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THE LONG-TERM REGULATION OF TYROSINE HYDROXYLASE ACTIVITY IN CULTURED SYMPATHETIC GANGLIA: ROLE OF GANGLIONIC NORADRENALINE CONTENT

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1 An organ culture system is described for the *in vitro* maintenance of superior cervical sympathetic ganglia taken from mice of any age. The relation of tyrosine hydroxylase (T-OH) activity to ganglionic noradrenaline (NA) content has been investigated under various culture conditions.

2 Depolarizing stimuli such as raised extracellular potassium and ouabain evoked increases of approximately 70% in the T-OH activity of cultured ganglia over a 48 h period. Exposure to a high concentration of potassium (high K^+) for 30 min at the start of a 48 h culture was sufficient to elicit significant increases in T-OH activity.

3 Depolarization-induced rises in T-OH activity were observed after culture in the presence or absence of nerve growth factor.

4 The NA content of ganglia, cultured for 48 h in the presence of high K^+, ouabain, reserpine, clorgyline and α-methyl-*p*-tyrosine, showed no constant relation to their T-OH activity.

5 Dibutyryl cyclic adenosine 3′-5′-monophosphate (dibutyryl cyclic AMP) mimicked high K^+ in its effect on ganglionic T-OH activity and NA content. Theophylline enhanced the potassium effects.

6 Rises in the T-OH activity of ganglia cultured in the presence of high K^+ and dibutyryl cyclic AMP were abolished if the protein synthesis inhibitors cycloheximide or actinomycin D were present in the culture medium.

7 It is concluded that the link between prolonged depolarization and rises in T-OH activity does not seem to depend upon changes in ganglionic NA content. In the intact animals, trans-synaptic modulation may take the form of a depolarization-induced rise in the cyclic AMP content of sympathetic ganglionic neurones leading to nuclear mediated synthesis of T-OH.

Introduction

The regulation of neurotransmitter synthesis is an essential adaptive property of nervous tissue. In the adrenergic nervous system there seems little doubt that tyrosine hydroxylase (T-OH), the rate-limiting enzyme for noradrenaline (NA) synthesis, can be rapidly activated to maintain transmitter supply in the event of acute increases in the activity of peripheral adrenergic neurones and the adrenal medulla (for review see Udenfriend & Dairman, 1971). These acute fluctuations in T-OH activity are rapidly reversible, do not seem to involve *de novo* enzyme synthesis and appear to be governed by the concentration of freely diffusible NA within the adrenergic neurone (Ikeda, Fahien & Udenfriend, 1966).

A second type of regulatory mechanism seems to exist through which adrenergic nervous tissue can react to long-term changes in transmitter demand. Evidence has recently accumulated that trans-synaptic influences play an important part in this long-term regulation which appears to operate through synthesis of new enzyme. Thus experimental situations which result in a prolonged rise in the impulse traffic in adrenergic neurones and adrenal medullary tissue in intact animals produce rises in tissue activity of biosynthetic enzymes such as T-OH (for reviews see Molinoff & Axelrod, 1971 and Thoenen, 1972). A characteristic of such enzyme induction is the ability of protein synthesis inhibitors to

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prevent the increases in enzyme activity (Thoenen, Mueller & Axelrod, 1969a; Mueller, Thoenen & Axelrod, 1969; Otten, Paravicini, Oesch & Thoenen, 1972).

The mechanism of the neurally mediated increases in enzyme activity is not yet understood, but recent reports of T-OH induction in isolated sympathetic ganglia (Mackay & Iversen, 1972a) and adrenal medulla (Silberstein, Lemberger, Klein, Axelrod & Kopin, 1971; 1972) in response to a high extracellular potassium concentration indicate that depolarization may be fundamental to this form of trans-synaptic modulation. Silberstein *et al.* (1972) have suggested that the release of NA in response to depolarization of adrenergic neurones might provide a means of linking depolarization with increased T-OH activity. Thus it seemed important to establish whether the NA content of adrenergic neurones bears any constant relation to their T-OH activity under long-term experimental conditions. It has also been reported that depolarization of a variety of nervous tissues, including sympathetic ganglia (Kakiuchi, Rall & McIlwain, 1969; McAfee, Schordoret & Greengard, 1971; Shimizu & Daly, 1972), leads to a rise in the cyclic adenosine 3′,5′-monophosphate (cyclic AMP) content of the tissue, and increases in T-OH activity in cultured sympathetic ganglia exposed to dibutyryl cyclic AMP have been described (Mackay & Iversen, 1972b). Increased intracellular production of cyclic AMP in response to depolarizing stimuli could thus provide an alternative mechanism linking membrane depolarization to T-OH synthesis.

This paper describes the use of the isolated mouse superior cervical sympathetic ganglion maintained in organ culture as a model system in which to investigate the relation of intracellular NA to T-OH activity under the influence of various long-term stimuli such as depolarization and cyclic AMP.

Methods

Mice, of various ages, were killed by exposure to ether and the superior cervical sympathetic ganglia were excised under sterile conditions. The ganglia were placed in Petri dishes containing Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland), and transferred onto autoclaved, rectangular Millipore filter rafts (1.0 x 5.0 cm) cut from inert mixed cellulose ester Millipore filter discs (Millipore GSWP 02500, pore diameter 0.22 μm plain white) and supported on stainless steel mesh bridges fashioned from sheets of expanded stainless steel mesh (stainless steel mesh grade 978, The Expanded Metal Co. Ltd, Caxton Street, London W1). Each raft supported a maximum of six ganglia. The rafts and bridges were placed in 5 ml borosilicate glass pots and medium was introduced to a level in each pot equal to the height of the bridge. Thus the ganglia were just bathed in culture medium, with approximately 180° C of their surface exposed for gas exchange. The pots were placed on moistened filter-paper in a Petri dish and the whole assembly transferred to an incubator kept at 37° C and perfused with a humidified gas mixture of 95% O_2 and 5% CO_2.

