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Relationship between acetylcholine synthesis and its concentration in rat cerebral cortex

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1. The synthesis of carbon-14 labelled acetylcholine (^{14}C-ACh) from carbon-14 uniformly labelled glucose (U.L. ^{14}C-glucose) under different conditions has been studied.

2. Increasing KCl concentration from 4 mM to 31 mM was associated with a large increase in the amount of ^{14}C-ACh in the medium and a significant decrease in the amount of ^{14}C-ACh in the tissue. This effect was observed in slices and minces but not in homogenate.

3. Increasing KCl concentration from 4 mM to 31 mM resulted in the release of pre-formed ^{14}C-ACh.

4. Elevated pre-incubation levels of ACh in slices incubated in 4 mM KCl medium was associated with a decrease in the formation of ^{14}C-ACh.

5. These findings are consistent with the hypothesis that the concentration of ACh in the vicinity of the site of synthesis regulates the rate of ACh formation.

The amount of acetylcholine (ACh) in the brain has been reported to change when the functional activity of the organ was altered by certain drugs. While increased levels of total brain ACh were associated with sedation or anaesthesia, decreased values were observed during periods of increased cerebral activity (Richter & Crossland, 1949; Crossland & Merrick, 1954). Giarman & Pepeu (1962) observed that the concentration of ACh in the brain appears to have a ceiling value and suggested that a control mechanism may maintain the levels of ACh within relatively narrow limits. They speculated that the imposition of a ceiling on the levels of ACh achievable in the brain may be related to a depression of the synthetic process by ACh itself.

In this study evidence is provided which suggests that the concentration of ACh in the vicinity of the site of synthesis may regulate the process of ACh formation. The formation of ^{14}C-ACh from carbon-14 uniformly labelled glucose (U.L. ^{14}C-glucose) has been studied in slices in which the ACh content has been elevated by prior administration of morphine (Giarman & Pepeu, 1962; Crossland & Slater, 1968) as well as under conditions in which the release of ACh from the slices was promoted.

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Methods

Male Sprague-Dawley rats weighing between 230 and 400 g were decapitated. The morphine-treated rats were decapitated 90 min after subcutaneous injection of morphine sulphate (50 mg/kg calculated as free base). The brains were immediately removed from the skulls and placed in ice-cold saline. Cerebral cortex slices were prepared as described by McIlwain & Rodnight (1962). Cortex minces were prepared after the method of Gardiner (1961). Homogenates of cerebral cortex were made in three volumes of ice-cold saline containing physostigmine sulphate (2×10^{-4}M) as a cholinesterase inhibitor.

Portions of 200 ± 10 mg (wet weight) of slices, 150 ± 10 mg (wet weight) of minces, or aliquots of homogenates containing 100 mg (wet weight) of cerebral cortex were placed in 50 ml. vessels containing 3 ml. of the incubation medium (pH 7·4). Two forms of incubation media were used. The composition of the first medium was as follows (mM): NaCl, 130; KCl, 4; $CaCl_2$, 2; $NaHCO_3$, 25; U.L. ^{14}C-glucose, 5. This medium will be referred to as 4 mM KCl medium. The composition of the second medium was as follows (mM): NaCl, 103; KCl, 31; $CaCl_2$, 2; $NaHCO_3$, 25; U.L. ^{14}C-glucose, 5. This medium will be referred to as 31 mM KCl medium. Both media contained physostigmine sulphate (2×10^{-4}M). The medium was equilibrated with an atmosphere of 95% oxygen and 5% carbon dioxide and the vessels were shaken at 37° C in a Dubnoff shaker for the time indicated.

To study the effect of KCl on the release of ACh, cortex slices were first incubated in 4 mM KCl medium for 60 min and weighed portions of these slices were reincubated in either 4 mM or 31 mM KCl media in the absence of glucose for periods of time varying between 5 and 60 min. Another portion of the slices was used for the estimation of the ^{14}C-ACh content of the re-incubated portions.

At the end of incubation the vessels were cooled to 0° C. Slices and minces were separated from the media by centrifugation at 2,200 *g* at 0° C for 20 min. The particulate fractions of the homogenates were separated by centrifugation at 13,000 *g* at 0° C for 20 min. The ^{14}C-ACh content of media, slices, minces or particulate fractions of the homogenate was estimated after the method of Browning & Schulman (1969). This method involves extraction of ACh from the tissue by homogenization in fresh incubation medium (pH 4) and heating in a boiling water bath for 10 min after the addition of ACh carrier. The mixture of labelled and unlabelled esters is purified by ion-exchange, after which ACh is isolated as the chloroaurate salt. ACh chloroaurate is plated on Pyrex planchets and its ^{14}C content is measured.

