CALCIUM UPTAKE BY MUSCLE SARCOPLASMIC RETICULUM FOLLOWING NEURAL APPLICATION OF BATRACHOTOXIN OR TETRODOTOXIN

K. K. WAN and R. J. BOEGMAN*
Department of Pharmacology, Queen's University, Kingston, Ontario K7L 3N6, Canada

Received 22 December 1979

1. Introduction

The nerve axon innervating mammalian skeletal muscle delivers trophic substances via axonal transport [1–3], in addition to electrical impulses which control the release of neurotransmitter [4].

Denervation of skeletal muscle causes marked structural and physiological changes of the affected muscle including the sarcoplasmic reticulum (SR) which is believed to play an important role in regulating the intracellular [Ca^{2+}] [5,6]. Accumulation of Ca^{2+} by vesicles of fragmented sarcoplasmic reticulum (FSR) increases after a relatively short period of denervation [7–10]. Ca^{2+}-uptake by FSR vesicles from slow and fast muscle fibres also changes upon changing the innervation to the muscle [11,12]. Thus, it seems that the SR is under some form of neurotrophic control.

Certain neurotoxins have become important tools in studying neural trophic regulation [13]. Among these are batrachotoxin (BTX) and tetrodotoxin (TTX). BTX, when applied to a nerve, blocks axonal transport as well as impulse transmission whereas TTX only blocks impulse transmission and has no effect on axonal transport [14–17]. Therefore, following application of either BTX or TTX to a nerve the importance of axonal transport in exerting a neurotrophic control on muscle can be determined. Here we present evidence supporting the concept that axonal transport of neurotrophic factors plays a role in the regulation of Ca^{2+}-uptake by muscle SR.

Abbreviations: EGTA, [Ethylene-bis (oxyethlenenitrilo)] tetraacetic acid; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; POPOP, p-bis [2-(5-phenyloxazole)]-benzene; PPO, 2,5-diphenyloxazole

* To whom correspondence should be addressed

2. Materials and methods

2.1. *Animals*

Adult female Sprague-Dawley rats (200–250 g) were used. One μl of BTX (10^{-12} mol) or TTX (10^{-9} mol) in 0.9% NaCl–10% dextrose was injected with a fine glass micropipette into the subperineural space of the peroneal nerve ~15 mm before the nerve enters the muscle [16]. The contralateral side was injected with the same volume of vehicle and was used as the experimental control. At various time intervals, the animals were sacrificed and the extensor digitorum longus (EDL) muscles removed. In each experiment FSR was prepared from a pool of 8–14 muscles.

2.2. *Chemicals*

TTX was purchased from Calbiochem-Behring Co., BTX was a gift from Drs E. X. Albuquerque and J. W. Daly, $^{45}CaCl_2$ was obtained from New England Nuclear and FCCP from Sigma Co. Other chemicals used were of reagent grade except POPOP and PPO which were of scintillation grade. Water used in all experiments was nanopure (NANOpure, Barnstead) and the equipment was rinsed with 1 mM EGTA before use.

2.3. *Method*

The microsomal fraction containing the FSR vesicles was isolated from the tissue homogenate by ultracentrifugation as in [18], and was used for Ca^{2+}-uptake on the same day of preparation. Ca^{2+}-uptake by the FSR vesicles in the absence and presence of

oxalate was determined by the millipore filtration method [19]. To inhibit mitochondrial Ca^{2+} uptake, FCCP was also included in the reaction mixture [20]. The Ca^{2+} concentrating ability and passive efflux of Ca^{2+} from the FSR vesicles was measured as in [21]. Protein was determined according to [22] and radioactivity was measured by liquid scintillation in a toluene base cocktail containing Triton X-114 and POPOP-PPO [23].

3. Results

The muscle wet weight and yield of FSR showed a progressive loss up to 12 days after application of BTX to the innervating nerve. However, the muscle weight recovered partially at longer time periods. A similar decrease in muscle weight and yield of FSR has been observed in denervated EDL [9,10]. In contrast, when the nerve was poisoned with TTX there was no marked decrease in muscle weight (table 1).