The culture medium was Eagle's minimum essential medium enriched with D-glucose to 6 mg/ml and containing 10% (v/v) neonatal calf serum and penicillin/streptomycin (100 i.u./ml). The pH of the medium was maintained at 7.4 ± 0.1 by variation of the ambient gas glow.

Nerve growth factor was prepared from adult male mouse submaxillary glands according to the method of Varon, Nomura & Shooter (1967) which yields the pure 7S fraction. This purification was kindly performed for us by the Wellcome Research Laboratories.

Tyrosine hydroxylase activity

At the end of the culture period, ganglia were harvested and washed thoroughly for 5 min in a large volume of Earle's basic salt solution (Flow Laboratories) to remove exogenous tyrosine present in the culture medium, and any experimental agents contained in the medium. Individual ganglia were then placed in 10 μl of distilled water in 15 ml conical glass tubes and frozen at −20° C. It was found that freezing and thawing sympathetic ganglia in distilled water released as much tyrosine hydroxylase activity as homogenization in distilled water. Enzyme activity was stable at −20° C for periods of up to twelve days. T-OH activity of individual ganglia was assayed according to the sensitive radiochemical technique described by Hendry & Iversen (1971) in which [^{3}H]-L-DOPA formed from sub-saturating concentrations of side-chain tritiated L-tyrosine is extracted by adsorption on alumina columns. The assay procedure was relatively insensitive to the presence of endogenous L-tyrosine in ganglion extracts, since the concentration of labelled substrate (8 μM) was below the saturating range for the enzyme. However, the fact that L-tyrosine was present in the culture medium at a concentration of 40 μg/ml (280 μM) made it necessary to investigate the possibility that carry-over of medium into the assay system could cause a reduction in apparent enzyme activity. Ganglia were frozen in various

dilutions of the culture medium so that the concentration of non-radioactive L-tyrosine present in the final 20 μl reaction volume ranged from 4-293 μM. A 50% saturation of enzyme activity was observed when the L-tyrosine concentration was 50 μM, representing 125 ng of L-tyrosine in excess of that contributed by the endogenous amino acid content of the ganglion and the added tritiated tyrosine. In order to avoid such contamination ganglia were routinely rinsed in tyrosine-free salt solution as described above.

Noradrenaline assay

Ganglia were harvested and rinsed as described above. Groups of two ganglia were immediately homogenized in 25 μl of potassium phosphate buffer 5 mM, pH 7.0 and the NA content of 10 μl portions of the homogenate estimated by the enzyme radiochemical method of Iversen & Jarrott (1970). The sensitivity of the method in our hands was reproducibly 1 ng of NA.

Choline acetylase assay

Pairs of harvested, rinsed ganglia were homogenized in 50 μl of distilled water and choline acetylase activity assayed according to the method of Fonnum (1969) with the minor modifications described by Black, Hendry & Iversen (1971).

Ganglionic cell counts

Estimations of the number of nerve cells per sympathetic ganglion were performed by a method similar to that described by Black, Hendry & Iversen (1972). Ganglia were fixed by immersion in 5% glutaraldehyde in Earle's basic salt solution, dehydrated in ethanol and embedded in paraffin. Serial 7 μm thick sections were cut through each ganglion and mounted as ribbons on clean glass slides. The sections were stained with cresyl violet and total ganglion volume was estimated by measuring the area of tissue in each section. The number of cells with prominent basophilic cytoplasm (presumptive adrenergic neurones) were estimated in four fields (350 x 250 μm) selected at random from different sections. Total cell numbers were calculated by extrapolating these values to the total ganglion volume, applying a correction for cell diameter in relation to section thickness as described by Abercrombie (1946).

Statistical Analysis

Student's *t* test was used to assess statistically significant differences between groups of results.

Results

Effects of culture on enzyme activities, noradrenaline content and cell survival

Maintenance of ganglia in organ culture was routinely for 48 hours. The effects of culture under basal conditions on T-OH activity, noradrenaline content and nerve cell survival in superior cervical ganglia from neonatal (6 days old) mice are shown in Table 1. Variation of T-OH activity with the time under basal culture conditions can be seen in Figure 1. There was no significant change up to 16 h but between 16 and 48 h in culture T-OH activity usually fell to approximately 65% of that present at the time of explantation (*in vivo* control). The NA content of the ganglia showed no significant change, but choline acetylase activity, a marker for pre-synaptic nerve remnants, disappeared completely within 24 h of commencing culture. Over the 48 h culture period the average number of nerve cells per ganglion had fallen by approximately 40%; the surviving cells being predominantly near the surface of the ganglion.

Table 1 Effect of 48 h culture on tyrosine hydroxylase (T-OH) and choline acetylase (ChA) activities, noradrenaline (NA) content and neuronal population of individual superior cervical ganglia taken from neonatal mice

	In vivo *control*	*Culture control*
T-OH activity (pmol DOPA formed per hour and per ganglion)	3.3 ± 0.4 (16)	2.1 ± 0.4 (18)
Choline acetylase activity (nmol ChA formed per hour and per ganglion)	1.1 ± 0.2 (6)	0 (6)
NA content (ng per ganglion)	1.7 ± 0.2 (16)	2.0 ± 0.1 (16)
Cell count (neurones per ganglion)	12,007 ± 1,204 (6)	7,076 ± 354 (6)

'In vivo control' refers to values at the time of explantation, 'culture control' refers to values after culture. Results are means with s.e. mean for numbers of ganglia in parentheses.

