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EFFECT OF POTASSIUM DEPOLARIZATION AND PREGANGLIONIC NERVE STIMULATION ON THE METABOLISM OF [^{3}H]-CHOLINE IN RAT ISOLATED SYMPATHETIC GANGLIA

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1 The effects of potassium depolarization and preganglionic nerve stimulation on the metabolism of [^{3}H]-choline in the isolated superior sympathetic ganglion of the rat have been studied.

2 When unstimulated (resting) ganglia were incubated for 10 min with a low concentration (0.1 μM) of [^{3}H]-choline (high affinity uptake), approximately 75% of the accumulated radioactivity was present as [^{3}H]-phosphorylcholine, 11% was [^{3}H]-acetylcholine ([^{3}H]-ACh) and the remainder was unchanged [^{3}H]-choline.

3 Depolarization of the ganglia with K (46 mM) before their incubation with [^{3}H]-choline, increased [^{3}H]-choline uptake by 70% and increased [^{3}H]-ACh synthesis by more than 700%, so that [^{3}H]-ACh represented almost 50% of the total radioactivity recovered. In contrast, the proportion of [^{3}H]-phosphorylcholine fell to 36% of the total radioactivity recovered.

4 The striking effect of K-depolarization on [^{3}H]-ACh synthesis in ganglia occurred at a concentration of 30 mM or above, and the maximum effect was seen at 45–50 mM.

5 Chronic denervation of the ganglia abolished all the effects of high-K on [^{3}H]-choline metabolism. In resting ganglia, [^{3}H]-ACh formation was reduced by over 80% but [^{3}H]-phosphorylcholine synthesis and the level of unchanged [^{3}H]-Ch were not affected by denervation.

6 Exposure of the ganglia to low-Na or hemicholinium-3 (HC-3) greatly reduced [^{3}H]-ACh synthesis in control resting ganglia and almost abolished the effects of high-K on [^{3}H]-ACh synthesis.

7 Prevention of transmitter release with high-Mg or low-Ca medium also prevented K-depolarization from stimulating [^{3}H]-ACh synthesis.

8 Preganglionic nerve stimulation had an effect on [^{3}H]-choline metabolism similar to that of K-depolarization. Thus, at all the frequencies studied (1–30 Hz), [^{3}H]-ACh synthesis was greatly increased and [^{3}H]-phosphorylcholine was reduced, the maximum effects occurring at 3 Hz.

9 When ganglia were incubated with a high concentration (100 μM) of [^{3}H]-choline (low affinity uptake), a different pattern of metabolism was observed. Most of the radioactivity in resting ganglia was present as unchanged [^{3}H]-choline (70%) with [^{3}H]-phosphorylcholine and [^{3}H]-ACh representing 23% and 6% of the total radioactivity respectively. K-depolarization decreased [^{3}H]-choline uptake but increased the proportions of [^{3}H]-phosphorylcholine and [^{3}H]-ACh to 32% and 24% of the total radioactivity respectively.

10 It is concluded that in unstimulated (resting) rat sympathetic ganglia most of the [^{3}H]-choline transport and metabolism occurs in postsynaptic structures. However, depolarization of the presynaptic nerve terminals appears to trigger a sodium-dependent, HC-3 sensitive, high-affinity uptake process, and causes a dramatic increase in presynaptic [^{3}H]-ACh synthesis together with a fall in postsynaptic [^{3}H]-phosphorylcholine synthesis. These changes in choline metabolism cannot be due to the depolarization of the nerve terminals *per se*, because they were abolished by high-Mg or low-Ca, i.e. when transmitter release was prevented. Thus, the increase in ACh synthesis may be triggered by a fall in the intraterminal concentration of ACh or by the changes in Ca flux induced by depolarization. Our experiments do not provide evidence on these possible mechanisms.

Introduction

It has long been known that in the cat superior cervical ganglion, stimulation of the preganglionic nerve increases acetylcholine (ACh) synthesis (Brown & Feldberg, 1936; Birks & MacIntosh, 1961) and that this is associated with an increase in choline uptake (Collier & Lang, 1969). Conversely,

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perfusion of cat sympathetic ganglia with choline-free medium decreases ganglionic ACh levels (Collier & MacIntosh, 1969). The importance of choline transport and metabolism in the formation of ACh was also suggested by the effects of hemicholinium (HC-3), a competitive inhibitor of choline transport, which was found to reduce ACh synthesis in the perfused sympathetic ganglion of the cat (MacIntosh, Birks & Sastry, 1956; Birks & MacIntosh, 1961).

In perfused cat ganglia, it has not been possible to investigate in detail the kinetics of the choline transport process, but studies using isolated sympathetic ganglia of the rat revealed the presence of both high and low affinity choline transport systems (Bowery & Neal, 1975). Similar multiple transport systems for choline have been reported in the central nervous system, where a high affinity, sodium-dependent, choline uptake process is believed to be associated with ACh synthesis and localized in cholinergic nerve terminals (Kuhar, Sethy, Roth & Aghajanian, 1973; Kuhar, Dehaven, Yamamura, Rommelspacher & Simon, 1975; Suszkiw & Pilar, 1976; Kuhar & Murrin, 1978). However, in rat sympathetic ganglia, chronic denervation did not affect the high affinity uptake process for choline (Bowery & Neal, 1975), apparently indicating that in ganglia, high affinity choline transport is not associated with cholinergic nerve terminals. An explanation for this inconsistency is provided by our studies on the effects of K-depolarization on choline uptake, which suggest that in ganglia, there may be two high affinity processes, one of which is localized in presynaptic nerve terminals and only becomes operational after the terminals are depolarized (Higgins & Neal, 1982).