The pre-incubation ACh content of aliquots of brain cortex slices from morphine-treated and unmedicated rats was assayed using the frog rectus abdominus muscle sensitized with physostigmine sulphate (10^{-5}M). Extraction of ACh was performed as described by McIntosh & Perry (1950).

Results

Formation of ^{14}C-ACh by cortex slices, minces and homogenates in 4 mM and 31 mM KCl media

When the K^+ concentration in the incubation medium was increased from 4 mM to 31 mM there was a 4–5 fold increase in the total amount of ACh formed by slices and minces. This increase was due primarily to a marked increase in the

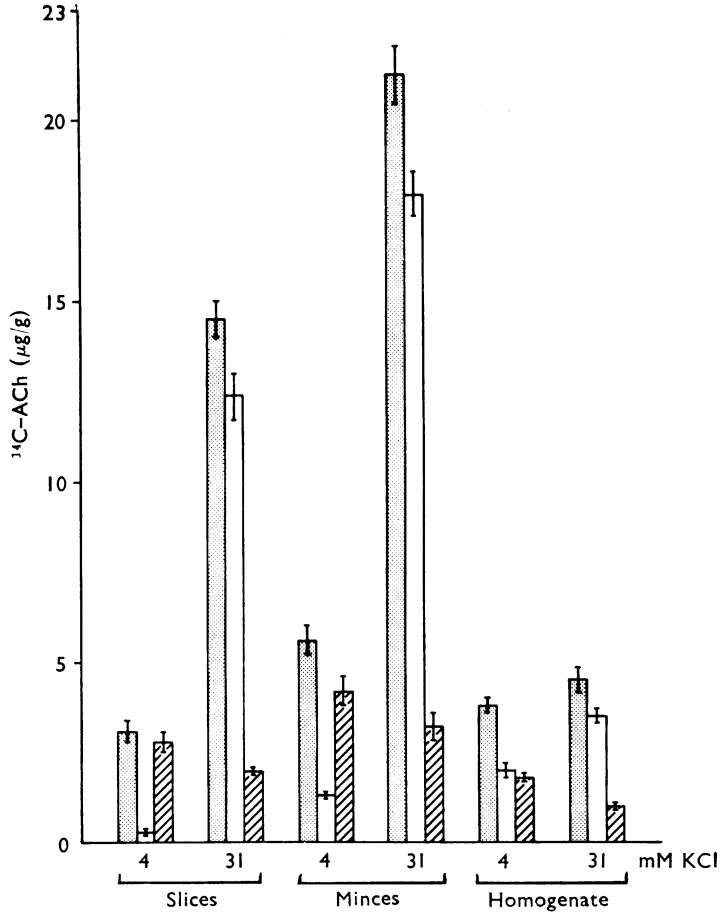

FIG. 1. Formation of ^{14}C-ACh by cortex slices, minces and homogenates. Portions of 200±10 mg (wet weight) of slices, 150±10 mg (wet weight) of minces or aliquots of homogenates containing 100 mg (wet weight) of cerebral cortex were incubated for 60 min at 37° C in 3 ml. of incubation medium. The composition of the 4 mM and 31 mM KCl media are described in **Methods.** ▨, Total ACh ; □, ACh in medium ; ▧ ACh in tissue.

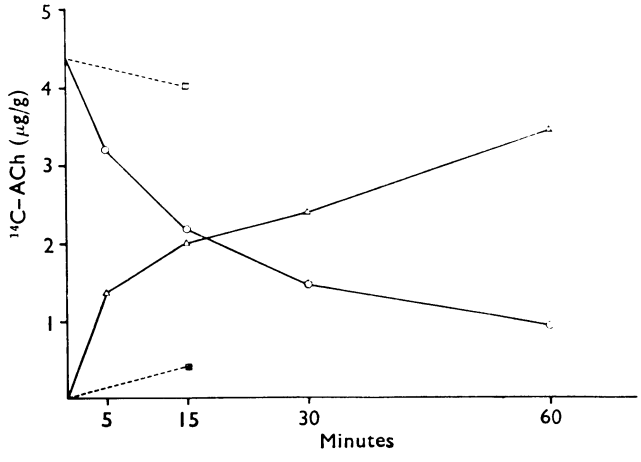

FIG. 2. Release of ^{14}C-ACh from cortex slices. Preincubation (60 min in 3 ml. of 4 mM KCl medium) and incubation in 3 ml. of either 4 mM or 31 mM KCl medium from which glucose was omitted for the time indicated. □- - -□, Slices, 4 mM KCl ; ■- - -■, medium, 4 mM KCl ; △——△, medium 31 mM KCl ; ○——○, slices, 31 mM KCl.

amount of ACh found in the incubation medium. There was about a 25% decrease in the amount of ^{14}C-ACh in the tissue. Synthesis of ^{14}C-ACh by cortex homogenates was the same as in slices in low K^+ medium. The stimulation by high K^+ seen in slices and minces was not observed in the homogenate (Fig. 1).