Ca^{2+} uptake in the absence of oxalate by FSR vesicles isolated from muscles innervated by BTX-treated nerves decreased gradually, reaching 59% of control at ~12 days (table 2). At subsequent time intervals there was a partial recovery in the Ca^{2+}-uptake capacity. However, at 24 days it was still 29% below control values. The uptake of Ca^{2+} by FSR from 4-day denervated muscle shows a similar trend by being 20% lower than that obtained from the contralateral control side. In contrast, FSR isolated from muscles in which TTX was applied to the nerve showed only a small (10%) decrease in their ability to accumulate Ca^{2+} in the absence of oxalate. Ca^{2+} uptake in the presence of oxalate, which has been termed 'calcium loading' [24] and is believed to simulate the in vivo sequestering of Ca^{2+} by the SR during muscle relaxation, was also examined. As shown in table 2 the Ca^{2+}-loading by FSR from muscles innervated by BTX-treated nerves showed an increase during the first 12 days after application of the toxin to the nerve. A similar increase in Ca^{2+}-loading has been obtained in FSR from denervated muscle ([7–10], table 2). In sharp contrast, following TTX application to the nerve the FSR did not show a comparable increase in Ca^{2+}-loading.

The total Ca^{2+}-loading capacity of FSR does not necessarily bear any relationship to the function of the FSR, the latter being to reduce the intracellular $[Ca^{2+}]$ to $\sim 10^{-7}$ M [21]. A measure of the Ca^{2+}-concentrating ability of the FSR at sub-saturating $[Ca^{2+}]$ would supply kinetic characteristics of the Ca^{2+}-uptake process equivalent to that seen in vivo. Using this approach we found that the Ca^{2+}-concentrating ability of FSR from EDL in which the nerve had been exposed to BTX 12 days in advance was lower than that of the contralateral control preparation (fig.1a). In addition to showing a decreased Ca^{2+}-uptake the passive efflux of Ca^{2+} was greater in the BTX treated preparations (fig.1b).

Table 1
Muscle wet weight after exposing the innervating nerve to BTX, TTX or denervation

Days after treatment	Muscle (EDL) wet weight (mg)								
	Contralateral	BTX	% change	Contralateral	TTX	% change	Contralateral	Denervation	% change
4	115.5 (2)	104.2 (2)	− 9.8	142.9 (2)	136.7 (2)	−4.0	112.0 (1)	115 (1)	+ 2.0
8	165.7 (3) ±17.5[a]	139.3 (3) ±17.8[a]	−15.9	117.8 (3) ± 8.2[a]	113.9 (3) ± 4.2[a]	−3.3	191.7 (1)	141.7 (1)	−26.1
12	134.8 (5) ± 2.87[a]	95.64 (5) ± 5.29[a]	−29.0		–			–	
16	108.4 (2)	88.2 (2)	−18.6		–			–	
24	130.8 (1)	121.7 (1)	− 7.0		–			–	

[a] Mean ± SEM

The peroneal nerve innervating one extensor received toxin (BTX or TTX) while the contralateral received vehicle and served as control. Numbers in parenthesis indicate the number of experiments performed. In each experiment, 8–12 pairs of muscle (EDL) were removed and the average wet weight per muscle determined

Table 2
Effect of neural application of BTX, TTX or denervation on Ca^{2+}-transport activities of FSR

Days after treatment	No. expt.	$^{45}Ca^{2+}$-uptake by FSR (μmol Ca^{2+}/mg protein)						$^{45}Ca^{2+}$ loading (oxalate) by FSR (μmol Ca^{2+}/mg protein)					
		Contra-lateral	BTX	Contra-lateral	TTX	Contra-lateral	Denerva-tion	Contra-lateral	BTX	Contra-lateral	TTX	Contra-lateral	Denerva-tion
4	2	0.103	0.095 (92.2%)	0.117	0.106 (90.6%)	0.128	0.103 (80.0%)	0.520	0.683 (131%)	0.496	0.545 (110%)	0.537	0.948 (176%)
8	3	0.134 ± 0.006[a]	0.109 ± 0.006[a] (81.3%)	0.128 ± 0.003[a]	0.115 ± 0.004[a] (90.0%)	–		0.442 ± 0.029[a]	0.625 ± 0.052[a] (141%)	0.618	0.572 ± 0.050[a] (92.6%)	–	
12	3	0.162 ± 0.002[a]	0.096 ± 0.006[a] (59.3%)	0.126 ± 0.001[a]	0.118 ± 0.002[a] (94.0%)	–		0.253 ± 0.005[a]	0.410 ± 0.003[a] (162%)	–		–	
16	1	0.098	0.075 (76.5%)	–		–		0.516	0.825 (159%)	–		–	
24	1	0.131	0.093 (71.0%)	–		–		0.470	0.658 (140%)	–		–	