In order to obtain further information on the functional significance of these different high affinity choline uptake processes, we have studied the metabolism of [^{3}H]-choline in unstimulated ganglia and in ganglia subjected to K-depolarization or preganglionic nerve stimulation.

A preliminary report of some of these results has been presented (Higgins & Neal, 1978).

Methods

Male Lister rats (200–400 g) were anaesthetized with urethane (1.5 g kg^{-1} intraperitoneally). The superior cervical ganglia together with pre- and post-ganglionic nerve trunks were excised and desheathed.

Incubation conditions

Ganglia were given a preliminary incubation for 30 min in 50 ml of Krebs Ringer bicarbonate medium at 37°C and then incubated for a further 10 min in medium containing [^{3}H]-choline, usually at a concentration of 0.1 mM. Each ganglion was then washed at room temperature, blotted on filter paper, and weighed on a Beckman microbalance, a procedure which was usually completed within 60 s. Ganglia were then transferred to 0.1 ml glass homogenizers and homogenized in 50 μl of 15% formic acid (1.0 M) in acetone containing [^{14}C]-butyrylcholine (0.1 μCi/ml) as an internal standard and choline, butyrylcholine and ACh (each 1 mg/ml) as carriers.

Separation and estimation of metabolites

Aliquots (10 μl) of the supernatant together with 10 μg of phosphorylcholine were applied to Whatman No. 1 paper. The paper was wetted with buffer (1.5 M acetic acid, 0.75 M formic acid, pH 2) and electrophoresis carried out using a Camay high voltage electrophoresis system at 4000 V for 20 min (Potter & Murphy, 1967). The paper was then sprayed with a saturated solution of iodine in *n*-heptane to visualize the choline metabolites. The paper was decolourized by exposure to steam and the stained areas were then cut out and placed in scintillation vials. The radioactivity was extracted with distilled water (0.2 ml). The insoluble pellet formed by centrifuging the homogenate (1000 *g* × 5 min) was washed twice with formic acid/acetone and dried in an oven at 80°C. Each pellet was then dissolved in 0.5 ml of Soluene (Packard) and neutralised with glacial acetic acid (0.1 ml). Ethoxyethanol (4 ml) and butyl-PBD (10 ml, 0.5% w/v in toluene) were added to each vial and the radioactivity was determined by liquid scintillation spectrometry. The radioactivity in samples of incubation medium (50 μl) and supernatant (10 μl) were measured in a similar way. The amounts of ^{14}C and ^{3}H in each sample were determined by the double label external ratio method. The amount of each radioactive metabolite was corrected for losses during electrophoresis (as estimated by recovery of the [^{14}C]-butyrylcholine) and expressed as d min^{-1} mg^{-1} wet tissue weight. It was considered inappropriate to quantitate metabolites in terms of picomoles synthesized since the specific activity of intracellular [^{3}H]-choline was not known.

The radioactivity contained in the homogenate supernatant was separated into five fractions by high voltage paper electrophoresis; [^{3}H]-choline moved furthest from the origin followed by [^{3}H]-ACh and [^{3}H]-betaine. [^{3}H]-phosphorylcholine moved with a low mobility and was incompletely separated from a further radioactive metabolite which remained at the origin. This last metabolite was present in small quantities and may have been phosphatidylcholine although it was not identified. The unseparated low mobility fractions (including phosphorylcholine) will be referred to hereafter as [^{3}H]-phosphorylcholine,

since this metabolite accounted for more than 90% of the radioactivity near the origin. The homogenization procedure extracted more than 90% of the total accumulated radioactivity. The radioactivity remaining in the pellet was not identified.

Preganglionic denervation

Preganglionic denervation was performed two weeks prior to excision by removing a 5 mm length of preganglionic nerve under halothane anaesthesia.

Preganglionic nerve stimulation

The preganglionic nerve trunk was drawn into a miniature suction electrode and stimulated supramaximally with square wave electrical pulses (0.5 ms duration). The postganglionic nerve trunk was drawn into a similar suction electrode and the signals amplified differentially and displayed on a storage oscilloscope.

Solutions

Krebs Ringer bicarbonate of the following composition was used (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.4, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 9.5. The medium was continuously gassed with 5% CO_2 in O_2.

Low-Na medium (25 mM Na^+) Low-Na medium was prepared by substituting LiCl for NaCl.

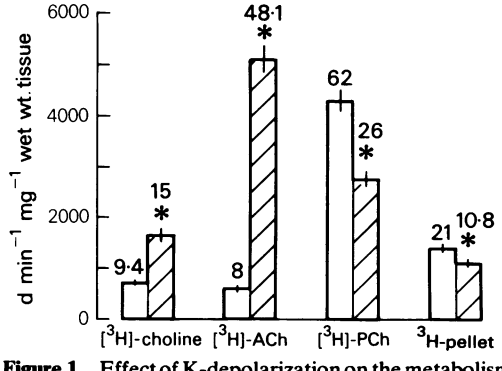

Figure 1 Effect of K-depolarization on the metabolism of [^{3}H]-choline (0.1 μM, high affinity uptake). Ganglia were given a preliminary incubation for 30 min in normal medium (controls, open columns) or in medium containing KCl (46 mM, hatched columns). All ganglia were then incubated for 10 min in fresh medium containing [^{3}H]-choline (0.1 μM). Each column represents the mean of at least 20 experiments; vertical lines show s.e.mean. The figures above the histobars give the percentage of each metabolite: PCh = phosphorylcholine. *Significantly different from controls: P at least < 0.05.

High-K medium High-K solutions were prepared by increasing the KCl concentration. No other ions were omitted to compensate for the increased ionic strength.

High-Mg solution was prepared by increasing the concentration of $MgSO_4$ to 20 mM.

Ca-free solution was prepared by omitting $CaCl_2$.