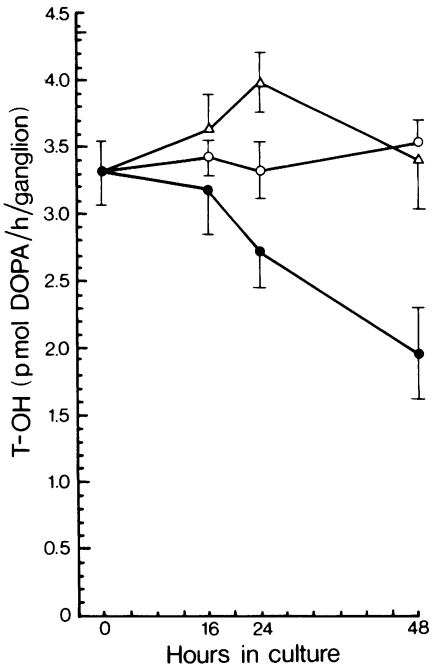

Fig. 1 Time course of tyrosine hydroxylase (T-OH) activity in individual superior cervical ganglia taken from neonatal mice and maintained for 48 h in organ culture under basal conditions (•, culture control), in the presence of nerve growth factor (○) at a concentration of 1.6 μg/ml and in the presence of a ten-fold increase in the potassium concentration (54 mM) of the culture medium (△). Points are means and s.e. mean (vertical bars) for groups of at least six ganglia.

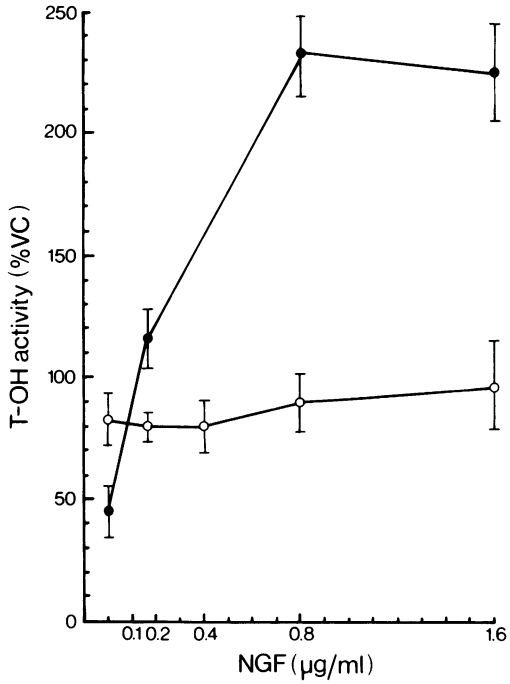

Fig. 2 Tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia taken from mice of 6 days (•) or 4 weeks (○) of age and cultured for 48 h in the presence of a range of concentrations of nerve growth factor (NGF). T-OH activity is expressed as a percentage of the activity measured at the time of explantation (VC). Points are means with s.e. mean (vertical bars) for groups of at least six ganglia.

Ganglia from adult mice (28 days old) showed a T-OH activity of 13.1 ± 0.9 pmol DOPA formed per hour and per ganglion and a NA content of 4.1 ± 0.3 ng per ganglion after 48 h in culture, neither parameter differing significantly from the *in vivo* control. Removal of the connective tissue sheath of the ganglia, whether neonatal or adult, had no significant effect on the maintenance of T-OH activity in culture.

In order to investigate the possible presence of soluble toxic components in the Millipore filter rafts that supported the cultured ganglia, some groups were maintained for 48 h on rafts that had been immersed in boiling water for 1 h prior to use in the culture system. Maintenance of enzyme activity in ganglia cultured on rafts treated in this way was no different from that observed on rafts that had simply been autoclaved in the normal manner.

Response to nerve growth factor (NGF)

The NGF protein isolated from adult male mouse submaxillary glands has been shown to increase the T-OH activity of sympathetic ganglia when administered to neonatal mice (Hendry & Iversen, 1971; Black *et al.*, 1972). Figure 2 shows the *in vitro* effect of NGF on the T-OH activity of cultured superior cervical ganglia taken from neonatal and adult mice. Cultured ganglia from 6 day old mice showed a steeply graded response to NGF whereas ganglia from adult animals were relatively insensitive. Neonatal ganglia responded to NGF (0.8 μg/ml) with a five-fold increase in total ganglionic T-OH activity relative to their culture controls. This response was abolished in the presence of the protein synthesis inhibitor cycloheximide (2 μg/ml). At a concentration of 0.16 μg/ml of NGF in the culture medium the T-OH activity of neonatal ganglia remained fairly constant throughout the 48 h culture period (Figure 1).

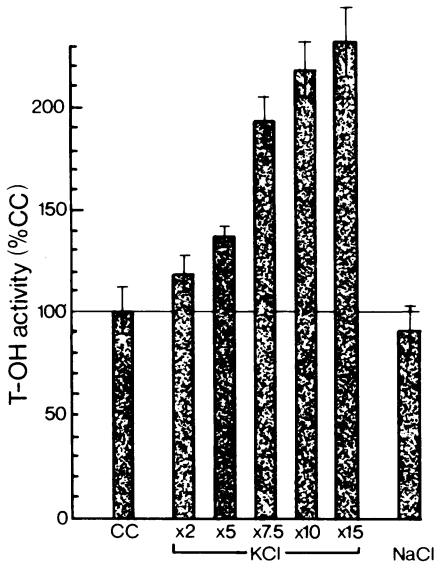

Fig. 3 Tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia taken from 6 day old mice and cultured for 48 h in the presence of increases in the molarity of potassium in the medium ranging from a doubling (10.8 mM KCl) to a fifteen-fold increase (81 mM KCl). Another group of ganglia was cultured for 48 h in the presence of an increased concentration of NaCl equivalent to the molarity increase caused by X 10K. T-OH activity is expressed as a percentage of that observed in ganglia cultured in medium of normal ionic composition (culture control; CC). Results are means with s.e. mean (vertical bars) for groups of six ganglia.

