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CHANGES IN SYNAPTOSOMAL HIGH AFFINITY CHOLINE UPTAKE FOLLOWING ELECTRICAL STIMULATION OF GUINEA-PIG CORTICAL SLICES: EFFECT OF ATROPINE AND PHYSOSTIGMINE

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1 Superfused guinea-pig cortical slices were electrically stimulated at different frequencies and the changes in acetylcholine (ACh) content measured. Synaptosomes were prepared at the end of the stimulation period and high affinity choline uptake (HACU) rate was measured.

2 The effect of increasing KCl concentrations was compared on ACh content of the slices and on synaptosomal HACU.

3 Electrical stimulation (2, 5, 10, 20 Hz) elicited a frequency-dependent linear increase in synaptosomal HACU rate and a decrease in ACh content of the slices.

4 The addition of atropine (1.5×10^{-8} M) to the slices enhanced and that of physostigmine (3×10^{-5} M) reduced the frequency-dependent increase in HACU rate. Atropine (1.5×10^{-6} M) not only antagonized the effect of physostigmine, but the HACU rate measured after treatment with both drugs was larger than that found after atropine alone.

5 These results indicate that in the cortical cholinergic nerve endings, depolarization caused by electrical stimulation is coupled with an increase in choline transport which can be modulated by the addition of atropine or physostigmine. Furthermore, within given experimental conditions a linear relationship exists between the reciprocal of ACh content in the slices and synaptosomal HACU.

Introduction

The high affinity choline uptake (HACU) system (Haga & Noda, 1973; Yamamura & Snyder, 1973) is considered the regulatory step in the synthesis of acetylcholine (ACh) (Jope, 1979).

In vitro studies attempting to correlate ACh release, content and HACU showed that when tissue was pretreated with high K^+ concentration and then HACU was measured in normal K^+ medium, HACU was activated (Barker, 1976; Murrin & Kuhar, 1976; Murrin, Dehaven & Kuhar, 1977; Roskoski, 1978; Weiler, Jope & Jenden, 1978). The activation was associated with a reduction in synaptosomal ACh content (Weiler *et al.*, 1978; Roskoski, 1978) and an increase in ACh synthesis (Barker, 1976; Weiler *et al.*, 1978).

It has been proposed (Kuhar, 1977) that HACU is directly coupled with the impulse flow in the cholinergic neurones and in support of this, HACU rate is enhanced in a frequency-dependent manner in the ciliary nerve-iris preparation of chicken *in vitro* (Vaca & Pilar, 1979).

In the present investigation this hypothesis has been further tested by measuring HACU rate in synaptosomes prepared from guinea-pig cortical brain slices submitted to direct electrical stimulation at different frequencies and for comparison to KCl depolarization. The effects of atropine and physostigmine on HACU were investigated and an attempt to correlate ACh content in the brain slices with synaptosomal HACU rate was also made.

A preliminary communication of the results was presented at the Joint Meeting of the British and Italian Pharmacological Societies in Verona (Antonelli, Beani, Bianchi, Pedata & Pepeu, 1981).

Methods

Brain slice preparation and perfusion

The experiments were carried out on adult guinea-pigs of either sex and of 400–500 g body weight. After decapitation, cortical slices were prepared according to the method of Beani, Bianchi, Giacomelli & Tamberi (1978). Briefly, the right and left parietal cortex were rapidly dissected out and plunged into cold oxygenated Krebs solution with the following composition (mM): NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10 and $NAHCO_3$ 25.

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The cortical samples were cut into slices 400 μm thick by means of a microtome for fresh tissue. The slices were kept floating for 30 min in Krebs solution gassed with 95% O_2 and 5% CO_2 and then transferred to Perspex superfusion chambers of 0.9 ml volume and perfused at the rate of 0.5 ml/min at 37°C with the same medium.

Stimulation parameters and protocols

The perfusion with normal Krebs solution was continued for 45 min. During the last 20 min of the perfusion, the slices were stimulated either by applying electrical pulses of alternating polarity (intensity 30 mA/cm^2, duration 5 ms) at different frequencies or by substitution of the Krebs solution with a KCl-enriched medium. When a drug effect was investigated, the drug was added to the perfusing medium 10 min before the electrical stimulation.