Radiochemicals

Methyl-[^{3}H]-choline chloride (13 Ci mmol^{-1}) was obtained from the Radiochemical Centre, Amersham. Methyl-[^{3}H]-choline (84 Ci mmol^{-1}) was obtained from New England Nuclear, W. Germany. 1-[^{14}C]-butyrylcholine was obtained from New England Nuclear, and was purified prior to use by high voltage paper electrophoresis.

Results

Metabolism of [^{3}H]-choline in unstimulated and K-depolarized ganglia

When unstimulated (resting) ganglia were incubated with a low concentration of [^{3}H]-choline (high affinity uptake), only 8% of the accumulated radioactivity was present as [^{3}H]-ACh, the major metabolite being [^{3}H]-phosphorylcholine (62%). The pellet accounted for 21% of the total radioactivity and only 9.4% represented unchanged [^{3}H]-choline (Figure 1). [^{3}H]-betaine usually accounted for less than 1% of the radioactivity.

In a previous study (Higgins & Neal, 1982), we found that depolarization of the ganglia by high-K increased the high affinity uptake of [^{3}H]-choline and that this effect was maximal at $[K]_o = 45$ mM. We therefore examined the effect of this concentration of K on the metabolism of [^{3}H]-choline.

It can be seen from Figure 1 that depolarization of the ganglia with high-K (46 mM) during the preliminary (but not the actual incubation with [^{3}H]-choline) dramatically altered the pattern of [^{3}H]-choline metabolism. Thus, the synthesis of [^{3}H]-ACh was increased by 850%, but [^{3}H]-phosphorylcholine formation and the [^{3}H]-pellet were decreased by 35% and 22% respectively. The unchanged [^{3}H]-choline was increased by 230% (Figure 1). K-depolarization also increased the total accumulation of radioactivity by 68% ($P < 0.001$) confirming previous experiments (Higgins & Neal, 1982). The amount of each ^{3}H-metabolite expressed as a percentage of the total accumulated radioactivity in both unstimulated and depolarized ganglia is shown in Figure 1.

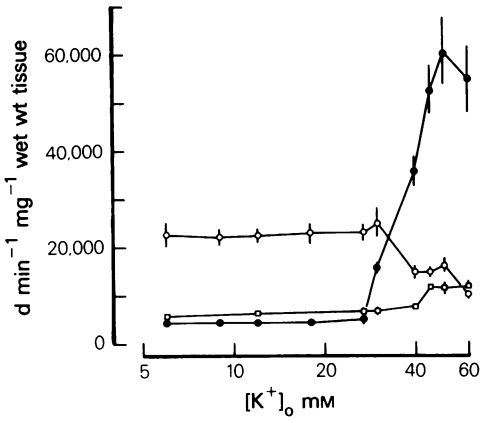

Figure 2 Effect of varying $[K]_o$ on [^{3}H]-choline metabolism. Ganglia were preincubated for 30 min with medium in which $[K]_o$ was varied (6–60 mM). Ganglia were then incubated for 10 min with unmodified medium containing [^{3}H]-choline (0.1 μM). The amount of each radioactive metabolite (mean of 6 experiments) is plotted against $[K]_o$; vertical lines show s.e.mean. [^{3}H]-choline (□); [^{3}H]-acetylcholine (●); [^{3}H]-phosphorylcholine (○).

Effects of different potassium concentrations on the metabolism of [^{3}H]-choline

The effects of different concentrations of $[K]_o$ (6–60 mM) are illustrated in Figure 2 and show that high-K did not affect [^{3}H]-choline metabolism at concentrations less than 30 mM. However, at this and higher concentrations, [^{3}H]-ACh and unmetabolized [^{3}H]-choline levels were increased whilst [^{3}H]-phosphorylcholine decreased. The maximum increase was seen when $[K]_o = 45–50$ mM and in all subsequent experiments 46 mM K was used.

Effect of high-K on the time course of [^{3}H]-choline metabolism

Ganglia were given a preliminary incubation with either unmodified medium (controls) or with medium containing high-K and then transferred to fresh medium containing [^{3}H]-choline (0.1 μM) in which the incubations were continued for a further 10–60 min. Preincubation with high-K caused a striking increase in the subsequent rate of [^{3}H]-ACh formation compared with controls (Figure 3) but after 20 min the level of [^{3}H]-ACh in ganglia exposed to high-K did not increase further with time. Preincubation with high-K depressed the subsequent synthesis of [^{3}H]-phosphorylcholine for up to 30 min when the rate increased to a value similar to that of control ganglia.

Metabolism of [^{3}H]-choline by denervated ganglia

When ganglia were preincubated with unmodified medium (resting ganglia), denervation reduced [^{3}H]-ACh synthesis by 82% compared with controls but had no effect on the levels of unchanged [^{3}H]-choline or on the synthesis of [^{3}H]-phosphorylcholine and [^{3}H]-pellet (Figure 4).