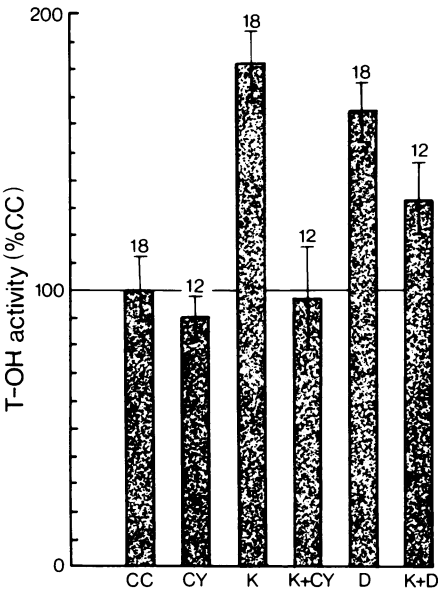

Fig. 4 Effects of cycloheximide (CY 2 μg/ml) and actinomycin D (D 5 μg/ml) on the tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia taken from 6 day old mice and cultured for 48 h in the presence of a ten-fold increase in the potassium concentration (K) of the medium. Drugs were present throughout the culture period. T-OH activity is expressed as a percentage of the activity in culture control (CC) ganglia, and values are means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

Effects of drugs which stimulate acetylcholine receptors

In an attempt to reproduce as closely as possible in culture the effects of pre-synaptic nerve activity, superior cervical ganglia were maintained for 48 h in the presence of a range of concentrations of acetylcholine (1 mM-100 pM) and of carbachol (1 mM-10 nM) in the presence and absence of the irreversible acetylcholinesterase inhibitor echothiopate (phospholine; 10 μM). Small but insignificant rises were observed in the T-OH activity of the cultured ganglia. Possible explanations for this failure to respond are discussed later.

Effects of raised extracellular potassium

In order to cause a prolonged neuronal depolarization which did not depend upon activation of nicotinic receptors, ganglia were cultured in media with raised potassium (K^+) concentrations and their T-OH activity, NA content and cell survival were determined.

Isolated superior cervical ganglia from neonatal mice were maintained for 48 h in media containing a range of increases in K^+ concentration with no added NGF. A graded response was observed (Fig. 3) with a maximal increase in T-OH activity of 100% elicited by K^+ (54 mM) (x 10 normal). This concentration of potassium was used in all subsequent experiments in which the K^+ stimulus was applied. Exposure of ganglia to a similar rise in the molarity of sodium produced no significant change in T-OH activity. Results in Fig. 1 shows that by 24 h ganglia taken from neonatal mice have significantly ($P < 0.05$) higher T-OH activity when cultured in the presence of high potassium. Figure 4 shows the effects of the protein synthesis inhibitor cycloheximide (2 μg/ml) and the RNA synthesis inhibitor actinomycin D (5 μg/ml) on the response of neonatal ganglia cultured for 48 h in the presence of a ten-fold rise in K^+ concentration.

The results show that inhibition of nuclear directed protein synthesis prevents the rise in T-OH in response to the K^+ stimulus. The presence of actinomycin D by itself over the 48 h culture period resulted in an increased T-OH activity, but no further response was elicited by K^+.

Table 2 shows the effects of high K^+ on the T-OH activity of superior cervical ganglia from mice of various ages cultured for 2 days. It is evident that ganglia from mice of ages ranging from 2 days to 4 weeks were capable of responding to the depolarizing stimulus. Results observed in ganglia taken from adult mice are worthy of particular note. These ganglia showed little deterioration in their T-OH activity over a 48 h culture under basal conditions, and thus the 70% rise in T-OH activity elicited by culture in high K^+ represented a highly significant increase in enzyme activity relative to that found in the intact animal.

Further experiments were performed in order to define the minimum period of exposure to the depolarizing stimulus necessary to induce T-OH synthesis (Figure 5). Exposure to high K^+ for as little as 30 min at the start of a 48 h culture, with subsequent change to control medium, was sufficient to stimulate a significant ($P < 0.05$) rise in T-OH activity. A maximal rise of T-OH activity was elicited by a 3 h exposure to high K^+, and this rise could be prevented if cycloheximide (2 μg/ml) was present in the culture medium during the entire 48 h culture period, but not if cycloheximide was present only for the initial 3 h period of K^+ exposure.

The NA content of neonatal ganglia doubled after exposure to high K^+ for 48 h in culture (Figure 8). Similar results were obtained with ganglia from adult mice.

