterograde axonal transport. It is difficult to know the meaning of this transport, but it may be that calmodulin is incorporated into lysosomes which carry the protein back to the pericaryon to be degraded or reutilized.

Effects of colchicine on the axonal transport of calmodulin

Colchicine is known to depolymerize the microtubules and block the fast axoplasmic transport of noradrenergic vesicles in peripheral sympathetic neurones (Dählstrom, 1970). The drug has also an inhibitory effect on the slow axonal transport, probably because the growth of microtubules and filaments constitutes the major part of the slowly growing axoplasm (Dählstrom, 1971) from which calmodulin might form a part.

The local injection of colchicine into the inferior mesenteric ganglion caused an inhibition of the anterograde, but not the retrograde transport of calmodulin. A similar effect of cochicine when applied to cell bodies has been previously found (Frizzel *et al.*, 1975). The fact that control (injected with saline) and colchicine-treated ganglia contained similar amounts of calmodulin and that its axonal transport was inhibited only in the nerve from the colchicine-treated ganglion, suggests that the injection of fluid by Hamilton syringe had no damaging effect.

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