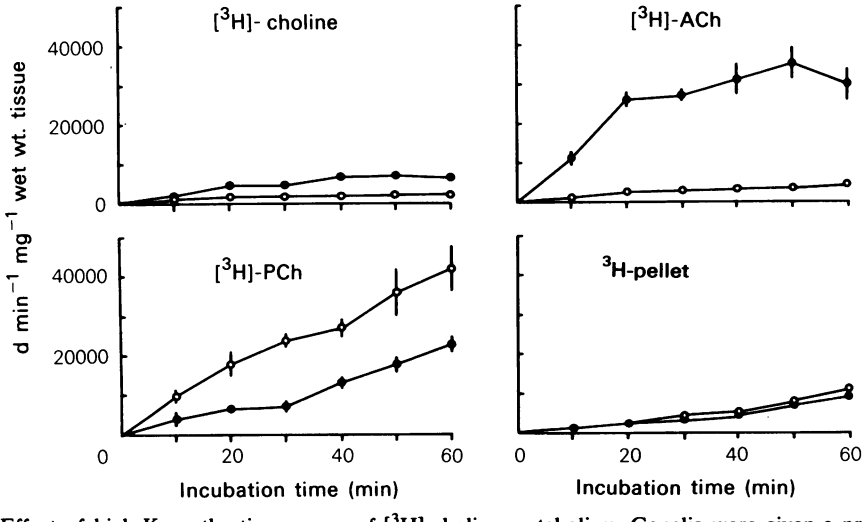

Figure 3 Effect of high-K on the time course of [^{3}H]-choline metabolism. Ganglia were given a preliminary incubation for 30 min in either unmodified medium (controls) or with medium containing KCl (46 mM). The ganglia were then transferred to fresh medium containing [^{3}H]-choline (0.1 μM) and the incubations were continued for 10 to 60 min. Each point represents the mean of 6 experiments; vertical lines show s.e.mean. (○) Incubations with unmodified medium; (●) K 46 mM present in preliminary incubation.

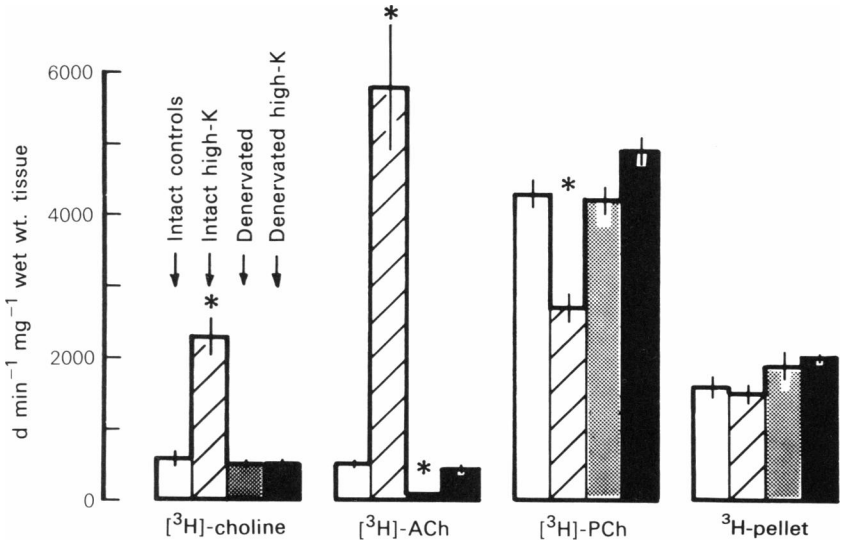

Figure 4 Comparison of [^{3}H]-choline metabolism by intact and denervated ganglia. Ganglia were given a preliminary incubation for 30 min with either unmodified medium or with medium containing KCl (46 mM). Incubations were then continued for 10 min in unmodified medium containing [^{3}H]-choline (0.1 μM). Each column represents the mean of 6 experiments; vertical lines show s.e.mean. For each metabolite, the first (open) column shows the result from unstimulated, intact (control) ganglia: the second (hatched) column, from intact, K-stimulated ganglia; the third (stippled) from unstimulated, denervated ganglia and the fourth (solid) from K-stimulated, denervated ganglia. *Significantly different from controls: $P < 0.01$. Note: in Figures 4 to 7, the different conditions for [^{3}H]-choline only are also given on the Figure itself.

The effects of high-K on [^{3}H]-choline metabolism were almost abolished by chronic denervation, thus, the rise in [^{3}H]-choline levels and fall in [^{3}H]-phosphorylcholine were absent in denervated ganglia. K-stimulation did increase [^{3}H]-ACh synthesis compared with unstimulated denervated ganglia but this 'stimulated' level was not significantly different from the levels in unstimulated (resting) intact ganglia.

The percentage increase in [^{3}H]-ACh synthesis in response to K-depolarization in denervated ganglia was similar to that in intact ganglia, suggesting that some presynaptic terminals still exist in denervated ganglia.

Effect of hemicholinium-3 on [^{3}H]-choline metabolism

When HC-3 (1 μM) was included in the preliminary incubation and incubation, the synthesis of [^{3}H]-ACh was reduced by 60% and unmetabolized [^{3}H]-choline levels were reduced by 30%. The synthesis of [^{3}H]-phosphorylcholine was unaffected by this concentration of HC-3 (Figure 5). In the presence of HC-3, high-K caused a small increase in [^{3}H]-ACh synthesis, but this still remained less than that seen in the unstimulated control ganglia.

Effect of low $[Na]_o$ on [^{3}H]-choline metabolism

Ganglia were given a preliminary incubation for 30 min in medium with a low concentration of sodium ($[Na]_o = 25$ mM). They were then incubated for a further 10 min with low-Na medium containing [^{3}H]-choline. Under these conditions the total accumulation of radioactivity was reduced to 62% of controls incubated in normal medium. Compared with control ganglia, [^{3}H]-ACh synthesis was reduced by 78%, [^{3}H]-phosphorylcholine synthesis was reduced by 37% and unmetabolized [^{3}H]-choline was reduced by 26% (Figure 6).

When ganglia were depolarized with high-K in the preliminary incubation (low-Na medium), [^{3}H]-ACh synthesis was increased by 320% compared with ganglia incubated with low Na medium. The amount of [^{3}H]-ACh synthesized under these conditions was similar to that synthesized by unstimulated control ganglia incubated in normal medium (Figure 6).

Effect of high-K on [^{3}H]-choline metabolism when transmitter release is inhibited

The presence of high Mg (20 mM) or zero Ca during the preincubation period greatly reduced or abolished the effects of high-K on [^{3}H]-choline metabolism (Figure 7).

