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POTASSIUM ACTIVATION OF [^{3}H]-CHOLINE ACCUMULATION BY ISOLATED SYMPATHETIC GANGLIA OF THE RAT

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1 The effect of K-depolarization on the uptake of low and high concentrations of [^{3}H]-choline by isolated superior sympathetic ganglia of the rat has been studied.

2 In unstimulated ganglia, the uptake of [^{3}H]-choline (0.1 μM) ('high affinity uptake') was unaffected by denervation or by hemicholinium-3 (HC-3), suggesting uptake by structures other than cholinergic nerve terminals.

3 K-depolarization of the ganglia increased [^{3}H]-choline accumulation by the high affinity uptake process but in contrast the 'low affinity' accumulation of [^{3}H]-choline (100 μM) was decreased.

4 The K-activated, 'high affinity' component of choline uptake was highly sodium-dependent, inhibited by HC-3, and was abolished by denervation.

5 In incubation conditions designed to prevent transmitter release (Ca-free medium and high-Mg medium), the K-activated uptake of [^{3}H]-choline was abolished.

6 It is concluded that in unstimulated ganglia, there is little choline uptake by nerve terminals. However, when the terminals are depolarized, choline uptake is increased by the activation of a sodium-dependent, HC-3-sensitive transport process. The activation of this uptake process is apparently associated with the release of acetylcholine from the terminals, or by changes in ionic fluxes, and not by the depolarization *per se*.

Introduction

The transport process responsible for the uptake of choline in the central nervous system possesses both high and low affinity components (Yamamura & Snyder, 1972; 1973; Haga & Noda, 1973; Dowdall & Simon, 1973; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973; Kuhar, Sethy, Roth & Aghajanian, 1973; Carroll & Butterbaugh, 1975a; 1975b). The high affinity process is highly sodium-dependent and is believed to be associated with cholinergic nerve terminals. Furthermore, it has been suggested that the presence of a high affinity choline uptake process might be useful as a marker for cholinergic nerve terminals (Kuhar, 1973; 1976; Sorimachi & Kataoka, 1974; 1975). However, choline uptake into parasympathetic (Suszkiw, Beach & Pilar, 1976) and sympathetic (Collier & Katz, 1975; Bowery & Neal, 1975) ganglia was unaffected by preganglionic denervation suggesting that in these experiments there could be little choline accumulation by the cholinergic nerve terminals. In the present experiments, we have examined in more detail the accumulation of choline by the rat isolated superior cervical ganglion and have confirmed that in unstimulated ganglia, the cholinergic nerve terminals accumulate little if any choline from the external medium. However, when the terminals are depolarized, a sodium-dependent, high affinity uptake process is activated and choline uptake into the nerve terminals occurs. The metabolism of the [^{3}H]-choline accumulated in unstimulated and depolarized ganglia is described in the following paper (Higgins & Neal, 1982).

Some of these results have been published in a preliminary form (Higgins & Neal, 1977).

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 postganglionic nerve trunks were excised and desheathed.

Incubation conditions

Ganglia were given a preliminary incubation in 50 ml of Krebs Ringer bicarbonate medium at 37°C and then incubated individually for 10 min in 1 ml of medium containing [^{3}H]-choline (1 μCi ml^{-1}) at a

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final concentration of either 0.1 μM or 100 μM. Using kinetic parameters previously determined (Bowery & Neal, 1975), it was calculated that at 100 μM, choline uptake was mainly by the low affinity process whilst at 0.1 μM, the uptake was about 50% high affinity and 50% low affinity. The latter uptake is referred to as 'high affinity'. Each ganglion was then washed at room temperature, blotted on filter paper, and weighed on a microbalance as previously described (Brown, Halliwell & Scholfield, 1971). The ganglia were dissolved in 0.5 ml Soluene (Packard) in plastic scintillation vials and the Soluene was then neutralized with 0.1 ml glacial acetic acid to reduce chemiluminescence. 2-Ethoxyethanol (4.0 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. Samples were corrected for quenching using the channels ratio method.

