FUNCTIONAL DIFFERENTIATION IN sB AND sC NEURONS OF TOAD SYMPATHETIC GANGLIA

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It is said that the sympathetic postganglionic fibres in mammals are nonmyelinated (Gaskell, 1886) with many exceptions (Langley, 1922). Some trials were made to classify the neurons in the sympathetic ganglia (de Castro, 1932; Dogiel, 1896) and the nerve fibres of the sympathetic nervous system (Bishop and Heinbecker, 1932; Eccles, 1935), and it was pointed out that two groups of nerve fibres out of four were distributed to different effectors according to conduction velocity (Eccles, 1935).

In the sympathetic nervous system of the bull frog, the myelinated B fibres were found in addition to the nonmyelinated in both the pre- and postganglionic fibres (Bishop and O'Leary, 1938). The presence of postganglionic B fibres was also found in the toad sympathetic nervous system. Intracellular recording revealed that the lumbar sympathetic ganglia consisted of two kinds of neurons; named sB and sC neurons after referring to the conduction velocities of their postganglionic fibres, and the membrane characteristics of these two neurons (Nishi, Soeda and Koketsu, 1965).

The present studies were carried out to see the relation between the conduction velocity and the size of sB and sC neurons, and the functional differences, if any, between these fast and slow efferent conduction systems in the toad's sympathetic nervous system.

METHODS

For the study of the intracellular electrical activities, only the tenth sympathetic ganglion of the toad (Bufo vulgaris japonica) was used at room temperature (17-23°C). Both male and female toads were employed throughout the year. In winter they were awakened from hibernation by warming with tepid bathing solution for a suitable time. The ganglion was dissected from pithed toads together with the tenth spinal nerve and sympathetic chain above the ganglion. It was mounted on a small platform in a plastic chamber, lifting with two pairs of platinum wires both pre- and postganglionic fibres up into the mineral oil covering the thin layer of Ringer's solution, which soaked the region.

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of the tenth ganglion. Two pairs of platinum wires were used as the stimulating and recording electrodes and could be switched, if necessary, to record ortho- and antidromic responses. Orthodromic responses were obtained by applying the stimulus to the sympathetic chain at the level of the seventh or eighth ganglion, and antidromic ones to the tenth spinal nerve. Intracellular recording was done under direct visual control by means of glass microelectrodes filled with 3 M KCl or 1 M CH₃COOK saturated with methyl blue, which was connected to the cathode-follower preamplifier, after removing as much connective tissues around the ganglion as possible. Ringer's constituents were as follows; NaCl 102.6, KCl 2.7, CaCl₂ 1.8, NaHCO₃ 2.4 mM/l respectively.

To record sympathetic components of action potentials from the skin or muscle nerve, the gastrocnemius nerve and N. cutaneus cruris medialis inferior were chosen. After each branch was freed from the skin or muscle, the origin of each one was traced back to the sciatic nerve and further to the tenth sympathetic ganglion retrogradely and removed from the toad as a whole, and set up in the chamber mentioned above.

Stimulation and recording was made in mineral oil, and the position of the stimulating electrode was the same as in the previous description.

For investigation of the secretory activities of the toxic glands or changes in drop intervals in perfusing the vascular systems of the lower limb, stimulation and recording were carried out in the pithed and totally eviscerated toads as follows; the tenth postganglionic fibre entering the eleventh spinal nerve was cut off and electrical potentials were monitored from the cut central end of it, stimulation being applied to the chain at the level as in the previous description with various intensities of 1 msec square pulse. A fine glass cannule was inserted into the unilateral communicating iliac artery as input and another into the communicating iliac vein as output. The communicating iliac artery and vein of the other side were ligated. The former was connected to a Mariot vessel by a silicone tube. Perfusate was of Ringer's solution, the constituents of which were described before, and perfusing pressure was about H₂O. Stimulating frequencies were 10 to 40 cps and drop intervals of perfusate were monitored by a drop counter.