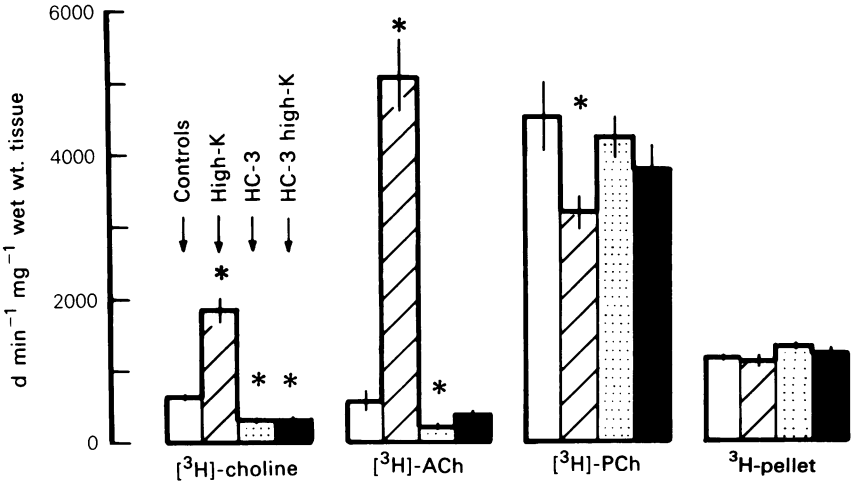

Figure 5 The effects of hemicholinium-3 (HC-3) on the metabolism of [^{3}H]-choline. Ganglia were given a preliminary incubation for 30 min with either unmodified medium (controls) or with medium containing KCl (46 mM). Incubations were then continued for 10 min in medium containing [^{3}H]-choline (0.1 μM). The effect of 1 μM HC-3 was examined by adding it to both of the incubations. Each column represents the mean of 6 experiments; vertical lines show s.e.mean. Controls, open columns; K-stimulated, hatched columns; 1 μM HC-3 in both incubations, stippled columns; 46 mM K stimulated and 1 μM HC-3 in both incubations, solid columns. *Significantly different from controls: $P<0.001$, except [^{3}H]-PCh where $P<0.02$.

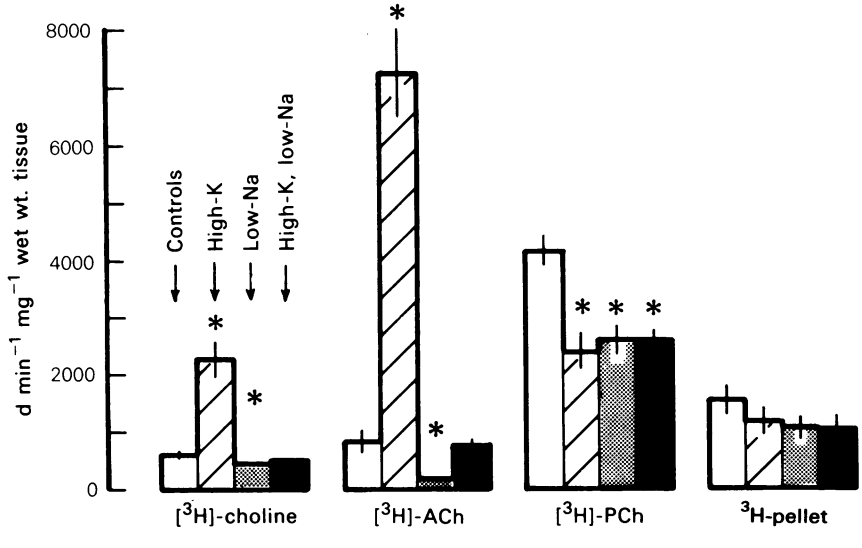

Figure 6 The effect of low-Na on [^{3}H]-choline metabolism. Ganglia were given a preliminary incubation for 30 min with either unmodified medium (controls) or with medium containing KCl (46 mM). Ganglia were then incubated for 10 min with medium containing [^{3}H]-choline (0.1 μM). Sodium chloride was replaced by lithium chloride in both incubations. Each column represents the mean of 6 experiments; vertical lines show s.e.mean. Controls, open column; 46 mM-KCl in preincubation, hatched columns; both incubations with low-Na medium, stippled columns; 46 mM-K in preincubation plus low-Na in both incubations, solid columns. *Significantly different from controls: $P<0.01$.

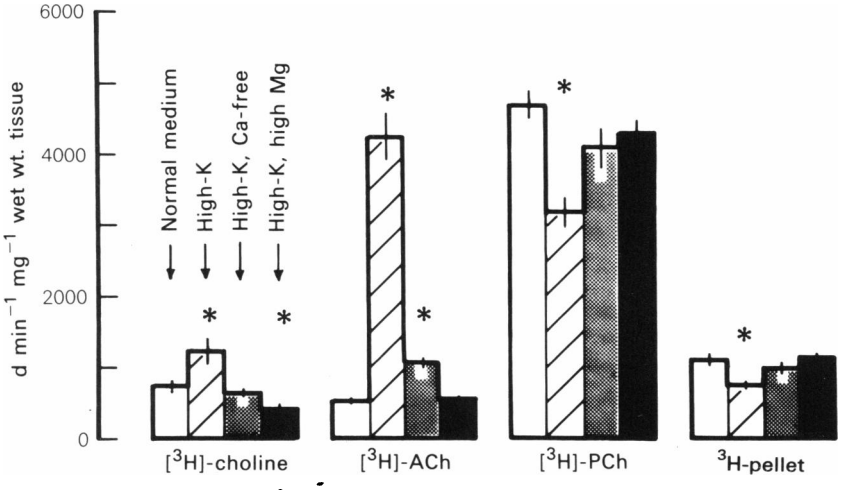

Figure 7 Effect of K-depolarization on [^{3}H]-choline metabolism when transmitter release is inhibited. Ganglia were given a preliminary incubation for 30 min with either unmodified medium (controls) or with medium containing 46 mM-KCl. This incubation was further modified, either by omission of Ca-ions, or by increasing the $[Mg]_o$ to 20 mM. Ganglia were then incubated with unmodified medium containing [^{3}H]-choline (0.1 μM). Each column shows the mean of 6 experiments; vertical lines show s.e.mean. Controls, open columns; 46 mM-KCl in preincubation, hatched columns; 46 mM-KCl and Ca-free preincubation, stippled columns; 46 mM-KCl and 20 mM Mg in preincubation, solid columns. *Significantly different from controls: $P<0.01$.