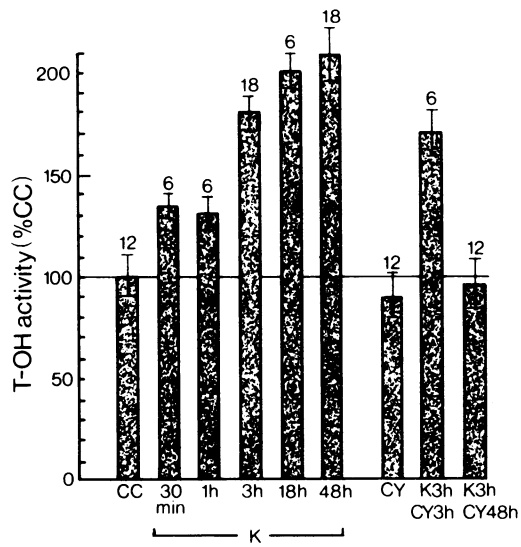

Fig. 5 Tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia excised from neonatal mice and maintained in organ culture for 48 h under control conditions (CC) or after exposure to medium containing a ten-fold increase in potassium concentration (K) for periods ranging from 30 min to 48 h in culture. Ganglia exposed to high potassium for periods less than the total 48 h culture period were transferred to medium of normal ionic composition for the remainder of the culture. Also shown are the effects of cycloheximide (CY) present alone for 48 h at a concentration of 2 μg/ml, present only during a 3 h potassium exposure and present over a total 48 h culture period, the initial 3 h of which was in the presence of high K. Results are expressed as percentages of the culture control (CC) and are means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

Table 2 Increases in tyrosine hydroxylase (T-OH) activity of superior cervical ganglia excised from mice of various ages when maintained in organ culture for 48 h in the presence of a ten-fold increase in the potassium concentration of the culture medium

Age (days)	*T-OH activity (% control)*
2	143 (6) ± 16
4	135 (6) ± 9
5	147 (6) ± 12
6	178 (18) ± 10
14	146 (6) ± 16
28	174 (12) ± 8

Results are expressed as percentages of the T-OH activity of ganglia cultured under control conditions and are means with s.e. mean for numbers of ganglia in parentheses.

Neonatal ganglia usually exhibited a 40% reduction in cell numbers over a 48 h period under control culture conditions (Table 1). In the presence of a ten-fold increase in the K^+ concentration of the medium the average cell number per ganglion (5,898 ± 397, mean and s.e. mean for 6 ganglia) was not significantly different from the values in control cultures ($P > 0.05$). Thus the depolarizing stimulus increased the T-OH activity per cell.

The interaction of NGF with the depolarizing stimulus was also investigated (Figure 6). Neonatal ganglia were exposed to high K^+ in the presence of increasing concentrations of NGF in the culture medium. In this situation the 'culture control' ganglia were maintained in media of normal ionic composition but containing a range of NGF

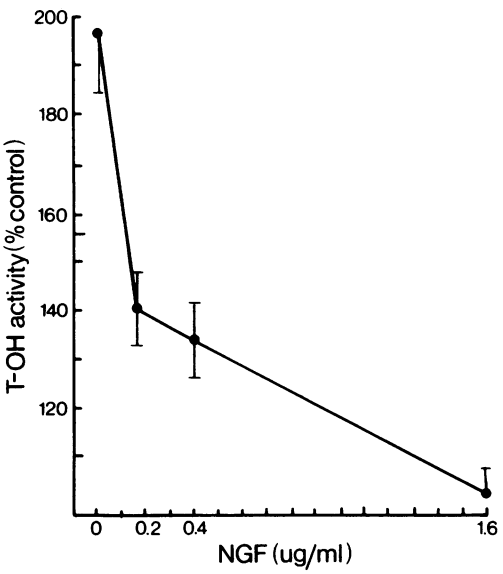

Fig. 6 Tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia taken from neonatal mice and cultured for 48 h in the presence of a ten-fold increase in the potassium concentration of the medium, with or without nerve growth factor (NGF) at various concentrations. The rises in T-OH activity elicited by potassium are expressed as percentages of culture control values in ganglia exposed to various concentrations of NGF in the culture medium (see Figure 3). Each point is the mean with s.e. mean (vertical bars) for at least six values obtained from ganglia taken from mice of ages ranging from 2 to 6 days.

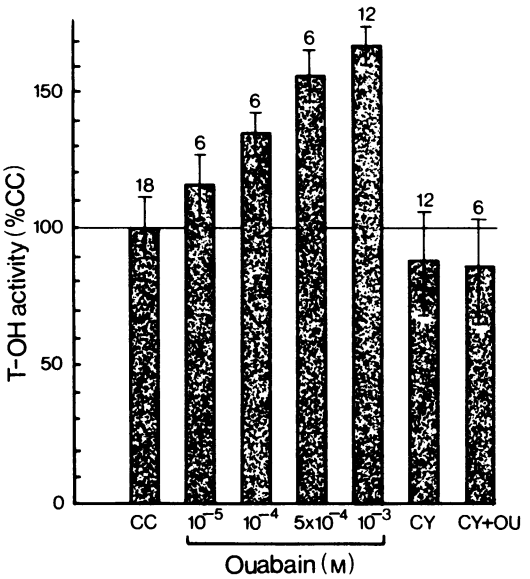

Fig. 7 Tyrosine hydroxylase (T-OH) activity of individual superior cervical ganglia taken from neonatal mice and maintained in organ culture for 48 h in the presence of ouabain, cycloheximide (CY; 2 μg/ml) and cycloheximide plus ouabain (CY + OU; cycloheximide 2 μg/ml + ouabain 1 mM). T-OH activity is expressed as a percentage of culture control (CC) and columns represent the means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

concentrations from 0-1.6 μg/ml. Therefore K^+ responses were gauged relative to control T-OH activity governed by the prevailing NGF concentration (cf. Figure 2). In the absence of added NGF the T-OH response to K^+ was greater than 80%, but this response diminished as the NGF content of the medium was increased (Fig. 6), and once ganglia had reached the point of maximum stimulation by NGF, at a concentration of 1.6 μg/ml, no further response to increased potassium was elicited.

Exposure to ouabain

The cardiac glycoside ouabain is thought to reduce the resting transmembrane potential of excitable cells by inhibiting the Na^+/K^+ activated ATP-ase present in the cell membrane. The effects of this form of depolarizing stimulus on the T-OH activity and NA content of cultured superior cervical ganglia were investigated.