Results were expressed as the tissue:medium ratio (T/M ratio) which was defined as:

$$\frac{\text{d min}^{-1}\ \text{mg}^{-1}\ \text{wet weight tissue}}{\text{d min}^{-1}\ \mu\text{l}^{-1}\ \text{incubation medium}}$$

Denervation

Ganglia were denervated two weeks before excision by removing a 5 mm length of preganglionic nerve, under halothane anaesthesia.

Solutions

Krebs Ringer bicarbonate medium 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 95% O_2 and 5% CO_2.

Sodium-free medium contained (mM): sucrose 286, Tris (hydroxy methyl) aminomethane 50, KCl 4.8, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 9.5. The pH of the medium was adjusted to 7.4 with concentrated HCl and was continuously gassed with oxygen.

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

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

Calcium free solution was prepared by omitting $CaCl_2$.

[Methyl-^{3}H] choline (10.1 Ci mmol^{-1}) was obtained from The Radiochemical Centre, Amersham.

Results

Time course and temperature-dependence of choline uptake

When ganglia were incubated with [^{3}H]-choline (0.1 μM) at 37°C, radioactivity was rapidly accumulated and at 60 min a tissue/medium (T/M) ratio of approximately 16 was achieved. At this temperature, accumulation of radioactivity was linear for 20 min after which time it began to level off (Figure 1). At 25°C, accumulation was linear for 40 min and a maximum T/M ratio of about 6 was achieved after 50 min. Accumulation of radioactivity at 0°C was linear for at least 60 min when T/M ratio of only 1.5 was obtained (Figure 1). Accumulation of radioactivity by the tissue did not reduce the concentration of radioactivity in the incubation medium by more than 2% in any one incubation.

Subsequent incubations were carried out at 37°C and the incubation with [^{3}H]-choline was of 10 min duration when the initial rate of accumulation of radioactivity was linear.

Choline uptake by chronically denervated ganglia

The uptake of choline by chronically denervated ganglia was studied in order to find out whether a significant proportion of choline accumulation was occuring in cholinergic nerve terminals. Ganglia were

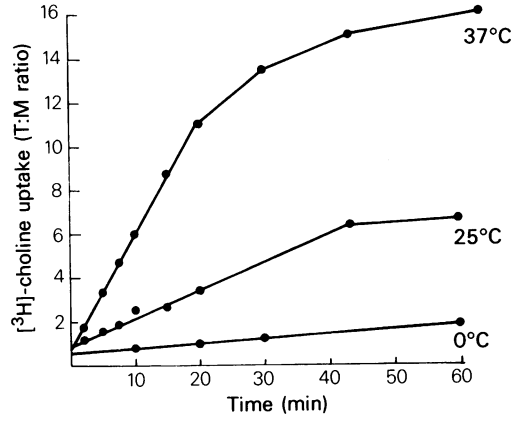

Figure 1 The time course and temperature-dependence of choline uptake. Ganglia were incubated for 30 min at the appropriate temperature and then transferred to medium containing [^{3}H]-choline (0.1 μM; 10 Ci mmol^{-1}) and further incubated for up to 60 min. The radioactivity accumulated was expressed as the tissue:medium (T:M) ratio. Each point is the mean of at least 6 determinations and the s.e.means were less than 10%.

denervated 14 days before excision. This time was chosen because most of the nerve terminals have been shown to degenerate during this period (Raisman, Field, Ostberg, Iversen & Zigmond, 1974). Choline uptake was measured at concentrations of either 0.1 μM or 100 μM (Figure 2). Chronic denervation did not affect choline uptake at either of the choline concentrations tested, indicating that at most, only a very small proportion of the choline was accumulated by preganglionic structures.

Effect of incubation with Na-free medium

Oxygenated Tris-buffer medium was used for both the preliminary incubations and the incubations with [^{3}H]-choline. The medium contained either 286 mM sucrose (Na-free incubation), or 143 mM sodium chloride (control incubations). When ganglia were incubated with 0.1 μM [^{3}H]-choline, those incubated in Na-free medium accumulated 30% ($P<0.05$) less radioactivity than control ganglia (Figure 3). This Na-dependent component of choline uptake was not localized in preganglionic structures, since a similar degree of sodium dependence (24%) was observed when the ganglia had been chronically denervated. When the concentration of choline in the final incubation was 100 μM, accumulation of radioactivity was reduced ($P<0.05$) by 24% in sodium-free medium (Figure 3).