Effect of preganglionic nerve stimulation on the metabolism of [^{3}H]-choline

Preganglionic nerve stimulation did not increase the accumulation of total radioactivity by ganglia, but produced striking changes in the pattern of [^{3}H]-choline metabolism, very similar to those produced by K-depolarization (Figure 8). Thus, nerve stimulation increased the synthesis of [^{3}H]-ACh by 445%. Unchanged [^{3}H]-choline was also increased, whilst the proportions of [^{3}H]-phosphorylcholine and [^{3}H]-pellet were decreased (Figure 8). The synthesis of [^{3}H]-ACh was increased maximally at 3 Hz, when the percentages of [^{3}H]-choline, [^{3}H]-ACh, [^{3}H]-phosphorylcholine and ^{3}H-pellet were 21(14), 40(8.8), 31(58) and 8(18) respectively; the figures in parentheses giving the corresponding percentages obtained from control ganglia.

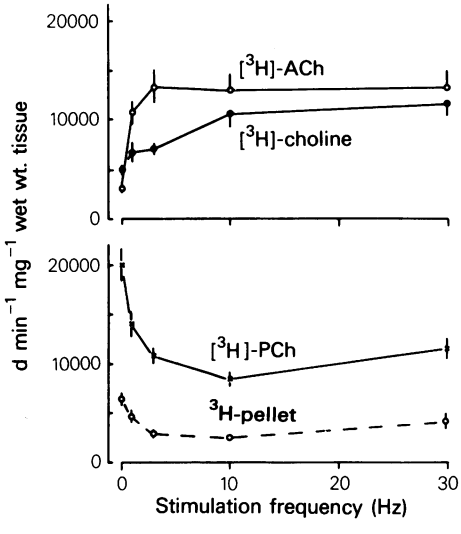

Figure 8 Effect of preganglionic nerve stimulation on the metabolism of [^{3}H]-choline. Ganglia were mounted in suction electrodes and given a preliminary incubation of 30 min. Ganglia were then transferred to medium containing [^{3}H]-choline (0.1 μM) and the preganglionic nerve stimulated supramaximally at the frequencies shown for 10 min. Each point represents the mean of 6–8 determinations; vertical lines show s.e.mean. [^{3}H]-choline, (●); [^{3}H]-ACh, (○); [^{3}H]-phosphorylcholine, (x); ^{3}H-pellet, (○).

Choline metabolism during incubation with 100 μM [^{3}H]-choline

In a previous study (Higgins & Neal, 1982), we found that when ganglia were incubated with a high concentration of [^{3}H]-choline (low affinity uptake), K-depolarization actually decreased the accumulation of radioactivity. This decrease in uptake was confirmed in the present study and the pattern of [^{3}H]-choline metabolism resulting from low affinity uptake was found to be different from that occurring after high affinity uptake. Thus, when ganglia were

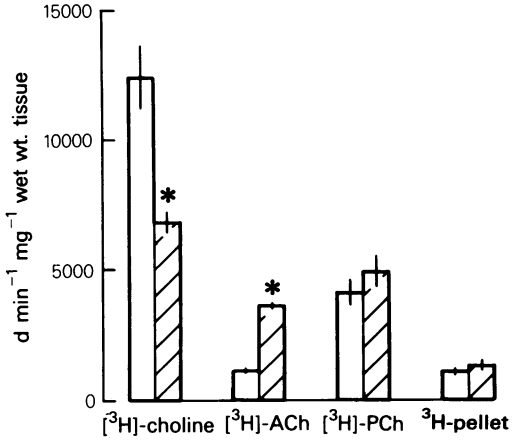

Figure 9 Effect of K-depolarization on the metabolism of 100 μM choline (low affinity uptake). Ganglia were given a preliminary incubation for 30 min with normal medium (controls, open columns) or in medium containing KCl (46 mM), hatched columns. The ganglia were then incubated for 10 min with medium containing [^{3}H]-choline (100 μM). Each column represents the mean of 6 experiments; vertical lines show s.e.mean. *Significantly different from controls: $P < 0.002$.

incubated with 100 μM choline, most of the radioactivity was recovered as unchanged [^{3}H]-choline (70% of total), followed by [^{3}H]-phosphorylcholine (23%) and [^{3}H]-ACh (6%). Small amounts of betaine (less than 1%) were also recovered (Figure 9).

When both the preliminary incubation and final incubation contained K 46 mM, the total accumulation of radioactivity was reduced by 30% ($P < 0.05$) compared with controls. In addition to this decrease in accumulation, K-depolarization also altered the pattern of metabolites from that seen in resting ganglia. Thus, the level of unmetabolized [^{3}H]-choline was reduced to 44% of the total radioactivity recovered and the proportion of [^{3}H]-ACh formed was increased to approximately 24% by incubation with high-K. The proportion of [^{3}H]-phosphorylcholine was increased to 32% of the total (Figure 9).