Pharmacological agents were injected into the abdominal vein or sometimes into the ascending aorta to observe the secretory activities of skin toxic gland. To elicit the changes in drop intervals in perfusing the vascular systems of the lower limb by pharmacological agents, they were injected into the tube which connected the artery and the Mariot vessel.

Histological examinations were made on the ninth and tenth sympathetic ganglion which were fixed in 10% formaldehyde solution or in 1 to 2% osmium tetroxide solution. To identify the chromaffine tissues in the ganglion, fixation was done in chromic-bichromate solution and then formaldehyde solution for about 12 hours respectively at about 4°C in a refrigerator. After fixation, ordinary dehydration with ethyl alcohol was followed by clearing with xylene and then it was embedded in paraffine. Sectioning was made at 7 micra thickness. Staining was selected from the following methods as occasion arose: hematoxylin-eosin, van Gieson, Azan and Nissl staining. After fixation in osmium tetroxide solution, serial sections of 7 micra thickness were stained with Azan or van Gieson method and used for measurement of the neuron size.

In making the neuron size histogram, every seven sections was taken on photomicrographs and size measurement were made on all cut surface of neurons. The neuron soma showed near round or oval shape, so the mean value of long and short axes was supposed as an indicator of the soma size. It was intended that one histogram would be composed of about five hundred observations. Class intervals of the neuron size were determined as a step of 2 micra.
After determining whether the recording neuron was B or C, intracellular marking was carried out by means of microelectrode filled up with 1M CH₃COOK saturated with methylblue (Thomas and Wilson, 1966). The ganglion with marked cell was cut off from the other remaining tissues and fixed in 10% formaldehyde solution, and made into serial sections. As the counter staining, van Gieson or carmine staining was used. If the marked neuron was contained in more than one section in succession, the maximum value was selected and plotted on a cumulative diameter histogram.

Direct stimulation of individual B neuron was tried in situ to see the effect of the discharge of that particular neuron on secretions from the toxic gland. Bridge circuit for direct stimulation was that of Pilar and Martin (1963). Stimulation was applied to the chain at 1 cps before penetrating the B neuron with a microelectrode, and under these conditions secretion was not seen from the toxic gland. After confirmation of the B neuron by the chain stimulation, a cathodal current pulse of a suitable duration was applied intracellularly with enough strength to elicit a spike. Direct stimulation was carried out with 3 to 5 cps for 5 to 30 min.

RESULTS

Electrical activities of sympathetic ganglia

By presynaptic stimulation, two components were easily recorded from the tenth spinal nerve separately (Fig. 1A). Exact threshold measurement was not made but the fast component was activated by a much weaker stimulus than that needed to observe the slow component. Taking conduction velocities into account, the fast component was supposed to be the excitation of B fibres and the slow one to be of C fibres. Conduction velocities of B and C component were about 5 m/sec. and 50 cm/sec. respectively.

With the intracellular recording, two kinds of neurons were recognized by their conduction rates of pre- and postganglionic fibres. Almost all neurons identified to have B axons by antidromic stimulation were considered to have B preganglionic fibres by orthodromic stimulation, i.e. sB neurons (Fig. 1B a, b), and the neurons, the axons of which were C, were considered to have C preganglionic fibres, i.e. sC neurons (Fig. 1B c, d).

In trials to insert the microelectrode into the aiming neuron, each neuron was clearly seen in most preparations under the stereoscopic microscope. Relatively large or small neurons were seen as yellow center with a jelly-like halo; the former may be nuclei and the latter cytoplasm (Fig. 1C).

In the course of many trials to insert the pipette into the aiming neurons, the impression was that the relatively large neurons were usually innervated by B fibres and the small neurons by C fibres with a few exception.