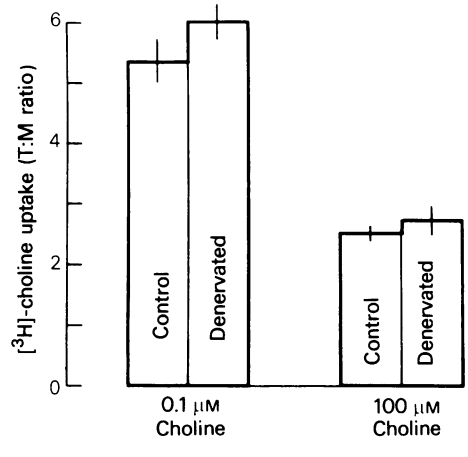

Figure 2 The effect of chronic denervation on [^{3}H]-choline uptake. Ganglia were given a preliminary incubation for 30 min at 37°C and then transferred to fresh medium containing [^{3}H]-choline (0.1 or 100 μM). The incubations were continued for 10 min. Each column represents the mean and vertical lines indicate s.e.mean of 6 determinations.

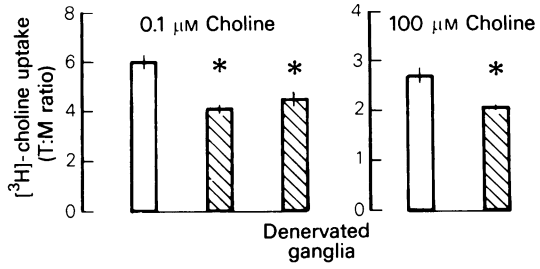

Figure 3 The effect of Na-free medium on [^{3}H]-choline uptake. Ganglia were given a preliminary incubation for 30 min in oxygenated Tris buffer (pH 7.4) medium containing either 286 mM sucrose (Na-free) (hatched columns) or 143 mM NaCl (controls) (open columns). Ganglia were then transferred to fresh medium containing [^{3}H]-choline (0.1 or 100 μM) and the incubations continued for a further 10 min. The reduction of [^{3}H]-choline (0.1 μM) uptake in Na-free medium was the same in both normal and denervated ganglia indicating that this Na-dependent component of choline uptake was not localized in nerve terminals. Each column shows the mean and vertical lines indicate s.e.mean of at least 6 experiments. *Significantly different from controls ($P<0.05$) Student's t test.

Effect of hemicholinium-3 (HC-3)

When ganglia were incubated with [^{3}H]-choline (0.1 μM), the accumulation of radioactivity was not affected by 1 μM HC-3 (when added to both the preliminary and final incubations). This concentration of HC-3 has been shown to inhibit selectively high affinity choline transport by synaptosomes (Yamamura & Snyder, 1973).

Effect of incubation with high-K

When ganglia were given a preliminary incubation and final incubation in medium containing 44.8 mM KCl (total $K^+ = 46$ mM) the effect on choline uptake differed according to the concentration of choline in the final incubation (Figure 4). When the concentration of choline was 0.1 μM, high-K increased the accumulation of radioactivity by 40% ($P<0.001$). In contrast, when the concentration of choline was 100 μM, incubations with high-K reduced the accumulation of radioactivity by approximately 30% ($P<0.01$). When high-K was present in the preliminary incubation only, the subsequent accumulation of 0.1 μM choline was increased by 68% ($P<0.001$) compared with controls. The inhibition of uptake of 100 μM choline was observed only when high-K was included in the incubation with [^{3}H]-choline. It did not occur when the tissue was exposed to high-K medium only during the preincubation stage.

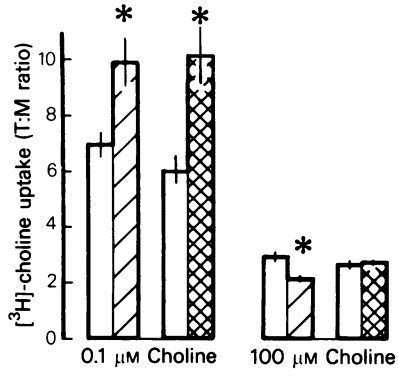

Figure 4 The effect of high-K on [^{3}H]-choline uptake. Ganglia were given a preliminary incubation for 30 min in either unmodified medium or with medium containing 46 mM K. Ganglia were then incubated for a further 10 min with medium containing [^{3}H]-choline (0.1 or 100 μM). Each column denotes the mean and vertical lines show s.e.mean of 6 to 24 experiments. *Significantly different from controls ($P<0.01$ at least). Open columns: controls; hatched columns: high-K in both preliminary incubation and incubation with [^{3}H]-choline; cross-hatched columns: high-K in preliminary incubation only.