Discussion

This study shows that K-depolarization of sympathetic ganglia produces a dramatic increase in [^{3}H]-ACh synthesis and a concomitant fall in [^{3}H]-phosphorylcholine synthesis. These changes in [^{3}H]-choline metabolism occur in the preganglionic nerve terminals since they were abolished by chronic preganglionic denervation. The increase in [^{3}H]-ACh synthesis produced by K-depolarization appears to be associated with the K-activated, high affinity uptake process for [^{3}H]-choline described in the previous study (Higgins & Neal, 1982). Thus, factors which inhibited the K-activated uptake of [^{3}H]-choline such as exposure to low $[Na]_o$, low $[Ca]_o$, high $[Mg]_o$, HC-3 or chronic denervation also prevented or reduced the increase in [^{3}H]-ACh synthesis caused by K-depolarization.

Metabolism of [^{3}H]-choline by unstimulated (resting) ganglia

In resting ganglia incubated with a low concentration of [^{3}H]-choline a high proportion (77%) of the [^{3}H]-choline was incorporated into phosphorylcholine and only a little was present as unchanged [^{3}H]-choline (12%) and [^{3}H]-ACh (11%). The latter finding is consistent with our uptake studies (Higgins & Neal, 1982) in which we found that the high affinity uptake of [^{3}H]-choline in presynaptic nerve terminals was almost undetectable in resting ganglia.

When ganglia were incubated with [^{3}H]-choline (100 μM) (low affinity uptake), most of the radioactivity was recovered as unchanged [^{3}H]-choline (71%), although the major metabolite was still [^{3}H]-phosphorylcholine (23%). The rate of choline transport under these conditions presumably exceeded the rate at which choline was metabolized. Similar results have been reported previously in synaptosomes (Diamond & Kennedy, 1969; Yamamura & Snyder, 1973).

The synthesis of [^{3}H]-ACh by ganglia incubated with [^{3}H]-choline (0.1 μM) was reduced by 85% after chronic denervation showing that synthesis of [^{3}H]-ACh in resting ganglia occurred mainly in preganglionic structures. In contrast, the synthesis of [^{3}H]-phosphorylcholine was not affected by denervation, presumably indicating that synthesis of [^{3}H]-phosphorylcholine by nerve terminals is relatively insignificant. Similarly, the proportion of radioactivity not extracted by homogenization and the amount of unchanged [^{3}H]-choline were unaffected by denervation and were not, therefore, located in preganglionic structures.

The results of these denervation experiments show that virtually all of the [^{3}H]-choline transported into preganglionic structures was acetylated to form [^{3}H]-ACh (since only [^{3}H]-ACh levels were altered by denervation) and this is in agreement with the suggestion that choline transport can be rate limiting for ACh synthesis (Haubrich & Chippendale, 1977; Kuhar & Murrin, 1978). The finding that ACh synthesis and phosphorylcholine synthesis occurred in different ganglionic structures suggests that the concentration of ACh in nerve terminals is unlikely to influence the rate of phosphorylcholine synthesis in the manner suggested by Haubrich (1973). Haubrich found that ACh stimulated choline kinase *in vitro* and

suggested that the concentration of ACh in nerve terminals might regulate the availability of choline for subsequent ACh synthesis in a similar manner (Haubrich & Chippendale, 1977).

Effects of high-K on [^{3}H]-choline metabolism

When ganglia were incubated with a low concentration of [^{3}H]-choline after preincubation with high-K, there was a striking increase in the synthesis of [^{3}H]-ACh, a smaller increase in unchanged [^{3}H]-choline levels and the synthesis of [^{3}H]-phosphorylcholine was reduced.

These results are in close agreement with those of Polak, Molenaar & van Gelder (1977) who examined ACh synthesis by rat cortical slices. All of the effects of high-K were almost or completely abolished by chronic denervation, thus the increase in [^{3}H]-ACh synthesis must have occurred in preganglionic structures. Preincubation with high-K must also have caused an accumulation of unchanged [^{3}H]-choline in preganglionic structures, perhaps because the increased rate of choline transport exceeded the rate of ACh synthesis. This result suggests that choline transport may not always be rate limiting for ACh synthesis.

The inhibition of [^{3}H]-phosphorylcholine synthesis in postganglionic structures by high-K was abolished by denervation and hence was dependent on the presence of intact preganglionic structures. This suggests that inhibition of [^{3}H]-phosphorylcholine synthesis was a consequence rather than a cause of increased [^{3}H]-ACh synthesis. Possibly increasing the rate of choline transport into nerve terminals resulted in less choline being available for transport into other structures; thus, when ganglia were incubated with 100 μM choline (low affinity uptake), high-K increased the synthesis of [^{3}H]-ACh but did not affect [^{3}H]-phosphorylcholine synthesis.

Effect of preganglionic nerve stimulation on [^{3}H]-choline metabolism

As with K-depolarization, preganglionic nerve stimulation increased [^{3}H]-ACh synthesis and unchanged [^{3}H]-choline levels, and reduced the synthesis of [^{3}H]-phosphorylcholine. However, unlike K-depolarization, these changes in [^{3}H]-choline metabolism were unaccompanied by an increase in the total accumulation of radioactivity. The rate of [^{3}H]-ACh synthesis increased with stimulation frequencies of 1–3 Hz, but was not increased by higher stimulation frequencies (10 and 30 Hz). This plateau effect was probably due to a steady state situation where [^{3}H]-ACh synthesized = [^{3}H]-ACh released. Furthermore, at high rates of stimulation the radiolabel was probably diluted by unlabelled choline formed by hydrolysis of released endogenous ACh. It has been estimated that about half of this choline can be reaccumulated by ganglionic nerve terminals (Collier & Katz, 1974).