[a] Mean ± SEM

Calcium uptake by FSR was done as in [15] in Tris–maleate (pH 6.8) 20 mM; KCl 0.1 M; $MgCl_2$ 5 mM; $^{45}CaCl_2$ 148 μM (spec. act. 4000–7000 dpm/74 μmol Ca^{2+}); ATP 1.16 mM, FCCP 2 μg/ml and 0.1–0.2 mg FSR protein/ml. The reaction was started at 25°C by adding ATP and terminated after 30 s. The conditions for total loading capacity of FSR were the same; however, 5 mM of potassium oxalate was included in the reaction mixture. FSR in a reaction medium without ATP served as the reaction blank. The values in each experiment represent the average of at least 3 analyses on the FSR isolated from pooled muscles of 8–12 rats. The contralateral EDL was used as the experimental control. The values in parenthesis are % of control

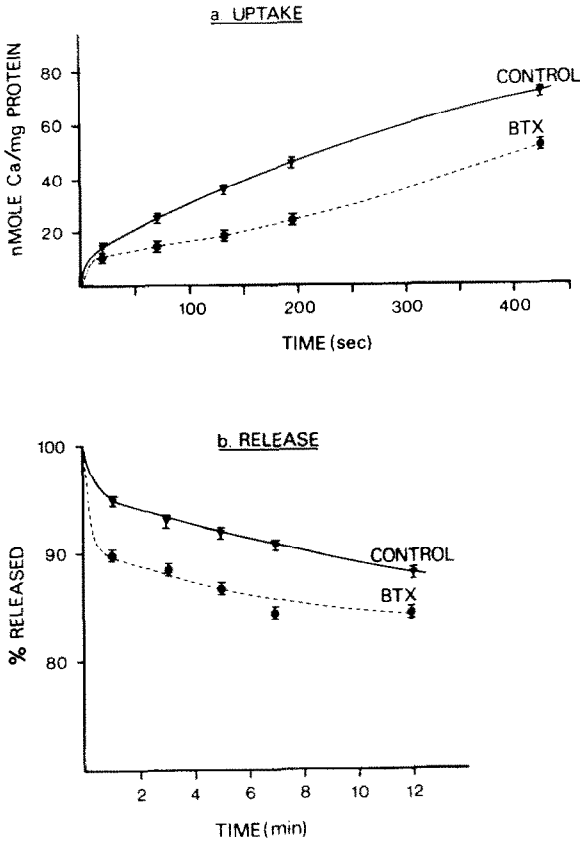

Fig.1. Calcium uptake and efflux from FSR 12 days following neural application of BTX. Assay procedure was that in [21], in Tris–maleate (pH 6.8) 20 mM; KCl 0.1 M; $MgCl_2$ 5 mM; $^{45}CaCl_2$ 10 μM (1000 dpm/nmol); ATP 1.16 mM; FCCP 2 μg/ml; potassium oxalate 5 mM and FSR protein 0.1 mg/ml.

4. Discussion

Experimental evidence has accumulated showing neurogenic control of many biochemical properties of muscle. Thus denervation or cross-innervation of a slow or fast muscle by a fast or slow motor nerve, respectively, leads to changes in the Ca^{2+}-transporting activity of the FSR; EGTA-sensitive Ca–Mg, K-ATPase; lactic dehydrogenase isoenzyme pattern and the molecular properties of myosin [3,6,11,12]. The neurogenic basis for these changes is not clear and could reside in the frequency of muscle contraction which is under neural control or alternatively in trophic factors supplied to the muscle by axonal transport [1,3]. By exploiting the selective action of neurotoxins such as TTX and BTX on impulse conduction and axonal transport we have obtained evidence that Ca^{2+}-mobilization by FSR is under trophic control by neural factors supplied to the muscle by axonal transport.