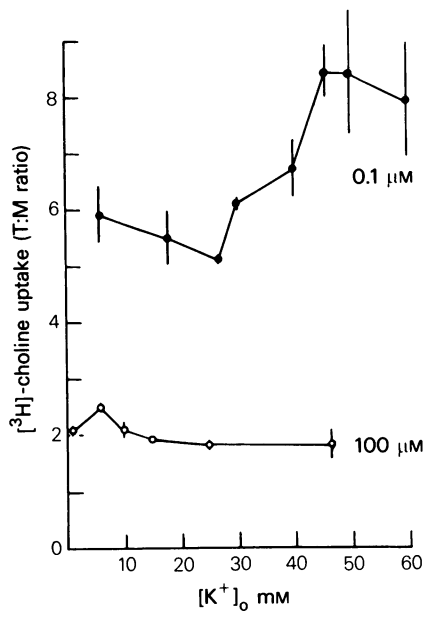

Figure 5 The effect of varying $[K]_o$ on [^{3}H]-choline uptake. Ganglia were given a preliminary incubation for 30 min and then transfused to fresh medium containing [^{3}H]-choline (0.1 or 100 μM). The K concentration in both incubations was varied between 6 and 60 mM. Choline uptake (tissue:medium ratio) is plotted against $[K]_o$. Each point is the mean and vertical lines show s.e.mean of 4 to 6 experiments.

Effect of varying potassium concentration $[K]_o$ in medium

The preceding results showed that high-K (46 mM) exerted strikingly different effects depending on whether the choline concentration was 0.1 μM or 100 μM. It was of interest to know how the magnitude of each effect varied with changes in $[K]_o$. For these experiments $[K]_o$ was altered in both the preliminary incubation and the incubation with [^{3}H]-choline. The results are shown in Figure 5. The accumulation of radioactivity when ganglia were incubated with 0.1 μM choline was unaffected by K concentrations less than 27 mM. At higher K concentrations, the uptake of choline increased until a maximum was observed with 45–50 mM K. The accumulation of radioactivity when ganglia were incubated with 100 μM choline was reduced by relatively small increases in K concentration and inhibition was maximum when $[K]_o = 25$ mM.

Effect of high-K on choline uptake in chronically denervated ganglia

The purpose of these experiments was to show whether or not the activation of choline (0.1 μM) uptake occurred in preganglionic structures. Chronically denervated ganglia were given a preliminary incubation and final incubation in medium containing 46 mM K. No increase in choline uptake was observed in these ganglia (when compared with control or denervated ganglia incubated in normal medium). Instead, inhibition ($P<0.05$) of choline uptake was observed (Figure 6). The inhibition of choline uptake by K (46 mM) which occurs when normal ganglia are incubated with high concentrations of choline (100 μM) also occurred in denervated ganglia.

These experiments show that when ganglia were incubated with 0.1 μM choline, high-K activated choline uptake in preganglionic structures, presumably the cholinergic nerve terminals). When denervated ganglia were incubated with 0.1 μM choline, or when control or denervated ganglia were incubated with 100 μM choline, high-K inhibited choline transport, suggesting that high-K inhibited choline transport into postganglionic cells and/or glial cells.