Exposure of neonatal ganglia to a range of ouabain concentrations over a 48 h period resulted in graded increases in their T-OH activity (Figure 7). Ouabain at a concentration of 1 mM evoked a 70% rise in T-OH activity, and this response was abolished when the protein synthesis inhibitor cycloheximide (2 μg/ml) was present in the medium during the 48 h culture period. Similar results were obtained with ganglia from adult mice. The effect of ouabain (1 mM) on the NA content of neonatal ganglia cultured for 48 h was to cause a 70% fall (Fig. 8), the reduction in NA being accompanied by a 70% rise in T-OH activity.

When cultured in the presence of both raised K^+ and ouabain (1 mM), the T-OH activity of neonatal ganglia rose by some 100%, and their NA content was virtually identical to that of the culture control (Figure 8).

Effects of reserpine, clorgyline and α-methyl-p-tyrosine

The effects on T-OH activity of agents expected to exert an effect primarily on NA content were

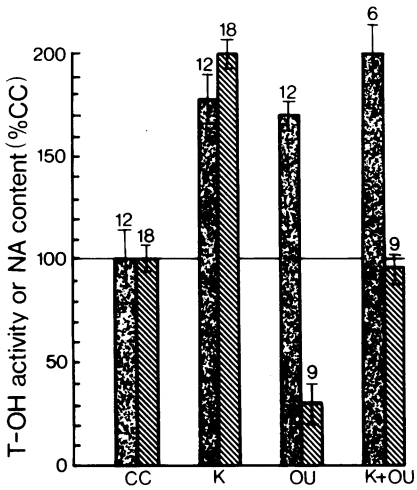

Fig. 8 Tyrosine hydroxylase (T-OH) activity (stippled columns) and noradrenaline (NA) content (hatched columns) of individual superior cervical ganglia taken from neonatal mice and maintained in organ culture for 48 h in the presence of high potassium (K 54 mM), ouabain (OU 1 mM) and potassium plus ouabain (K 54 mM + OU 1 mM). Results are expressed as a percentage of culture control (CC) and columns represent means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

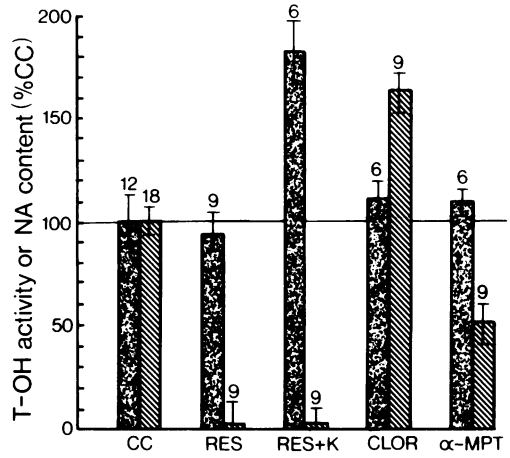

Fig. 9 Tyrosine hydroxylase (T-OH) activity (stippled columns) and noradrenaline (NA) content (hatched columns) of superior cervical ganglia taken from neonatal mice and maintained in organ culture for 48 h in the presence of reserpine (RES 25 μM), reserpine plus high potassium (RES 25 μM + K 54 mM), clorgyline (CLOR 1 μM) and α-methyl-*p*-tyrosine (αMPT 20 μM). Results are expressed as a percentage of culture control (CC) and columns represent means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

explored. Reserpine is known to prevent intracellular binding of NA, and when present in the culture medium at a concentration of 25 μM produced a virtually complete depletion of ganglionic NA, but had no significant effect on T-OH activity (Figure 9). Combining reserpine with raised K^+ produced an 80% rise in T-OH activity, but the ganglia were still severely depleted of NA.

The monoamine oxidase inhibitor clorgyline at a concentration of 1 μM produced a 60% rise in the NA content of cultured ganglia without any significant effect on T-OH activity (Figure 9).

The reversible T-OH inhibitor, α-methyl-*p*-tyrosine at a concentration of 25 μM produced a 50% reduction in the NA content of cultured ganglia, but had no significant effect on T-OH activity. This lack of effect on T-OH activity was to be expected, since enzyme activity was routinely assayed *in vitro* after thorough rinsing of the cultured ganglia.

Effects of dibutyryl cyclic adenosine 3',5'-monophosphate and theophylline

We have already reported that the potassium-induced rise in T-OH activity is enhanced in the presence of theophylline, and that exposure to 1 mM dibutyryl cyclic AMP over a 48 h period caused a rise in T-OH activity similar to that evoked by high K^+ (Mackay & Iversen, 1972b). Exposure of neonatal ganglia to dibutyryl cyclic AMP (1 mM) for an 8 h period with subsequent change to control medium for the remainder of a 48 h culture period produced a 75% rise in T-OH activity. Exposure of ganglia to dibutyryl cyclic AMP for periods of less than 8 h produced no significant change in T-OH activity. The rises in T-OH activity evoked by dibutyryl cyclic AMP present for 8 h or more were prevented by cycloheximide (2 μg/ml) or actinomycin D (5 μg/ml).

