The Effect of Preganglionic Stimulation on the Incorporation of L-[U-¹⁴C]Valine into the Protein of the Superior Cervical Ganglion of the Guinea Pig

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(Received 30 January 1970)

1. Superior cervical ganglia from the guinea pig carry out an energy-dependent incorporation of L-[¹⁴C]valine into protein *in vitro*. 2. Stimulation of the preganglionic nerve at a physiological frequency for more than a few minutes decreases the ability of the ganglia to incorporate labelled valine into protein.

Stimulating the preganglionic nerve *in vitro* causes a variety of metabolic changes in mammalian sympathetic ganglia. The rates of oxygen consumption, CO₂ evolution, lactate production, glucose uptake (Larrabee, 1958; Horowicz & Larrabee, 1958, 1962*a,b*) and glycogen breakdown (Härkönen, Passonneau & Lowry, 1969) are increased and there is an increased rate of incorporation of ¹⁴C from [¹⁴C]glucose and of ³²P_i into phosphatidylinositol located in some postsynaptic structure (Larrabee, Klingman & Leicht, 1963; Larrabee & Leicht, 1965; Larrabee, 1968). In this paper the effect of stimulating the preganglionic nerve on the incorporation of tracer amounts of L-[¹⁴C]valine into ganglionic protein is described and compared with similar findings obtained from an electrically stimulated preparation of guinea-pig cerebral cortex (Jones & Banks, 1970*a*).

METHODS

Excision of superior cervical ganglia. Male guinea pigs (350–500 g) were anaesthetized with an intraperitoneal injection of urethane (1500 mg/kg) before an incision was made in the neck to expose the sterno-hyoid muscle. The animals were then killed by perforating the diaphragm and cutting the heart. This allowed both superior cervical ganglia to be removed rapidly from a bloodless field after fine silk threads [Davis Geck. Cyanamid (G.B.), London W.C.2, U.K.] had been tied to their pre- and post-ganglionic nerves. Immediately after excision the ganglia were placed in ice-cold, sterile, bicarbonate saline, pH 7.4, [NaCl, 136 mM; KCl, 5.6 mM; NaHCO₃, 16.2 mM; NaH₂PO₄, 1.2 mM; MgCl₂, 1.2 mM; CaCl₂, 2.2 mM; glucose, 5.5 mM; gassed with O₂ + CO₂ (95:5)], where they remained for up to 10 min before being placed in the stimulation chamber. In a few experiments (indicated in the text) a longitudinal incision was made through the centres of both the control and stimulated ganglia with a sterile Swan Morton no. 11 scalpel blade to decrease the possibility of anoxia at the centre of the ganglion. The left and right

superior cervical ganglia used in these studies were of a very similar shape and had wet weights of 3.34 ± 0.19 mg (11) and 3.29 ± 0.13 mg (11) respectively. (Results are given as means $\pm$ s.e.m., with numbers of observations in parentheses.) If, as happened occasionally, the excised ganglia differed markedly in size or shape, they were discarded. The protein content of the ganglia was 0.35 ± 0.04 mg (16) or 10.6% of the wet weight and their dry weight was 0.83 ± 0.03 mg (8) or 25.1% of the wet weight.

Stimulation chamber. The stimulation chamber was similar to that described by Roch-Ramel (1964) except that it allowed the control and stimulated ganglia to be incubated together and was provided with a water jacket (Fig. 1). The vessel contained 75 ml of bicarbonate saline. Each ganglion was supported by fine silk threads drawn into two glass pipettes; the two pipettes supporting the stimulated ganglion contained platinum electrodes that made contact with the saline within the tubes (Fig. 1). The other three electrodes consisted of platinum wires fused into the ends of sealed glass tubes. Before each experiment the assembled stimulation chamber and the three stock solutions used to make the saline were sterilized in an autoclave at 15 lb/in² for 15 min.

Stimulation of ganglia. The silk threads tied to the pre- and post-ganglionic nerves were joined to threads passing through the appropriate pipettes and the ganglia (both obtained from the same animal) were pulled into the position shown in Fig. 1 so that the pre- and post-ganglionic nerves were drawn into the pipettes. The nerves fitted the narrow apertures of the pipettes tightly so that a high-resistance path was established between the two recording electrodes and between the two stimulating electrodes. The earth electrode made contact with the pre-ganglionic nerve between the ganglion and the pair of stimulating electrodes.