Effect of incubation with high-K in sodium-free medium

One group of ganglia was preincubated and then incubated in Na-free Tris buffer. Another group of ganglia was preincubated and incubated in Na-free

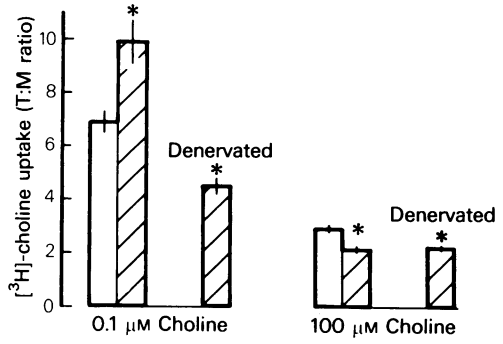

Figure 6 Comparison of the effects of high-K on [^{3}H]-choline uptake in intact and denervated ganglia. Ganglia were given a preliminary incubation for 30 min with either unmodified medium (controls) (open columns) or with medium containing 46 mM K (hatched columns). Each group of ganglia was then incubated for a further 10 min in identical medium to which had been added [^{3}H]-choline (0.1 or 100 μM), Each column represents the mean and vertical lines show s.e.mean of 6–12 experiments. *Significantly different from controls ($P<0.01$).

buffer containing 46 mM K. The concentration of choline in the incubation medium was 0.1 μM. In the absence of sodium ions, high-K did not increase choline uptake. Instead, uptake was reduced by 25% ($P<0.05$). The results are shown in Figure 7. Thus, the K-activated choline uptake into nerve terminals was highly sodium-dependent.

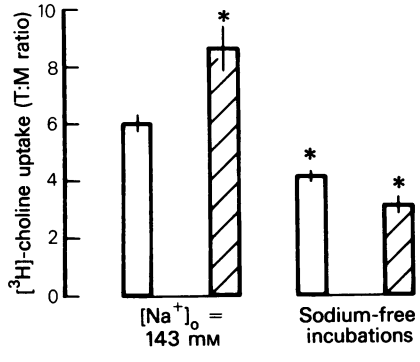

Figure 7 Sodium-dependence of K-stimulated choline uptake. Ganglia were given a preliminary incubation for 30 min at 37°C and then transferred to medium containing [^{3}H]-choline (0.1 μM) and the incubations were continued for 10 min. Each column represents the mean and vertical lines show s.e.mean of at least 6 experiments. *Significantly different from controls ($P<0.05$). Controls: open columns; high-K: hatched columns.

Effect of high-K on choline uptake in the presence of hemicholinium-3

Ganglia were preincubated and incubated in medium containing 46 mM K. The concentration of choline in the incubation was 0.1 μM. Addition of HC-3 (1 μM) to both the preincubation and the incubation abolished the K-activation of choline uptake. Instead, high-K inhibited choline transport when HC-3 was present (Figure 8).

Effects of incubation with high-K on the uptake of 0.1 μM choline when transmitter release is prevented

High concentrations of K evoke the release of acetylcholine (ACh) from ganglionic nerve terminals (Brown & Feldberg, 1936). It was possible that the effect of high-K on choline uptake by preganglionic nerve terminals, might be related to this effect on ACh release. For this reason the effect of high-K on the uptake of 0.1 μM choline was investigated in (a) Ca-free medium and (b) high-Mg medium ($[Mg]_o = 20$ μM). These two conditions were found to reduce ACh release evoked by high-K (46 mM) by more than 80% (Higgins & Neal, unpublished results). Ganglia were preincubated and incubated in medium containing 46 mM K from which the Ca ions had been omitted, or 20 mM Mg added. The concentration of choline was 0.1 μM. The results are shown in Figure 9 and were similar for each of the two conditions which inhibited ACh release. The activation of choline by high-K was abolished and instead, inhibition of choline uptake (compared with ganglia incubated with unmodified medium) was observed.

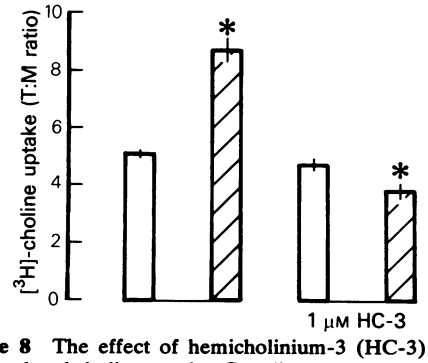

Figure 8 The effect of hemicholinium-3 (HC-3) on K-stimulated choline uptake. Ganglia were given a preliminary incubation for 30 min and then transferred to fresh medium containing [^{3}H]-choline (0.1 μM). The figure shows the effect of including 1 μM HC-3 (in both incubations) on the K-stimulated choline uptake. Each column is the mean and vertical lines show s.e.mean of at least 6 experiments. *Significantly different from controls ($P<0.05$). Controls: open columns; high-K: hatched columns.