Release of pre-formed ^{14}C-ACh

The release of pre-formed ^{14}C-ACh from slices previously incubated with U.L. ^{14}C-glucose is shown in Fig. 2. After 15 min re-incubation in 31 mM KCl medium about 50% of the amount of ^{14}C-ACh in the slices was released into the medium. Re-incubation for the same period of time in 4 mM KCl medium showed that only 10% of the ^{14}C-ACh was released into the medium. When the re-incubation was carried out for 1 hr in the 31 mM KCl medium only 20% of the initial

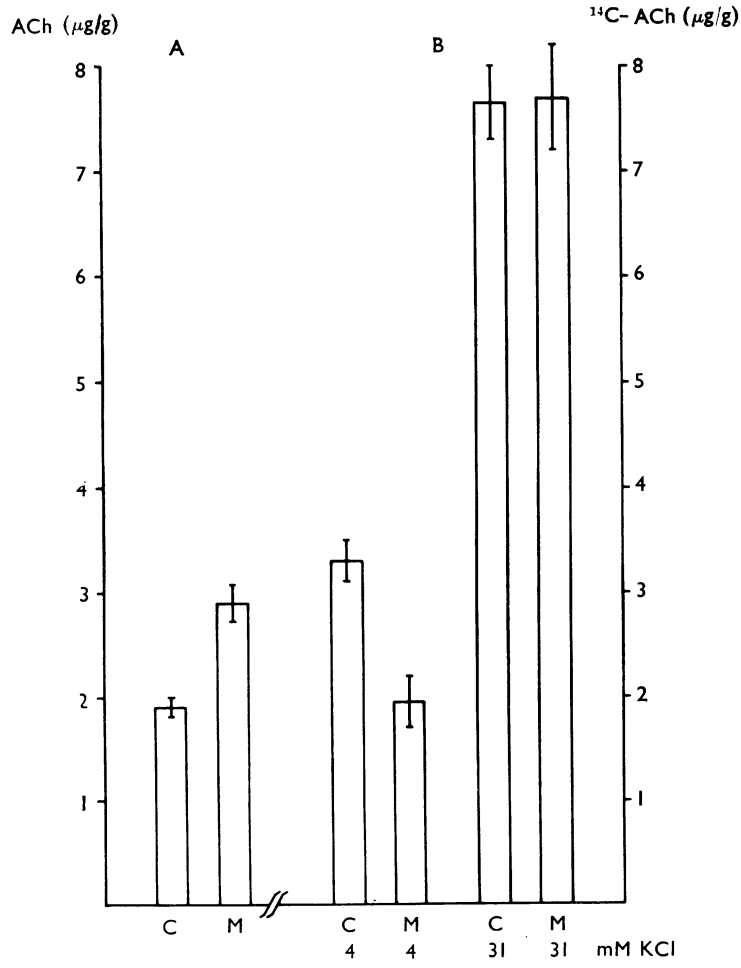

FIG. 3. Influence of morphine administration on the ACh content (A) and on the formation of ^{14}C-ACh (B) in rat cerebral cortex slices. ACh content of aliquots of cortex slices from control and morphine-treated rats before incubation was determined by bioassay. Portions of 200 ± 10 mg (wet weight) of slices were incubated in 3 ml. of 4 mM or 31 mM KCl medium for 30 min at 37° C. C, Control; M, morphine-treated.

amount of ACh remained in the slices. The sum of ^{14}C-ACh released into the medium and that remaining in the slices was almost the same at the four time points studied.

Influence of morphine on ACh content and ^{14}C-ACh formation

The ACh content in cortex slices from control and morphine treated rats is shown in Fig. 3. The amount of ACh in the slices from morphine treated animals was about 45% greater than in those from control rats. This is in agreement with values in whole brain observed by Giarman & Pepeu (1962). The amounts of ^{14}C-ACh formed by cortex slices from morphine-treated rats was markedly less than that formed by slices from untreated animals when incubated in 4 mM KCl medium for 30 min. This effect was not observed when the 31 mM KCl medium was used.