Once the ganglia had been introduced into the apparatus, they were preincubated for 1 h at 37°C (unless otherwise stated) before the simultaneous addition of L-[U-¹⁴C]valine (270 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) and starting of the electrical stimulation. For stimulation periods of less than 1 h 7.5 μ Ci of L-[¹⁴C]valine was added, but for periods of from 1 to 4 h only 1.5 μ Ci was used.

Fig. 1. Diagram of the stimulation chamber. The water-jacketed vessel contained 75 ml of bicarbonate saline gassed with $O_2 + CO_2$ (95:5); the ganglion preparations were drawn into narrow-mouthed pipettes by fine silk threads (dotted line). The two stimulating electrodes were attached to a square-wave stimulator (S) and the two recording electrodes were connected to a low-level preamplifier (PA) which amplified the action potential for display on an oscilloscope (O). An electrode at earth potential made contact with the preganglionic nerve between the stimulating electrodes and the ganglion.

The ganglia were stimulated supramaximally at 4 pulses/s by square waves of 0.1 ms duration generated by a Palmer Electronic Square-Wave Stimulator. The post-ganglionic action potential was amplified by a Tektronix type 122 low-level preamplifier and displayed on a Tektronix type 502A double-beam oscilloscope. A stimulation frequency of 4 pulses/s was chosen as it is within the physiological range for sympathetic nerves (Iversen, 1967; Blakely, 1968).

In some experiments not involving electrical stimulation single ganglia were incubated in large test tubes maintained at 37°C and containing 25 ml of saline gassed with $O_2 + CO_2$ (95:5) as shown in Fig. 2. Preincubation was carried out in one tube and at zero time the ganglion holder was transferred to another tube containing oxygenated saline, L-[^{14}C]valine and the drug whose action was being studied.

Determination of the specific radioactivity of the ganglion protein. To measure the specific radioactivity of the protein, ganglia from two incubation experiments were required. In one incubation the left ganglion was stimulated and the right acted as control and in the other incubation the roles were reversed. At the end of the incubation the pre- and post-ganglionic nerves were removed and the ganglia were placed in homogenizer

Fig. 2. Diagram of incubation tube. A single ganglion was supported by silk threads tied to hooks attached to the gassing tube. The vessel contained 25 ml of bicarbonate saline gassed with $O_2 + CO_2$ (95:5) and was incubated at 37°C in a water bath.

tubes containing 1 ml of ice-cold 5% (w/v) trichloroacetic acid + 0.5% casein hydrolysate. The pooled stimulated ganglia and the pooled control ganglia were homogenized by hand in glass homogenizers of the type described by Larabee *et al.* (1963). Each homogenate was diluted with a further 3 ml of 5% trichloroacetic acid + 0.5% casein hydrolysate and was centrifuged at 1500g for 3 min; the resulting pellet was washed once by resuspension in 4 ml of 5% trichloroacetic acid + 0.5% casein hydrolysate and twice by resuspension in 4 ml of 5% trichloroacetic acid. It was then resuspended in 5% trichloroacetic acid for the third time and incubated at 90°C for 30 min to hydrolyse nucleic acids. The tubes were then cooled in ice for 30 min before being centrifuged. The resulting pellets were extracted twice by resuspension in 4 ml of ice-cold chloroform-methanol (1:1, v/v) and once with 4 ml of ice-cold diethyl ether. The extracted pellets were finally resuspended in 1 ml of glass-distilled water.

Samples of the resuspended pellets (0.5 ml) were placed in glass vials together with 10 ml of liquid scintillator [NE 220+NE 221 (1:1, v/v); Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K.]. Their radioactivity was measured by using a Nuclear-Chicago liquid-scintillation counter. The counting efficiency, determined by the channels-ratio method, was about 75%.

Protein was determined in samples (0.2 ml) of the resuspended and extracted pellets by adding 1 ml of 2% Na_2CO_3 in 0.1 M-NaOH containing 0.02% sodium potassium tartrate and 0.01% $CuSO_4$, 10 min before adding 0.1 ml of a 1:1 (v/v) mixture of Folin reagent (BDH Chemicals Ltd., Poole, Dorset, U.K.) and water. The

resulting mixture was immediately heated at 50°C for 10 min and the extinction of the solution was read at 500 nm with a Zeiss PMQII spectrophotometer fitted with a 0.2 mm slit.