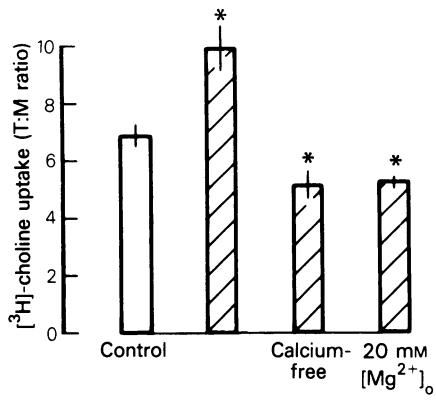

Figure 9 The effect of high-K on choline uptake when transmitter release is inhibited. Ganglia were given a preliminary incubation for 30 min and then transferred to fresh medium containing [^{3}H]-choline (0.1 μM) and incubated for a further 10 min. Transmitter release was inhibited by either omission of Ca or increasing the Mg concentration to 20 mM in both incubations. Each column is the mean and vertical lines show s.e.mean of at least 6 experiments. *Significantly different from controls ($P<0.01$). Controls: open columns; high-K: hatched columns.

Discussion

[^{3}H]-choline uptake by rat sympathetic ganglia

The results showed that ganglia accumulated radioactivity in a temperature-sensitive manner when incubated with a low concentration of [^{3}H]-choline (0.1 μM). This accumulation was unaffected by chronic denervation suggesting that under the present incubation conditions there was no detectable accumulation of [^{3}H]-choline by nerve terminals. The possibility that this result might be due to technical failure of the denervation experiments is unlikely since all the animals exhibited ptosis on the denervated side. Similar results have been reported for rat sympathetic ganglia (Bowery & Neal, 1975), cat sympathetic ganglia (Collier & Katz, 1974) and chick ciliary ganglia (Suszkiw *et al.*, 1976).

High affinity choline transport is characteristically sodium-dependent (for review see Kuhar & Murrin, 1978) but the accumulation of [^{3}H]-choline by rat ganglia was not highly sodium-dependent when ganglia were incubated with either a high or low concentration of choline. Furthermore, the proportion of the uptake which was sodium-dependent was similar (~25%) in each case, and was not altered by chronic denervation. Thus, the sodium-dependence observed in resting ganglia was characteristic of a low affinity transport process, not localized to cholinergic nerve terminals.

One of the most potent inhibitors of high affinity choline uptake is HC-3. This compound inhibits high affinity choline uptake in synaptosomes competitively with a K_i of approximately 0.1 μM (Dowdall & Simon, 1973; Guyenet *et al.*, 1973; Yamamura & Snyder, 1973; Haga & Noda, 1973; Rommelspacher & Kuhar, 1974; Holden, Rossier, Beaujouan, Guyenet & Glowinski, 1975). The uptake of [^{3}H]-choline by rat ganglia was unaffected by HC-3 (1 μM) and reinforces the conclusion that in unstimulated ganglia, sodium-dependent high affinity choline uptake by nerve terminals is not significant. Similar results have been obtained in chick ciliary ganglia in which the uptake of [^{3}H]-choline was also unaffected by HC-3 (Suszkiw *et al.*, 1976).

It has been suggested that the sodium-dependent, high affinity choline uptake system is found only in cholinergic nerve terminals, and that the presence of this system may be used as a sensitive and specific marker for cholinergic nerve terminals (Kuhar, 1973; 1976; Sorimachi & Kataoka, 1974; 1975). This idea seems to be applicable if uptake is studied in a synaptosomal preparation but choline uptake was not associated with nerve terminals in relatively intact preparations of peripheral nervous tissue (Chang & Lee, 1970; Potter, 1970; Collier & Katz, 1974; Suszkiw *et al.*, 1976). Furthermore, uptake of [^{14}C]-choline at low concentrations by rat cortical slices was not affected by HC-3, and only small amounts of radioactivity were incorporated into ACh (Polak, Molenaar & van Gelder, 1977). These experiments indicate that choline uptake by cholinergic nerve terminals is relatively small compared with uptake into other structures. Thus, high affinity choline uptake cannot be regarded as a sensitive marker for cholinergic nerve terminals, unless the uptake is studied in a purified preparation of nerve terminals (i.e. synaptosomes).