High affinity choline uptake

In order to measure HACU, at the end of the stimulation period the slices were rinsed with ice-cold 0.32 M sucrose, weighed and homogenized in a volume of ice-cold sucrose equal to ten times the weight of the tissue. HACU estimation was performed essentially according the method of Simon, Atweh & Kuhar (1976). The homogenate was centrifuged for 10 min at 1000 *g* to remove nuclei and cellular debris. The supernatant was centrifuged at 17,000 *g* for 20 min to obtain a crude mitochondrial pellet (P_2) according to Gray & Whittaker (1962). The pellet (P_2) was resuspended in a volume of ice-cold 0.32 M sucrose equal to the original volume of the homogenate and briefly rehomogenized. Aliquots (100 μl; 0.2 mg of protein) of the resuspended P_2 pellets were added in triplicate to 900 μl of a Krebs-Ringer phosphate medium. The samples were preincubated for 5 min at 37°C; 4 min incubation was then started with the addition of methyl-[^{3}H] choline chloride (6.4 Ci/mmol), obtained from the Radiochemical Centre (Amersham), to the tubes to obtain a final concentration of 0.06 μM. The Krebs-Ringer phosphate buffer had the following composition (mM): NaCl 122, KCl 4.9, $CaCl_2$ 1.3, Na_2HPO_4 15.8, $MgSO_4$ 1.2 and glucose 11.1, pH 7.4. Under the above conditions choline uptake was linear up to 6 min of incubation and up to 300 μl of the resuspended P_2 pellet. At the end of the incubation time the synaptosomes were recovered by Millipore filtration. Filters were dissolved in 1 ml of ethylenglycolmonomethylether (Merck), 10 ml of Instagel (Packard) added and radioactivity counted in a Packard Tricarb (model 577) scintillation spectrometer with a 40% counting efficiency determined by the external standard method. In routine experiments only total high affinity uptake was estimated and blanks were always run under identical experimental conditions in order to correct the uptake rates for filter retention of label. In some experiments HACU rate in a sodium-free medium was also measured.

The rates of choline uptake were expressed as picomoles of choline per 4 min incubation time and per milligram of protein (pmol 4 min^{-1} mg^{-1} protein).

Acetylcholine content

At the end of the stimulation period the slices were quickly transferred into boiling McIlwain buffer (citric acid-disodium phosphate buffer 0.014 M, pH 4.0) for the extraction of their ACh content according to the method of Beani & Bianchi (1963).

Protein determination

Protein content of the slices and homogenates was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.

Drugs

The following drugs were used: atropine sulphate, physostigmine sulphate and acetylcholine chloride purchased from BDH and tetrodotoxin purchased from Sankyo.

Statistical analysis

Student's two tailed *t* test was used to evaluate statistically significant differences. The regression lines were calculated by least squares analysis and tested for correlation coefficients (*r*).

Results

Effect of electrical stimulation, atropine and physostigmine on high affinity choline uptake rate and acetylcholine content

Table 1 shows the effect of different stimulation frequencies on ACh content of the slices at the end of the stimulation period and on HACU rate in the synaptosomes obtained from slices incubated under the same conditions. The changes induced by atropine and physostigmine are also shown.

A significant decrease in ACh content was found only after stimulation at 10 and 20 Hz. Conversely, starting from 5 Hz a significant increase in HACU rate occurred but stimulation periods shorter than 20 min did not significantly affect HACU rate. By

Table 1 Effect of 20 min electrical stimulation at different frequencies on acetylcholine (ACh) content in slices of guinea-pig cerebral cortex incubated with or without atropine and physostigmine and on high affinity choline uptake (HACU) rate in synaptosomes obtained from slices incubated in the same experimental conditions

Drug			*Stimulation frequency (Hz)*			
ACh content	*Concentration (M)*	*Value at rest (pmol/mg protein ± s.e.)*	*2*	*5*	*10*	*20*
					% changes	
None	—	386.7 ± 12.7	92 ± 7.3	95.3 ± 6.8	72.7 ± 5.6*	68.0 ± 5.2**
Atropine	1.5×10^{-6}	368.5 ± 23.7	—	—	58.2 ± 5.3**	79.7 ± 12.1
Physostigmine	3×10^{-5}	425.0 ± 27.6	—	—	79.0 ± 2.9*	79.0 ± 3.4*
Physostigmine plus atropine	3×10^{-5} + 1.5×10^{-6}	365.0 ± 17.6	—	—	83.0 ± 3.4*▲	81.8 ± 6.0*
HACU rate		*(pmol mg^{-1} protein 4 min^{-1} ± s.e.)*				
None		2.45 ± 0.10	100 ± 10	123 ± 15**	138 ± 20**	151 ± 20*
Atropine	1.5×10^{-8}	2.59 ± 0.15	136 ± 20°°	147 ± 22°°	150 ± 18°°	173 ± 13°°
Atropine	1.5×10^{-6}	2.77 ± 0.16	126 ± 20°	148 ± 22°	164 ± 30°	125 ± 20°
Physostigmine	3×10^{-5}	2.61 ± 0.16	85 ± 7°	104 ± 20	121 ± 7°	115 ± 10°
Physostigmine plus atropine	3×10^{-5} + 1.5×10^{-6}	2.73 ± 0.19	155 ± 15°°†	164.18 ± °°†	183 ± 25°°†	154 ± 15