In histological examinations relatively large and small neurons in size were also recognized. Some of the relatively large cut surfaces of the neurons had much larger nuclei and nucleolus than small cut surfaces of the neurons and some of the small cut surfaces of the neurons were stained much darker in the osmium fixed preparations (Fig. 1D). These relations seemed to be recognized in haematoxylin-eosin and Nissl stained preparations.
Fig. 1. Electrical activities and morphology of sympathetic neurons. A. Typical compound action potential recorded extracellularly. Stimulating and recording conditions; see Methods. Antecedent fast deflection; sB component. Successive slow deflection; sC component. Horizontal bar; 10 msec. Vertical bar; 100 μV. B. Typical intracellular responses of sB (a, b) and sC (c, d) neurons. a, c; orthodromic response. b, d; antidromic response. Horizontal bar; 10 msec. Vertical bar; 50 mV. C. Photomicrograph of a fresh sympathetic ganglion. Horizontal bar; 50 micra. D. Photomicrograph of a histological preparation of the tenth sympathetic ganglion. Two small neurons inserted with a large neuron are seen in upper region. Osmium fixation and Azan staining. Horizontal bar; 50 micra.
The size of the neurons in the sympathetic ganglia

A neuron size histogram was made of about five hundred cut surfaces of the neuron somata obtained from serial sections of the sympathetic ganglia. Neuron size, mean of the long and short axes, distributed from about 10 to 45 micra in every 12 histogram with skewness; the mode was present at about 20 micra and the median was about 28 micra (FIG. 2A, B). Another peak seemed to be present at about 35 micra. These tendencies were also recognized in every histogram regardless of weight, sex, or the level of the sympathetic ganglion, ninth or tenth. So the total of 12 histograms from 6 toads, 3 ninth and 9 tenth sympathetic ganglia, were added up into one histogram. Thus the total number of the cut surfaces of the neurons measured for their size were up to 6915. The tendencies seen in each histogram was emphasized as a consequence. Background continuous curves were obtained by a suitable method fitting the observed data (FIG. 3).

FIG. 2. Typical diameter histogram obtained from 280 g male toad. Diameter was used the same meaning as neuron size expressed by the mean of long and short axes of the cut surfaces of the neurons. A. Histogram of left tenth sympathetic ganglion from 500 cut surfaces of the neurons. B. Histogram of right sympathetic ganglion from 500 sections of the neurons. C. A + B. Cumulative histogram consisted of 1000 sections of the neurons. Ordinate; number of observations. Abscissa; diameter in micra. One class interval; 2 micra.
In these serial sections chromaffine tissues were often seen in various numbers in one ganglion. Isolated single chromaffine cells were difficult to recognize and usually they formed a mass or group, or sometimes were not recognized at all in one ganglion. Their cell size was smaller than the smallest neurons and identification was made by chrome-bichromate uptakeability (Fig. 4).

FIG. 3. Cumulative diameter histogram obtained from 12 ganglia and consisted of 6915 observations of neuron sections. Ordinate and abscissa; same as in Fig. 2.

The size of the intracellularly marked neurons

There were 42 cases of neurons marked intracellularly with methylblue including B and C neurons (Fig. 5). Eleven B neurons out of 26 marked B cells were able to record both ortho- and antidromic responses. Two cases were able to record only orthodromic response, and four cases, only antidromic response. They were estimated their latencies and their sizes were measured in serial sections. In 16 C neurons, the circumstances were as follows; both ortho- and antidromic response-2, only antidromic-1, orthodromic-12 respectively.

Fig. 5. Intracellularly marked sB (c) and sC (f) neuron and their ortho- and antidromic responses. Column A; sB neuron. Column B; sC neuron. a, d; orthodromic response. b, e; antidromic response. Horizontal bar; 10 msec. Vertical bar; 50 mV. (Horizontal bar at lower right side; 10 and 50 micra.)

When marked neurons were sectioned, they were contained in 3 to 6 sections in case of B neurons, and in 2 to 3 in case of C neurons in successful preparations. All marked neurons were plotted on the summed up histogram
From values indicated by background histogram, it was found that the maximum frequencies of marked neuron size was at the larger positions both in B and C neurons. It was difficult to obtain ideal marking of neurons showing good electrical activities especially in the case of C neurons. When a good spike is recorded, marking was not so good and in case of low voltage spike or a good spike followed by subsequent deterioration, fairly good marking was obtained.