The NA content of neonatal ganglia cultured for 48 h in the presence of dibutyryl cyclic AMP (1 mM) rose by approximately 110% (Figure 10). Theophylline alone at a concentration of 5 μM had no significant effect on either T-OH activity or NA content but produced a significant ($P < 0.05$) enhancement of the increase in T-OH activity elicited by high potassium. Dibutyryl cyclic AMP had a similar enhancing effect on the potassium response, while simultaneous exposure to high potassium, dibutyryl cyclic AMP and theophylline evoked a rise in T-OH not significantly different

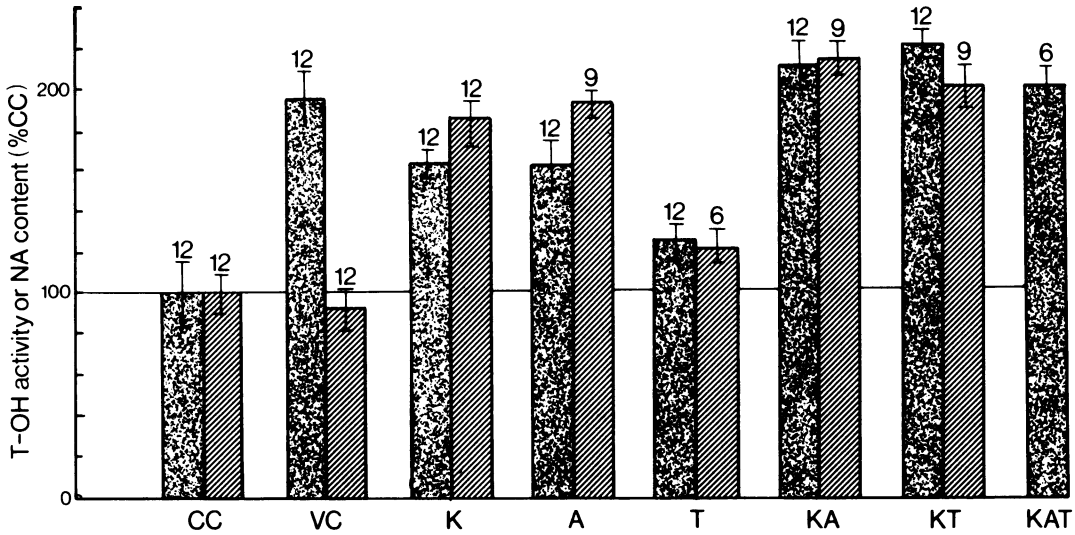

Fig. 10 Tyrosine hydroxylase (T-OH) activity (stippled columns) and noradrenaline (NA) content (hatched columns) of individual superior cervical ganglia taken from neonatal mice and maintained in organ culture for 48 h in the presence of high potassium (K 54 mM); theophylline (T 5 μM); high potassium + theophylline (K 54 mM + T 5 μM); dibutyryl cyclic 3'5'-AMP (A 1 mM); high potassium + dibutyryl cyclic 3'5'-AMP (K 54 mM + A 1 mM); and high potassium + dibutyryl cyclic 3'5'-AMP + theophylline (K 54 mM + A 1 mM + T 5 μM). Results are expressed as a percentage of the culture control (CC) and columns represent the means with s.e. mean (vertical bars) for numbers of ganglia shown above each column.

from that observed with potassium plus theophylline or potassium plus dibutyryl cyclic AMP. Exposure of cultured ganglia to sodium butyrate (1 mM) had no effect on T-OH activity. The number of nerve cells per ganglion surviving after culture for 48 h in the presence of dibutyryl cyclic AMP was 7,211 ± 325 (mean and s.e. mean for six ganglia) not significantly different from the value in culture controls. ($P > 0.1$). Thus dibutyryl cyclic AMP, like high potassium, resulted in increased enzyme activity per nerve cell.

Discussion

The use of organ culture techniques to investigate regulatory mechanisms for transmitter enzyme synthesis offers certain advantages over experiments in the intact animal. The maintenance of isolated organs allows an examination of the effects of parameters which are often extremely difficult to control *in vivo;* for example, the distinction between humoral and nervous influences on a peripheral nervous structure such as the superior cervical ganglion. However, the two approaches are best used in parallel to provide results that are complementary.

The culture system described here is simple in design, allowing ready access by drugs and ease of harvesting for biochemical or histological evaluation. Cell survival over the 2 day culture period was limited, but the remaining cells proved capable of adequate responses to environmental manipulation. The basic questions whose clarification was attempted with this system were firstly the nature of inter-neuronal influences on ganglionic T-OH activity and secondly the intracellular mechanism for enzyme regulation.

Evidence from many sources suggests that trans-synaptic events influence the T-OH activity in peripheral adrenergic neurones in intact mature animals. Exposure to environmental stress (Thoenen, 1970; Kvetnansky, Gewirtz, Weise & Kopin, 1970; Axelrod, Mueller, Henry & Stephens, 1970) and drugs such as reserpine (Thoenen, Mueller & Axelrod, 1969b, 1970) and phenoxybenzamine (Dairman & Udenfriend, 1970) are thought to produce either direct or reflex increases in sympathetic outflow. These procedures result in rises in T-OH activity in both sympathetic ganglia and adrenal medullae that can be prevented if these structures are previously deafferented (Thoenen *et al.*, 1969a, b) or, in sympathetic ganglia, if the animals are pretreated with ganglion-blocking drugs (Mueller, Thoenen &

Axelrod, 1970). The rises in T-OH activity can also be abolished by inhibition of protein synthesis (Mueller *et al.*, 1969; Thoenen *et al.*, 1969a; Otten *et al.*, 1972).

The work of Black *et al.* (1971) on the neonatal mouse superior cervical ganglion strongly suggests that a trans-synaptic message may also be necessary for the biochemical maturation of the adrenergic neurones in this ganglion during development. Rises in T-OH activity seen between the 6th and 13th day of life correlated closely with the establishment of synapses by the ingrowing preganglionic nerves. The developmental rises in T-OH activity could be prevented by decentralization of the ganglion or by treatment with ganglion blocking drugs (Black *et al.*, 1971; Hendry, 1973; Black & Geen, 1973).