Discussion

Increased KCl concentration in the incubation medium has been shown to increase several fold the amount of ACh detected by bioassay procedures (Mann, Tennenbaum & Quastel, 1939; Bertels-Meeuws & Polak, 1968). Present findings confirm these observations, and show that the increase is due entirely to increased amounts of ^{14}C-ACh in the medium.

Results presented in Fig. 1 show that the stimulating effect of increased KCl concentration requires the integrity of the cell membrane. The stimulating effect was observed with cortex slices and minces but not with homogenates. The amount of ACh found in the slices and minces after incubation in 31 mM KCl medium was consistently about 25% lower than that found after incubation in 4 mM KCl medium despite far greater synthesis in the 31 mM KCl medium. Bioassay techniques employed in previous studies did not allow the differentiation between the effect of increased KCl concentration on the formation and on the release of ACh. The use of ^{14}C-glucose as a precursor for the acetyl moiety of ACh has made it possible to distinguish between these two processes.

Enhanced release of ACh from the slices or minces into the medium may well account for the stimulating effect of 31 mM KCl. This is clearly seen in Fig. 2, in which the process of release of ^{14}C-ACh has been measured in 4 mM KCl and 31 mM KCl media. The effect of increased KCl concentration on the release of ACh has been demonstrated in other systems. For example, increasing KCl concentration of the medium from about 5 to 25–30 mM augmented the release of ACh from the isolated rat diaphragm (Mitchell & Silver, 1963). Also, increasing the KCl concentration in perfusion fluid increased the liberation of ACh from the superior cervical ganglion of the cat (Brown & Feldberg, 1936). It seems improbable that increased KCl concentration in the medium directly stimulated the synthetic process, because the rates of ACh formation were approximately equal in homogenates in both media.

While cellular integrity appears to be necessary for the stimulating effect of KCl, it is not required for ACh formation *per se*. This is deduced from the observation that the amount of ACh formed by homogenates is roughly the same as that formed by slices and minces in 4 mM KCl medium.

Furthermore, rubidium ions, and to a lesser extent caesium ions, have similar effects to KCl on the liberation of ACh from the perfused superior cervical ganglion of the cat (Brown & Feldberg, 1936) and from brain slices (Mann *et al.*, 1939). The similar action of these dissimilar monovalent cations argues for an effect of KCl on the cell membrane rather than on the synthetic process itself.

It seems reasonable to conclude that the stimulating effect of increased KCl on the synthesis of ACh is essentially due to accelerating the release of ACh into the medium, thus decreasing the ACh level in the vicinity of the site of formation. This is supported by the finding that the ^{14}C-ACh content of the slices and minces incubated in 31 mM KCl medium was consistently about 25% less than those found in slices and minces incubated in 4 mM KCl medium. This view is compatible with the concept that the level of ACh in the slice may regulate the rate of formation of the neurohormone. Thus, decreased levels of ACh may enhance the rate of ACh formation, while increased levels may depress the rate of synthesis. Such a regulating negative feedback mechanism between the ACh concentration in the tissue and the rate of ACh formation may explain the maintenance of the level of ACh in the brain within narrow limits.

In support of this concept is the finding of Kaita & Goldberg (1968), who observed that inhibition of choline acetyltransferase by ACh occurred at a concentration of 10 mM and progressively increased to 60% with a concentration of 100 mM ACh. Our finding that elevated pre-incubation levels of ACh in slices from morphine-treated rats were associated with a decreased rate of formation of the neurohormone in 4 mM KCl medium is in accord with the concept that the level of ACh in the vicinity of the site of synthesis may control the rate of formation of the neurohormone. That morphine did not alter the synthesis of ACh in 31 mM KCl medium despite elevated pre-incubation levels of ACh (Fig. 3) could be attributed to the releasing effect of KCl, which would lower the ACh level. This is rather likely in view of the finding that this concentration of KCl released about 50% of the ^{14}C-ACh of the slices within 15 min (Fig. 2).

The finding of Schuberth, Sollenberg, Sundwall & Sorbo (1966) that the dose of morphine used in the present studies did not alter the acetyl coenzyme A content of the rat brain also argues for the absence of a direct inhibition by morphine on the synthetic process of ACh formation. It would be anticipated that any depressant effect of morphine on glucose metabolism would be reflected in a change of the content of acetyl coenzymeA, which is an immediate precursor of ACh.

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