**Sympathetic components in skin and muscle nerve**

Action potentials recorded from skin and muscle nerve by applying stimulation to the sympathetic chain was supposed to be of B and C component, with respect to their conduction rates. From the skin nerve, N. cutaneus cruris medialis inferior, B and C component were recorded in most cases (Fig. 7A), and conduction velocities of B component was from 3.9 to 7.3 m/sec, mean of 32 cases was 5.6 m/sec. Of C, from 35 to 180 cm/sec, mean of 23 cases was 53 cm/sec. It was not always possible to record both B and C component at the same time, and B component was much more easily recorded than C component from the skin nerve; nerve bundles which contained both B and C fibres were 23; only B was 9; in both cases out of 32.

From the muscle branch, gastrocnemius, only action potential of the C component was recorded (Fig. 7B). It was difficult to observe sympathetic component from the muscle nerve; only 16 cases out of 32 tried preparations showed C component. In the other 16 cases nothing was observed.

**The effect of sympathetic nerve stimulation on the toxic gland and the vascular system of the lower limb**

With the stimulus intensity by which only B component was activated, train stimuli, 30 cps, consisting of 1 msec. square pulses were applied to the sympathetic chain. The action potentials were monitored from the central
Fig. 7. Typical sympathetic component recorded from skin nerve (A) and muscle nerve (B). N. cutaneus cruris medialis inferior and a branch to enter the gastrocnemius muscle were chosen as representative of skin and muscle nerve. | Horizontal bar; 20 msec. Vertical bar; 50 μV.

Fig. 8. Changes in drop intervals in perfusing the vascular system of the lower limb. Stimulating and recording conditions and procedure; see Methods. a; no interval changes with train stimuli each of which activated only sB component (A). b; interval changes with stimuli activated both sB and sC component at the same time (B). c; time marker: 1 division=1 sec. d; time mark of stimulation. Black bar expresses 42 sec. C; time course of interval changes seen in b. Ordinate; drop interval presented as percentage of control intervals before stimulation. Abscissa; time in sec. Horizontal bar shows stimulation time. A and B; horizontal bar; 10 msec. Vertical bar; 100 μV.
cut end of the tenth postganglionic fibre. Under these conditions, maximum 72 sec. stimulation caused no changes in drop interval in perfusing the vascular system, but clear secretions of the toxic gland of the lower limb was seen in all cases (Fig. 8A, a). With more intense stimulus enough to activate C component in addition to B component, it was possible to see the drop interval prolongation (Fig. 8B, b). Its time course was plotted in Fig. 8C. Decrease of prolonged drop intervals after reaching maximum value was scarcely recognized during the continuous train pulse stimulation in which 30 cps was continued for about 4 min.

When 1 ml of $2.5 \times 10^{-7} \text{g/ml}$ adrenaline (Nakarai, Kyoto) or noradrenaline (Nakarai) was injected into the iliac artery (see Methods), a similar prolongation of drop intervals was seen for about 15 sec. and maximum interval changes reached about 150% of the control interval value before stimulation or injection.

The relation of reached maximum drop interval and the stimulation time was obtained from several toads and plotted in Fig. 9. The reached maximum drop interval increased with an increase in the stimulation time until it reached saturation level.

![Graph](image)

**Fig. 9.** Relation between stimulation time and maximum interval. Ordinate and abscissa; same as in Fig. 8C.

**The effect of pharmacological agents on the toxic gland**

Pharmacological agents injected were acetylcholine (Tokyo Kasei, Tokyo), serotonin (Nakarai), tyramine (Nakarai), dopamine (Sigma), adrenaline (Nakarai) and noradrenaline (Nakarai) dissolved in Ringer's solution, from 1 to 5 mg in total, i.e. about 5 to 25 mg/kg and minimum doses tried were 0.4 mg/kg.
The agents which caused secretion from the toxic glands were acetylcholine, adrenaline and noradrenaline. But, after the sciatic nerve was cut off at the end of the tenth sympathetic ganglion, acetylcholine injection could not cause the secretion of the toxic gland. Under these conditions, further injection of adrenaline or noradrenaline could elicit secretory activity of the toxic gland in the same experimental toad.