ATP determination. ATP was determined by a modification of the firefly luminescence method as described by Banks, Mangnall & Mayor (1969).

Sources of drugs. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., cycloheximide from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., chloramphenicol from Parke, Davis and Co. Ltd., Hounslow, Middx., U.K. and actinomycin D from Merck, Sharp and Dohme Ltd., Hoddesdon, Herts., U.K.

RESULTS

Evidence for the effectiveness of stimulation. On increasing the stimulating voltage there first appeared a single biphasic action potential, due to type B cells (Fig. 3a), which increased in height before a second, slower, biphasic action potential, due to type C cells (Fig. 3b), developed. The profile of these potentials was similar to that found for the rat superior cervical ganglion by Dunant (1967) and confirmed in this laboratory, except that the B spike was higher, instead of smaller, than the C spike. The postganglionic origin of the action potentials was confirmed by their abolition in the presence of 10 μ M-hexamethonium bromide and by incubation in Ca²⁺-free saline. Action potentials in the pre-ganglionic nerve were not diminished by these treatments. The height of the post-ganglionic

Fig. 3. Postganglionic action potentials. (a) Potential from type B ganglion cells obtained by submaximal stimulation. (b) Potentials generated by type B and type C ganglion cells as a result of supramaximal stimulation. The ganglion preparation was incubated at 37°C in bicarbonate saline gassed with O₂ + CO₂ (95:5) and stimulated at 4 pulses/s with a pulse width of 0.1 ms.

action potentials increased during the course of some experiments, but diminished slightly in the majority.

Evidence for the incorporation of L-[¹⁴C]valine into protein. The observed incorporation of labelled valine into the trichloroacetic acid-washed lipid-extracted material obtained from ganglia incubated in the presence of L-[¹⁴C]valine can be equated with a genuine incorporation of label into protein for the following reasons. (1) The amount of label incorporated increased linearly with time (Fig. 4). (2) Digestion of samples of the extracted pellets with pronase at 37°C for 24 h liberated 93% of the radioactivity into solution. (3) Incubations carried out for 4 h in the presence of cycloheximide (100 μ g/ml), puromycin (100 μ g/ml) or chloramphenicol (100 μ g/ml) decreased the incorporation of label by 97.4, 94.0 and 15.1% respectively (Table 1).

Fig. 4. Time-course of the incorporation of L-[¹⁴C]valine into ganglion protein after incubation of ganglia in bicarbonate saline gassed with O₂ + CO₂ (95:5) at 37°C in the presence of 20 nCi of L-[¹⁴C]valine (270 μ Ci/mmol)/ml for varying periods of time. Before the addition of label the ganglia were preincubated for 1 h. Results are shown as means $\pm$ S.E.M. (indicated by vertical bars).

Table 1. *Effect of antibiotics on the incorporation of [14 C]valine into the protein of the superior cervical ganglion*

The ganglia were incubated in the presence of the antibiotic and [14 C]valine for 4 h after a 1 h preincubation.

Antibiotic added	[14 C]Valine incorporated		
	Control (c.p.m./mg of protein)	+ Antibiotic (c.p.m./mg of protein)	% inhibition
Cycloheximide (100 μ g/ml)	5752	150	97.4
Puromycin (100 μ g/ml)	6932	416	94.0
Chloramphenicol (100 μ g/ml)	7008	5950	15.1
Actinomycin D (1 μ g/ml)	7788	6150	21.0

Fig. 5. Effect of stimulation on the incorporation of L-[14 C]valine into protein of guinea-pig superior cervical ganglia. The ganglia were stimulated supramaximally at 4 pulses/s with a pulse width of 0.1 ms for various periods of time in bicarbonate saline gassed with $O_2 + CO_2$ (95:5). For incubation periods of less than 1 h the medium contained 75 nCi of L-[14 C]valine/ml and for periods of 1 h or longer, 20 nCi/ml. A preincubation period of 1 h in the absence of labelled valine preceded the onset of stimulation. ●, Normal ganglia incubated at 37°C; ○, normal ganglia incubated at 25°C; △, cut ganglia (see the Methods section) incubated at 37°C. The curve was drawn by eye through the points obtained with normal ganglia at 37°C.