BTX induces a state of denervation in muscle by blocking both axonal transport and impulse conduction. This is seen in the parallel weight loss observed in denervated muscle [9,10] and that obtained from BTX-treated nerves. In addition, following denervation there is an increase in total Ca^{2+}-loading by FSR in the presence of oxalate [7–10]. This is also observed in muscle innervated by BTX-poisoned nerves. While Ca^{2+}-uptake in the presence of oxalate increased in both denervated and BTX-treated preparations, a decrease in Ca^{2+}-uptake by FSR from both preparations was observed in the absence of oxalate ([10] table 2). In denervated muscle an increase in Ca^{2+}-release from Ca^{2+}-loaded FSR has been reported [10]. A similar increase in Ca^{2+}-release was observed in FSR preparations obtained from muscle innervated by a BTX-poisoned nerve (fig.1b). In marked contrast TTX, which only blocks impulse conduction, had minimal effects on Ca^{2+}-mobilization by FSR. The decrease in Ca^{2+}-uptake by FSR from muscle innervated by a BTX-poisoned nerve can be explained by assuming the formation of larger vesicles similar to that reported for denervated and dystrophic muscle which have a decreased Ca^{2+}-influx rate [10,21]. Alternatively the leakiness of the FSR could be due to changes in the organization of the membrane similar to that observed following exposure to ether which leads to increased leakiness of the FSR [25]. This is further supported by the observation that the protein electrophoretic pattern of the FSR does not change following neural application of BTX; there is, however, an increase in the FSR lipid content (unpublished).

A direct effect of BTX on muscle due to diffusion of the toxin has been ruled out on the basis of muscle membrane potential measurements and the lateral spread in the nerve of [^{3}H]BTX which is restricted to an area of 4–6 mm from the injection site [16]. In addition, when BTX (10^{-12} mol) was added to the Ca^{2+}-uptake incubation media no change in the amount of Ca^{2+} accumulated was observed.

In summary, by selectively blocking axonal transport and/or impulse conduction in nerves innervating fast skeletal muscle we have demonstrated that axonal transport plays a role in the control of the Ca^{2+}-transport system.

Acknowledgement

This work was supported by the Canadian Muscular Dystrophy Association.

References

[1] Miledi, R. (1960) J. Physiol. (London) 151, 1–23.
[2] Drachman, D. B. ed (1974) Ann. NY Acad. Sci., vol. 228.
[3] Gutmann, E. (1976) Ann. Rev. Physiol. 38, 177–216.
[4] Katz, B. and Miledi, R. (1965) Proc. Roy. Soc. B. 161, 483–498.
[5] Thesleff, S. (1974) Ann. NY Acad. Sci. 228, 89–104.
[6] Harris, A. J. (1974) Ann. Rev. Physiol. 36, 251–305.
[7] Brody, I. A. (1966) Am. J. Physiol. 211, 1277–1279.
[8] Howell, J. N., Fairhurst, A. S. and Jenden, D. J. (1966) Life Sci. 5, 439–446.
[9] Margareth, A., Salviati, G., DiMauro, S. and Turati, G. (1972) Biochem. J. 126, 1099–1110.
[10] Sreter, F. A. (1970) Exp. Neurol. 29, 52–64.
[11] Mommaerts, W. F. H. M., Buller, A. J., and Seraydarian, K. (1969) Proc. Natl. Acad. Sci. USA 64, 128–133.
[12] Margareth, A., Salviati, G. and Carraro, U. (1973) Nature 241, 285–286.
[13] Narahasi, T. (1977) Adv. Exp. Med. Biol. 84, 407–445.
[14] Albuquerque, E. X., Daly, J. W. and Witkop, B. (1971) Science 172, 995–1002.
[15] Ochs, S. and Worth, R. (1975) Science 176, 1087–1089.
[16] Boegman, R. J., Dephande, S. S., and Albuquerque, E. X. (1980) Brain Res. in press.
[17] Pestronk, A., Drachman, D. B. and Griffin, J. W. (1976) Nature 260, 352–354.
[18] MacLennan, D. H. (1974) Methods Enzymol. 32 B, 291–302.
[19] Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648–658.
[20] Scarpa, A. (1972) Methods Enzymol. 24, 343–351.
[21] Peter, J. B., Worsfold, M. and Fiehn, W. (1974) in: Exploratory Concepts in Muscular Dystrophy II (Milhorat, A. T. ed) Excerpta Medica, Amsterdam.
[22] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[23] Anderson, L. E. and McClure, W. O. (1973) Anal. Biochem. 51, 173–179.
[24] Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–78.
[25] Fiehn, W. and Hasselbach, W. (1969) Eur. J. Biochem. 9, 574–578.