**DISCUSSION**

Postganglionic fibres of toads are divided into three components, B, C₁ and C₂ by potential recording (Nishi, Soeda and Koketsu, 1965). With pre-synaptic stimulation, it was difficult to record three components clearly, and two deflections were usually able to record. It might be not so unreasonable to consider that the tenth sympathetic ganglion consisted of two efferent conduction systems, sB and sC system which might include C₁ and C₂ component. To distinguish B and C neurons by intracellular recording with respect to conduction velocities of pre- and postganglionic fibres, observations of Nishi et al. were used (Nishi, Soeda and Koketsu, 1965).

From the observations of fresh sympathetic ganglia, neurons in the ganglia might be divided into two groups by their soma size. It was difficult to consider that all the small cut surfaces were derived from those of small part of the large neurons, because of differences of the nucleus or nucleolus size and stainability of the cytoplasm between some of the relatively large and small cut surfaces of the neurons. Therefore it is probable that some of the small cut surfaces might belong to the small neurons. So it is suggested that the tenth sympathetic ganglion would consist of two kinds of neurons with respect to their soma size, though much more detailed histological investigations must be tried.

Neuron size histograms obtained from serial sections showed skewness and two peaks in cumulative histogram, which was supposed that two kinds of neurons would be present in the ganglion with respect to their soma size, i.e. large and small neurons. According to the curves obtained from suitable fitting, it was shown that there would be overlapping between the large and small neurons, and the transition from one to the other group is continuous. So it was impossible to divide all neurons into two groups clearly and to know the exact ratio of the number of B and C neurons in one ganglion.

Reconstruction of many neurons in a ganglion should be carried out to obtain the exact diameter of the neurons and classify the neurons in the ganglion according to their soma size.

In the frog sympathetic ganglia (Rana pipiens), chromaffine cells were not encountered (Pick, 1963). In the toad sympathetic ganglia, some groups of chromaffine cells were usually recognized, varying in mass, position and
number in each ganglion. It was proposed by R. M. ECCLES and B. LIBET that chromaffine cells might participate in P-potential of rabbit cervical sympathetic ganglia (ECCLES and LIBET, 1961), whilst a few or no chromaffine cells in the superior cervical ganglion of some mammals were reported (NORBERG, 1965). TOSAKA, CHICHIBU and LIBET also proposed the adrenergic unit as taking part in slow inhibitory potentials of the bullfrog sympathetic ganglia (TOSAKA, CHICHIBU and LIBET, 1968). The present author recorded spontaneous positive deflections from 0.5 to 3 mV together with miniature excitatory postsynaptic potentials at the same time in the same neurons of the toad sympathetic ganglia (HONMA and KOYANO, 1966) and this is now under the investigation. The functions of the chromaffine cells in the toad sympathetic ganglia are still yet unknown. Some tests on this problem were reported in rat cervical sympathetic ganglia (SIEGRIST, et al., 1968).

In measuring the membrane specific resistance of B and C neurons, NISHI et al. (1965) measured the neuron diameter in fresh preparations to obtain the surface area. They measured 5 B and 4 C neurons and showed that B neurons were larger than C neurons. It was also recognized in this examination that relatively large neurons showed B response and small neurons C response. To make more sure of this, the intracellular marking method was adopted and it seemed to become more certain that B system might consist of large neurons and C system of small neurons. The maximum frequencies of marked neuron size deviated to much large size from the values suggested by the suitably fitted curve. This may be derived from the additive action of shrinkage of the neuron somata; selectivity of the microelectrode liable to penetrate large neurons; extraction of neurons under direct visual control; selection of maximum value from the marked cells and so on.

The author had the belief that the skin might be supplied with B and C sympathetic component, and the muscle with only the C component, and then he found the report of HUTTER and LOEWENSTEIN that the same relations had been found in frog (Rana pipiens) (HUTTER and LOEWENSTEIN, 1955). It is not so unnatural to suppose that the B component would innervate the effector characteristic of the skin, and the C component the common one, and this was tested during perfusion of the vascular system of the lower limb and by stimulating the sympathetic chain. The B component was recorded from the central cut end of the sympathetic postganglionic fibre, when the toxic glands were activated and the C component was recorded only when the response of the vascular system was produced. These results suggest that the B system might innervate the toxic gland of the skin, and the C system the vascular system of the lower limb.