When, after preincubation for 1 h, ganglia were incubated for 17.5 min in the presence of cycloheximide (100 μ g/ml) and [14 C]valine was present

during the last 7.5 min of the incubation, the incorporation of label was decreased by 92.8%. These observations indicate that the incorporation of label into acid-precipitable material had the sensitivity to antibiotics to be expected of a mammalian rather than of a bacterial protein-synthesizing system. (4) Incorporation of label over a period of 4 h was decreased by 93% when the saline containing [14 C]valine lacked glucose and was gassed with a mixture of $N_2 + CO_2$ (95:5). This indicates that the incorporation of label is an energy-dependent process.

Effect of stimulating the preganglionic nerve on the incorporation of L-[14 C]valine into protein. When ganglia were stimulated supramaximally at 4 pulses/s for 7.5 min at 37°C the incorporation of label into protein was about 30% greater than the control value (Fig. 5). This enhanced incorporation was only transient, however, so that after 1 h of stimulation it had given way to an inhibition of about 30%, which persisted for the following 3 h (Fig. 5). A number of 3 h incubations at 4 pulses/s were also carried out at 25°C, after 1 h of preincubation at 25°C, in an attempt to decrease the possibility that anoxic conditions at the centre of the ganglion contributed to the inhibition of incorporation observed during prolonged periods of stimulation at 37°C (Larrabee, 1958). Fig. 5 shows that decreasing the temperature to 25°C did not diminish the extent of the inhibition of incorporation of [14 C]valine into protein caused by electrical stimulation. In a further attempt to eliminate the possibility that the existence of an anoxic core contributed to the inhibition of incorporation, ganglia that had had their centres pierced by a longitudinal incision were incubated and stimulated at 37°C. Fig. 5 shows that this procedure also failed to cause a significant change in the pattern of incorporation as a result of electrical stimulation.

The effect of stimulation on the ATP content of ganglia. Stimulating ganglia at 4 pulses/s for 4 h decreased the ATP content from a 4 h control of 3.6 ± 0.1 nmol/ganglion (6) (or 1.1 μ mol/g wet wt.) to 2.8 ± 0.1 nmol/ganglion (6) (or 0.85 μ mol/g wet wt.). The value of 1.1 μ mol/g wet wt. for the ATP content of the control ganglion agrees well with the values obtained by other workers (see Härkönen *et al.* 1969).

DISCUSSION

The results presented above show that stimulation of the preganglionic nerve leads first to an enhanced incorporation of L-[14 C]valine into the protein of guinea-pig superior cervical ganglia but subsequently causes an inhibition of incorporation. This pattern of incorporation is similar to that found in chopped cerebral-cortex tissue subjected to

electrical stimulation (Jones & Banks, 1970a). In the latter preparation the initial enhancement of incorporation correlated well with an increased rate of uptake of labelled valine into the tissue (Jones & Banks, 1970b). The increased incorporation observed during the first few minutes of stimulation in the present studies may occur for the same reason. The inhibition of incorporation that became evident after longer periods of stimulation may be a consequence of any or all of several changes that occur as a result of electrical activity. First, as shown in the present study, the ATP content of ganglia falls by some 30% during a 4h period of stimulation. This alone might decrease the ability of the stimulated ganglia to incorporate labelled valine into protein. The ATP content of electrically stimulated chopped cerebral cortex tissue is also diminished at a time when the incorporation of labelled valine into protein is inhibited (Jones & Banks, 1970a).

The diminished ATP content and the elevated rates of glycolysis and respiration observed in stimulated ganglia (Larrabee, 1958; Horowicz & Larrabee, 1958, 1962a,b) are probably consequences of an increase in the rate of consumption of ATP by the sodium pump, which in its turn is stimulated by the increased intracellular concentration of Na^+ and by the loss of intracellular K^+ that accompanies the propagation of action potentials. By analogy with other protein-synthesizing systems, the partial replacement of intracellular K^+ by Na^+ might be expected to decrease the rate of incorporation of labelled valine into protein by direct action on the protein-synthesizing machinery (Coleman, 1969; Smith, 1969). Further, the increase in intracellular Na^+ that accompanies electrical stimulation in chopped cerebral-cortex tissue is associated with an efflux of labelled valine from the tissues (Jones & Banks, 1970b). A similar loss of [^{14}C]valine from stimulated ganglia might also contribute to the decrease in the incorporation of label into protein.