Different from the respective value at rest: $*P<0.05$; $**P<0.001$.
Different from atropine 1.5×10^{-6} M at 10 Hz: $▲P<0.05$.
Different from the value obtained at the respective stimulation frequency without drugs: $°P<0.05$; $°°P<0.001$.
Different from the value obtained at the respective stimulation frequency in the presence of atropine 1.5×10^{-6} M; $†P<0.05$.

plotting the stimulation frequencies against the percentage increase in HACU rate, a linear relationship was obtained ($r = 0.99$).

When choline uptake was measured in a sodium-free medium its rate was 0.69 ± 0.03, pmol mg^{-1} protein 4 $min^{-1} \pm$ s.e. ($n = 4$). This value represents the 28.1% of the total uptake value at rest shown in Table 1 and underwent no significant changes after electrical stimulation at 5 Hz (0.80 ± 0.02) at 10 Hz (0.65 ± 0.03), KCl 32 mM depolarization (0.71 ± 0.07), or in the presence of atropine 1.5×10^{-8} M at 5 Hz (0.81, $n = 2$).

When atropine 1.5×10^{-8} M was added to the superfusion medium, the effect of electrical stimulation on HACU rate was enhanced and an increase in HACU rate was also detected at a stimulation frequency of 2 Hz; under these conditions a linear relationship ($r = 0.98$) between stimulation frequency and the percentage increase in HACU rate was also obtained.

Atropine 1.5×10^{-6} M also enhanced the effect of electrical stimulation on HACU rate up to 10 Hz stimulation frequency but it reduced the effect of electrical stimulation at 20 Hz. Similarly, in the presence of atropine there was a significant decrease in ACh content in the slices at 10 but not at 20 Hz stimulation frequency.

The addition of physostigmine 3×10^{-5} M to the superfusion medium strongly reduced the increase in HACU rate elicited by electrical stimulation; the linear relationship between stimulation frequency and HACU rate was lost since at 20 Hz HACU rate was smaller than that at 10 Hz. Atropine 1.5×10^{-6} M not only antagonized the effect of physostigmine but the HACU rate measured in the presence of both drugs together was larger than in the presence of atropine only and a 31% increase in HACU rate also could be found at 0.5 Hz stimulation frequency (data not shown). However, under these conditions HACU rate following stimulation at 20 Hz was also smaller than after that at 10 Hz.

The addition of physostigmine only slightly increased ACh content in the slices at rest, it reduced the decrease in ACh content following electrical stimulation and that induced by atropine 1.5×10^{-6} M at 10 Hz stimulation.

Effect of different K^+ concentrations on high affinity choline uptake rate and acetylcholine content

Table 2 shows the changes in ACh content in brain slices after 20 min incubation in increasing KCl concentrations from 4.7 to 62 mM and in HACU rate in the synaptosomes obtained from slices incubated under the same conditions. A marked decrease in ACh content and a large increase in HACU rate with a linear relationship with KCl concentration ($r = 0.98$) were found.

Discussion

Our results show a direct relationship between stimulation frequency and HACU rate in synaptosomes obtained from stimulated slices. We preferred to stimulate brain slices instead of synaptosomes since in our conditions, KCl-induced changes in HACU rate were almost three times greater than those observed by Murrin & Kuhar (1976) who depolarized the synaptosomes directly. On the other hand, HACU was measured in synaptosomes removed from the slices rather than in the slices since it has been shown (Polak, Molenaar & Van Gelder, 1977) that the differences in choline uptake brought about by incubation of slices in different media are greatest in the fraction P_2. According to Atweh, Simon & Kuhar (1975) the activity-induced changes in uptake were maintained *in vitro* as synaptosomal suspensions from treated animals were kept up to 3 h on ice without loss of uptake changes. A similar duration can be expected also when the changes are induced *in vitro*.