Direct stimulation of the single B neurons in situ was tested to see the effect of the discharge of one particular neuron on the toxic gland and to determine the innervation area, if possible. Thirteen neurons from 5 toads
were stimulated with various frequencies but secretion of the toxic gland was not observed.

To test the dependence of drop intervals on stimulation frequencies, 1 msec square pulses from 10 to 40 cps were applied to the chain, each of which had enough intensity to activate both B and C component at the same time. The initial rising phase of drop intervals increased its steepness gradually up to 30 cps. The effect with 40 cps was almost the same as 30 cps, and so the optimal stimulus frequency was considered to be about 30 cps to observe the drop interval changes in perfusing the vascular system.

To see the correlation between the size of a neuron and its axon diameter, sizes of the marked neurons were plotted against their axon diameter, which were calculated from the equation $V=2.05 \, D$, where $V$ is conduction velocity per sec., and $D$ is axon diameter in micron, though this is for a bull frog (TASAKI, 1953). This is only applicable to myelinated B fibres, and in the case of nonmyelinated C fibres, soma size was plotted against the conduction

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![Graphs](image.png)

**Fig. 10.** Relation between the soma size and axon diameter. A; axon diameter (ordinate) and soma size (abscissa) of 11 B neurons. B; diameter of preganglionic fibres (ordinate) and soma size innervated by them (abscissa) of 12 B neurons. C; conduction velocities of preganglionic fibres (ordinate) and soma size innervated by them (abscissa) of 14 C neurons.
velocity of preganglionic fibres instead of the axon diameter, because pre- and postganglionic fibres are mutually proportional in conduction velocity of both B and C neurons (Nishi, Soeda and Koketsu, 1965), and the antidromic response of marked C neuron was only one case. The impression from Fig. 10 was that in case of B neurons, soma size and its axon diameter probably might have much dense correlation than that of soma size and the diameter or conduction velocity of preganglionic fibres. The number of marked neurons were too small to do a statistical treatment to see these relationship and the presented graph could produce no conclusive correlations at all.

In histological sections of postganglionic fibres entering the eleventh spinal nerve, myelinated nerve fibres were recognized besides nonmyelinated ones in fairly good numbers. With anodal current passing on antidromic stimulation, Nishi et al. recorded the M, IS and SD spikes from B neurons (Nishi, Soeda and Koketsu, 1965). This was tried in several B neurons and the same responses were observed.

In mammals, the neurotransmitter in the sympathetic nervous system is considered to be noradrenaline, but in the frog, adrenaline would play the role (Euler, 1946; Azuma, Binia and Visscher, 1965; Angelakos, Glassman, Millard and King, 1965). To discover the transmitters in these B and C neurons of the toads, pharmacological agents tested were within narrow limits and impartial, and the determination of the substances in perfusate in stimulation of the sympathetic chain was not made. But the present examination suggested that myelinated adrenergic or catecholamine containing nerve fibres might be present in the toad sympathetic system. So the histochemical demonstration of catecholamines in the toad sympathetic ganglion and the toxic gland of the skin were tested after Falck et al. (Falck and Owman, 1965) and a part of the results were reported (Honma, 1969) and the rest will be prepared in the near future.

SUMMARY
1. The toad tenth sympathetic ganglion consisted of two efferent conduction systems according to conduction velocity and threshold, i.e. sB and sC systems.
2. Neurons in the ganglion might be classified into two groups at least with respect to their soma size, i.e. into large and small neurons.
3. It was suggested by the results of marking the neurons intracellularly that the large neurons might compose the fast sB conduction system, and small neurons sC system.
4. The possibility was presented that sB system might innervate the toxic gland of the skin, and sC system the vascular system of the lower limb.
5. Both sB and sC systems were assumed to consist of adrenergic neurons or catecholamine containing neurons.
SYMPATHETIC FUNCTIONAL DIFFERENTIATION

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