The results described with cultured ganglia taken from mice of ages ranging from 2 days to adulthood and exposed to a depolarizing stimulus in the form of high extracellular potassium suggest that such trans-synaptic effects could result from increases in the frequency of depolarization of ganglion neurones in response to the release of acetylcholine from pre-synaptic nerve endings. The failure of the pre-synaptic transmitter acetylcholine and carbachol to produce rises in the T-OH activity of isolated ganglia is of interest. Brown & Pascoe (1954) have shown that the sensitivity of the superior cervical ganglion of the cat to depolarization by acetylcholine is depressed following axotomy, however, the development of that depression was slow in onset. A more likely explanation of the apparent insensitivity of the cultured ganglia would seem to be the rapid development of receptor desensitization resulting from exposure to the agonists acetylcholine and carbachol in the culture medium. Direct membrane depolarization by potassium and ouabain offers a means of by-passing this difficulty. Not only have we found substantial increases in the T-OH activity of superior cervical ganglia maintained for periods of 48 h in the presence of such depolarizing stimuli, but also that the stimulus need only be applied for 30 min to trigger a rise in enzyme activity. On the other hand, the minimum time necessary for expression of increased enzyme activity is of the order of 24 hours. The prevention of these depolarization-induced rises in enzyme activity by the protein synthesis inhibitor cycloheximide required the presence of the inhibitor in the culture medium for the entire culture period, thus expression of increased T-OH activity evidently requires on-going protein synthesis for a time considerably greater than the initial stimulus period. These findings support the recent reports of Guidotti, Zivkovic, Pfeiffer & Costa (1973) and Guidotti & Costa (1973), who observed increases in the T-OH activity of the adrenal glands in intact rats 24 h after exposure to cold stress for a period of only 2 hours.

Actinomycin D produced a rise in the T-OH activity of cultured superior cervical ganglia, a phenomenon similar to that described by Tomkins, Levinson, Baxter & Dethlefsen (1972) as the 'paradoxical effect' of actinomycin D on the synthesis of enzymes and other proteins in a wide variety of mammalian cells. Ganglia exposed to high K^+ in the presence of actinomycin D failed to show a rise in T-OH activity over those exposed to actinomycin D alone, indicating that the increases in enzyme activity evoked by depolarization require nuclear directed protein synthesis for their expression.

In view of the reports (Scott & Fisher, 1970; Scott, 1971; Lasher & Zagon, 1972) of improved survival of cells cultured in the presence of raised potassium, it was important to establish that the observed rises in enzyme activity per ganglion did not simply reflect increased survival of nerve cells in the ganglia. Cell numbers were unchanged after 48 h in the presence of high potassium, thus the enzyme increases cannot be explained in this way.

On the basis of these results it would seem unnecessary to invoke any trophic pre-synaptic influence other than depolarization as the mediator of trans-synaptic modulation of T-OH activity. The role of NGF in the development of the mouse sympathetic nervous system is unclear. This hormone undoubtedly has potent effects on the T-OH activity of sympathetic ganglia both in the intact neonatal animal and in culture. However, in the intact animal, the administration of NGF cannot reverse the effect on T-OH of deprivation of the pre-synaptic input in surgically decentralized ganglia (Black *et al.*, 1972). Cultured ganglia can respond to high concentrations of NGF with a maximal increase in T-OH activity, but are quite capable of responding to a depolarizing stimulus in its absence. There seems no necessity to involve NGF in the scheme of depolarization-induced increases in T-OH activity in the developing superior cervical ganglion. Its role may lie either in the pre-natal morphogenesis of the ganglion, or in the interaction of postganglionic sympathetic nerves with their end organs (Hendry & Iversen, 1973).

The link between prolonged depolarization and rises in T-OH does not seem to depend upon changes in ganglionic NA. Our results show that under various long-term environmental manipulations the concentration of NA in ganglionic neurones bears no constant relation to their T-OH activity, and that increases in T-OH activity which are dependent upon protein synthesis can co-exist

with rises or falls in the NA content of the ganglia.

Depolarizing stimuli of various sorts, including exposure to high potassium have been shown by a number of workers to stimulate adenylate cyclase in nervous tissue. Guidotti *et al.* (1973) have inferred a causal relation between the rise in the cyclic AMP content of adrenal glands in the intact rat with the rise in T-OH activity in these organs observed after exposure of animals to either drugs (Guidotti & Costa, 1973) or cold stress. Cyclic AMP has been reported to induce tyrosine hydroxylase in clones of catecholamine containing cells of mouse neuroblastoma (Waymire, Weiner & Prasad, 1972; Richelson, 1973) and Keen & McLean (1972) have observed an increase in the dopamine-β-hydroxylase activity of rat superior cervical ganglia cultured in the presence of dibutyryl cyclic AMP. Our results show that exposure of superior cervical ganglia to dibutyryl cyclic AMP can mimic the effect of a depolarizing stimulus on their T-OH activity and NA content. The rises in T-OH activity due to dibutyryl cyclic AMP were blocked by both cycloheximide and actinomycin D, suggesting that like high potassium, the cyclic nucleotide expressed its effects through nuclear directed protein synthesis. The evidence seems to implicate cyclic AMP in the long-term control of T-OH in superior cervical ganglia.

Both in the case of biochemical maturation in the neonatal mouse and of environmental adaptation in the mature mouse, trans-synaptic modulation may take the form of a depolarization-induced rise in the cyclic AMP content of sympathetic ganglionic neurones leading to nuclear mediated synthesis of T-OH.

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