Effect of high-K on low affinity uptake

Low affinity choline uptake was studied by incubating ganglia with a high concentration of [^{3}H]-choline (100 μM). Incubation of ganglia with high-K reduced the accumulation of radioactivity by ganglia. Maximum inhibition of choline uptake occurred at a K concentration of 25 mM which was subsequently shown not to affect tritium release from ganglia which had been previously incubated with [^{3}H]-choline (Higgins & Neal, unpublished results). Inhibition of uptake by high-K was not, therefore, an apparent effect of increased release of radioactivity by ganglia. It has been suggested (Murrin & Kuhar, 1976; Vaca & Pilar, 1979) that choline uptake is partly driven by the membrane potential, and the

observation that ganglionic accumulation of [^{3}H]-choline was inhibited by depolarization with high-K is consistent with this theory.

Incubation of ganglia with low concentrations of [^{3}H]-choline (0.1 μM): effect of high-K

When both the preliminary incubation and the final incubation with [^{3}H]-choline (0.1 μM) were in medium containing high-K, the accumulation of radioactivity was increased by 40%. Similar results were obtained when only the preliminary incubation contained high-K, implying that this stimulation of choline uptake is not an effect of depolarization *per se*. Activation of choline uptake by high-K has also been demonstrated in synaptosomes and brain slices (Barker, 1976; Murrin & Kuhar, 1976; Polak, Molenaar & van Gelder, 1977; Murrin, Lewis & Kuhar, 1978; Roskoski, 1978; Weiler, Jope & Jenden, 1978). Stimulation of choline uptake by high-K in rat ganglia was only seen when $[K]_o$ was greater than 30 mM. This was later found to be the concentration of K required to stimulate ganglionic ACh release (Higgins & Neal, unpublished results).

Activation of [^{3}H]-choline uptake only occurred in preganglionic structures since this effect was abolished by chronic denervation. Accumulation of [^{3}H]-choline by denervated ganglia was reduced by 30% during incubations with high-K. This inhibition was quantitatively similar to inhibition of low affinity uptake by high-K, and provides further evidence that when ganglia were incubated with [^{3}H]-choline, radioactivity was accumulated mainly by a low affinity process.

Activation of choline uptake by high-K was abolished by incubations with Na-free medium or by HC-3 (1 μM). The K-stimulated component of [^{3}H]-choline uptake was therefore highly sodium-dependent and inhibited by a low concentration of HC-3. These are two features of high affinity (but not low affinity) choline transport systems which are associated with a high degree of ACh synthesis (see Kuhar & Murrin, 1978). Activation of choline uptake in synaptosomes or brain slices by high-K is due to an increased V_{max} of the high affinity transport process, the K_m being unchanged (Murrin & Kuhar, 1976; Barker, 1976; Polak *et al.*, 1977).

The activation of choline uptake by high-K was abolished when Ca was omitted from the incubation medium or when the Mg concentration was raised to 20 mM. Both of these procedures reduce the K-evoked ACh release from ganglia by more than 80% (Higgins & Neal, unpublished results). These results suggest that activation of choline uptake may be a consequence of increased ACh release. The requirement of Ca for choline uptake activation has been reported previously (Murrin & Kuhar, 1976; DeHaven & Kuhar, 1977; Polak *et al.*, 1977; Roskoski, 1978; Weiler *et al.*, 1978; Vaca & Pilar, 1979; but see Barker, 1976) but reports concerning the effects of high Mg are conflicting. Choline uptake activation was either inhibited (Barker, 1976; Roskoski, 1978; Vaca & Pilar, 1979) or unaffected (Murrin *et al.*, 1977); Weiler *et al.*, 1978) by high Mg. From the latter results, it was concluded that ACh release is not necessary for choline uptake activation, and that uptake activation may be a result of altered ion fluxes or intracellular ion concentrations (Murrin *et al.*, 1977). The present results in ganglia suggest that the release of ACh may be concerned with activating choline transport but do not exclude the possibility that changes in ion fluxes may be involved.

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