The effect of preganglionic nerve stimulation on [^{3}H]-ACh synthesis in rat ganglia was similar to that found for perfused cat ganglia (Collier & MacIntosh, 1969). Inhibition of phosphorylcholine synthesis by nerve stimulation has not been reported previously. Indeed, Collier & Lang (1969) found that preganglionic nerve stimulation did not affect the synthesis of [^{3}H]-phosphorylcholine when cat ganglia were perfused with [^{3}H]-choline. They used choline concentrations 100 times greater than those used in the present experiments and this may explain the discrepancy, if as suggested earlier, inhibition of [^{3}H]-phosphorylcholine synthesis was caused by decreased availability of [^{3}H]-choline.

Is activation of acetylcholine synthesis a consequence of increased ACh release?

The following results suggest that ACh release and activated ACh synthesis are closely related. (a) The minimum concentration of K required to either evoke [^{3}H]-ACh release or increase [^{3}H]-ACh synthesis was similar (30 mM). The K-concentrations required to produce maximum effects were not the same but this was probably because the duration of exposure to high-K was 30 min for measurement of [^{3}H]-ACh synthesis, but 2 min for measurement of [^{3}H]-ACh release. (b) Incubations with high-Mg or Ca-free medium almost abolished the increase in [^{3}H]-ACh synthesis caused by high-K. Either of these two conditions reduced the K-evoked [^{3}H]-ACh release by more than 80%. (c) The increase in [^{3}H]-ACh synthesis produced by preganglionic nerve stimulation (1 Hz) was reduced in high-Mg medium. The inhibition of nerve stimulation evoked [^{3}H]-ACh release by high-Mg was quantitatively similar (Higgins & Neal, unpublished observations).

These results suggest that increased [^{3}H]-ACh synthesis might be a direct consequence of ACh release, i.e. a decrease in the intraterminal concentration of ACh. However, we cannot exclude the possibility that ACh synthesis might be regulated by changes in Ca-fluxes or $[Ca]_i$ since there is no method available at present to alter Ca-metabolism without also affecting ACh release (Rubin, 1970).

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References

BIRKS, R. & MACINTOSH, R.C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Canad. J. Biochem. Physiol.*, **39**, 787–825.

BOWERY, N.G. & NEAL, M.J. (1975). Failure of denervation to influence the high affinity uptake of choline by sympathetic ganglia. *Br. J. Pharmac.*, **55**, 278P.

BROWN, G.L. & FELDBERG, W. (1936). The acetylcholine metabolism of a sympathetic ganglion. *J. Physiol.*, **88**, 265–283.

COLLIER, B. & KATZ, H.S. (1974). Acetylcholine synthesis from recaptured choline by a sympathetic ganglion. *J. Physiol.*, **238**, 639–655.

COLLIER, B. & LANG, C. (1969). The metabolism of choline by a sympathetic ganglion. *Can. J. Physiol. Pharmac.*, **47**, 119–126.

COLLIER, B. & MACINTOSH, F.C. (1969). The source of choline for acetylcholine synthesis in a sympathetic ganglion. *Can. J. Physiol. Pharmac.*, **47**, 127–135.

DIAMOND, I. & KENNEDY, E.P. (1969). Carrier-mediated transport of choline into synaptic nerve endings. *J. biol. Chem.*, **244**, 3258–3263.

HAUBRICH, D.R. (1973). Partial purification and properties of choline kinase (EC 2.7.1.3.2.) from rabbit brain: measurement of acetylcholine. *J. Neurochem.*, **21**, 315–328.

HAUBRICH, D.R. & CHIPPENDALE, T.J. (1977). Regulation of acetylcholine synthesis in nervous tissue. *Life Sci.*, **20**, 1465–1478.

HIGGINS, A.J. & NEAL, M.J. (1978). The effect of potassium on the metabolism of [^{3}H]-choline in sympathetic ganglion. *J. Physiol.*, **277**, 67–68P.

HIGGINS, A.J. & NEAL, M.J. (1982). Potassium activation of [^{3}H]-choline accumulation by isolated rat sympathetic ganglion. *Br. J. Pharmac.*, **77**, 573–579.

KUHAR, M.J., DEHAVEN, R.N., YAMAMURA, H.I., ROMMELSPACHER, H. & SIMON, J.R. (1975). Further evidence for cholinergic habenulo-interpeduncular neurones: pharmacological and functional characteristics. *Brain Res.*, **97**, 265–275.

KUHAR, M.J. & MURRIN, L.C. (1978). Sodium-dependent high affinity choline uptake. *J. Neurochem.*, **30**, 15–21.

KUHAR, M.J., SETHY, V.H., ROTH, R.H. & AGHAJANIAN, G.K. (1973). Choline: selective accumulation by central cholinergic neurons. *J. Neurochem.*, **20**, 581–593.

MACINTOSH, F.C., BIRKS, R.I. & SASTRY, P.B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature, Lond.*, **178**, 1181.

POLAK, R.L., MOLENAAR, P.C. & VAN GELDER, M. (1977). Acetylcholine metabolism and choline uptake in cortical slices. *J. Neurochem.*, **29**, 477–485.

POTTER, L.T. & MURPHY, W. (1967). Electrophoresis of acetylcholine choline and related compounds. *Biochem. Pharmac.*, **16**, 1386–1388.

RUBIN, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. *Pharmac. Rev.*, **22**, 389–428.

SUSZKIW, J.B. & PILAR, G. (1976). Selective localisation of a high affinity choline uptake system and its role in acetylcholine formation in cholinergic nerve terminals. *J. Neurochem.*, **26**, 1133–1138.

YAMAMURA, H.I. & SNYDER, S.H. (1973). High affinity transport of choline into synaptosomes of rat brain. *J. Neurochem.*, **21**, 1355–1374.

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