The present studies indicate that protracted stimulation of a sympathetic ganglion at a physiological frequency diminishes its ability to incorporate [^{14}C]valine into protein. However, it is still possible that the rates of synthesis of some proteins may increase as a consequence of stimulation although the synthesis of the majority must be impaired. Further, the present experiments do not enable a distinction to be made between changes occurring in the neurones and those taking place in neuroglia. In this context it is noteworthy that Pevzner (1965) has cytospectrophotometric evidence that during periods of electrical stimulation the protein content of neurones in cat superior cervical ganglia increases while protein is lost from the associated neuroglia. However, the physiological significance of this observation is not clear since the

preganglionic nerve was stimulated at the very high frequency of 300 pulses/s.

To date, three studies on the effect of electrical stimulation on protein synthesis by cell-containing preparations of brain have been carried out (Orrego & Lipmann, 1967; Jones & Banks, 1970a; Huttunen, 1969) and all agree that stimulation at about 100 pulses/s for several minutes diminishes the rate of incorporation of labelled amino acids into protein. The findings of the present study with a neuronal preparation that is more physiologically intact than the chopped cortical tissue is in general agreement with these observations.

There are, however, two reports that electrical stimulation of isolated nerve trunks (presumably containing few neurone cell bodies) leads to an increased incorporation of labelled amino acids into protein (Luxoro, 1960; Fischer & Litvak, 1967). In both instances the measured incorporation of label into protein could possibly be accounted for by a stimulation-induced loss of amino acids from the axons providing radioactive precursors for protein synthesis by the adjacent Schwann cells. However, it cannot be excluded that the increased incorporation of label did occur within the axons, since there is good evidence that synaptosomal mitochondria can incorporate labelled amino acids into protein and some indication that a typical eukaryotic ribosomal system for protein synthesis may exist within synaptosomes (Austin & Morgan, 1967; Morgan & Austin, 1968).

This work was supported by a grant from the Medical Research Council. I am most grateful to Mr B. Christian and Miss R. Grigas for their assistance.

REFERENCES

- Austin, L. & Morgan, I. G. (1967). *J. Neurochem.* **14**, 377.
- Banks, P., Mangnall, D. & Mayor, D. (1969). *J. Physiol., Lond.*, **200**, 745.
- Blakeley, A. G. (1968). *Proc. R. Soc. B*, **171**, 201.
- Coleman, G. (1969). *Biochim. biophys. Acta*, **174**, 395.
- Dunant, Y. (1967). *J. Physiol. Paris*, **59**, 17.
- Fischer, S. & Litvak, S. (1967). *J. cell. Physiol.* **70**, 69.
- Härkönen, M. H. A., Passonneau, J. V. & Lowry, O. H. (1969). *J. Neurochem.* **16**, 1439.
- Horowicz, P. & Larrabee, M. G. (1958). *J. Neurochem.* **2**, 102.
- Horowicz, P. & Larrabee, M. G. (1962a). *J. Neurochem.* **9**, 1.
- Horowicz, P. & Larrabee, M. G. (1962b). *J. Neurochem.* **9**, 407.
- Huttunen, M. (1969). Precirculated Academic Dissertation: University of Helsinki.
- Iversen, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*, p. 85. Cambridge University Press.
- Jones, C. T. & Banks, P. (1970a). *Biochem. J.* **118**, 791.

- Jones, C. T. & Banks, P. (1970b). *Biochem. J.* **118**, 801.
- Larrabee, M. G. (1958). *J. Neurochem.* **2**, 81.
- Larrabee, M. G. (1968). *J. Neurochem.* **15**, 803.
- Larrabee, M. G., Klingman, J. D. & Leicht, W. S. (1963). *J. Neurochem.* **10**, 549.
- Larrabee, M. G. & Leicht, W. S. (1965). *J. Neurochem.* **12**, 1.
- Luxoro, M. (1960). *Nature, Lond.*, **188**, 1119.
- Morgan, I. G. & Austin, L. (1968). *J. Neurochem.* **15**, 41.
- Orrego, F. & Lipmann, F. (1967). *J. biol. Chem.* **242**, 665.
- Pevzner, L. Z. (1965). *J. Neurochem.* **12**, 993.
- Roch-Ramel, F. (1964). *Helv. physiol. pharmac. Acta*, Suppl. no. 8, p. 1.
- Smith, D. W. E. (1969). *J. biol. Chem.* **244**, 896.