The present results demonstrate therefore that the increase in neuronal impulse flow in the cortical cholinergic nerve endings induced by electrical stimulation is directly coupled to an increase in HACU rate. However, only stimulation frequencies higher than 2 Hz and lasting 20 min trigger HACU

Table 2 Effect of different KCl concentrations on acetylcholine (ACh) content in slices of guinea-pig cerebral cortex and on high affinity choline uptake (HACU) rate in synaptosomes obtained from the slices after incubation

KCl (*mM*)	*ACh content* (*pmol/mg protein ± s.e.*)	*% change*	*HACU rate* (*pmol mg^{-1} protein 4 min^{-1}*)	*% change*
4.7	390.6 ± 12.7	100	2.5 ± 0.12	100
25	159.6 ± 22.0*	40.8	2.9 ± 0.4 *	148
32	77.3 ± 14.4*	20.3	4.73 ± 1.2 *	184
62	61.8 ± 5.5*	16.2	6.02 ± 0.72*	259

Each value is the mean ± s.e. of at least 5 experiments.
*Significantly different from slices in KCl 4.7 mM Krebs solution; $P < 0.001$.

activation. This seems to indicate that basal choline transport normally allows a wide range of responses. A direct relationship was also found between K^+ concentration in the superfusion medium and HACU rate, confirming previous observations (Barker, 1976; Murrin & Kuhar, 1976; Murrin *et al.*, 1977; Weiler *et al.*, 1978) that a prior increase in K^+ concentration is followed, after removal, by HACU activation. It appears therefore that in the cortical cholinergic nerve endings depolarization caused either by electrical stimulation or by high K^+ concentration is coupled with an increase in choline transport.

Vaca & Pilar (1979) showed a frequency-dependent increase in HACU rate in the chick ciliary nerve. According to these authors the depolarization of the nerve endings stimulates HACU rate through a Ca^{2+}-dependent increase in ACh output followed by a decrease in the level of intraterminal ACh which in turn activates ACh synthesis by mass action, allowing further accumulation of choline.

Our results also show a frequency and K^+ concentration-dependent decrease in ACh content in the slices.

It is well known that brain ACh is contained mostly within the synaptosomes (Hebb & Whittaker, 1958) and therefore changes in ACh content in the cortical slices can be assumed to correspond to changes in synaptosomal ACh content.

Following this assumption, the reciprocal of ACh content of the brain slices in all experimental conditions obtained from Table 1 by converting the percentage changes of the values at rest and directly from Table 2, was plotted against the respective synaptosomal HACU rate. Two different correlations were found if the changes in ACh content and HACU rate elicited by electrical stimulation were kept separate from those induced by K^+ depolarization. As shown in Figure 1, a regression line with an $r = 0.98$ ($P < 0.01$) was found by plotting the reciprocal of ACh content against the respective changes in HACU rate induced by K^+ depolarization and a regression line with $r = 0.97$ ($P < 0.001$) by plotting the changes induced by electrical stimulation without and with drugs, excluding the two experiments in which stimulation occurred during superfusion with atropine and physostigmine together. However, if these experiments were also included, an r of 0.72 ($P < 0.05$) was found. Jenden, Jope & Weiler (1976) reported that there is an inverse correlation of choline uptake and ACh content, altered by treatments *in vivo*, in mouse brain synaptosomes. A similar correlation was found *in vitro* by Polak *et al.* (1977) and Roskoski (1978).

Physostigmine (Paton, 1954; Wright, 1956; Dettbarn & Bartels, 1968) and large concentrations of atropine (Paton, 1954) may cause a partial

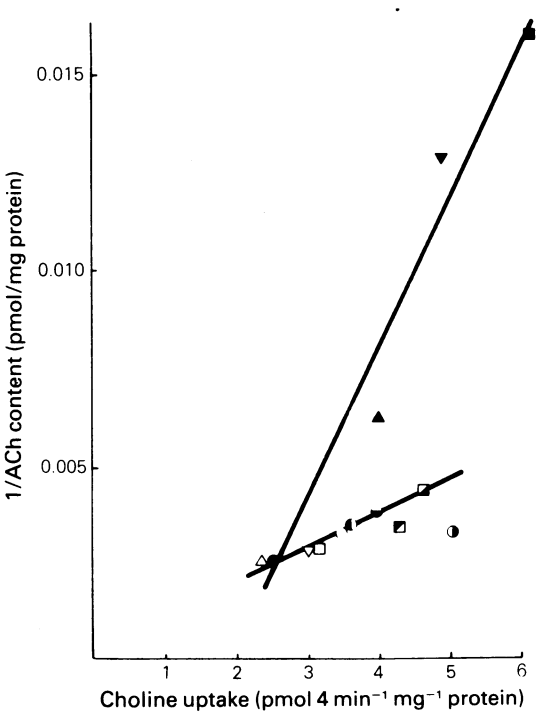

Figure 1 Correlations between the reciprocal of acetylcholine (ACh) content in the brain slices and synaptosomal high affinity choline uptake (HACU) rate. (●) Control; KCl mM: (▲) 25, (▼) 32, (■) 62; (△) 2 Hz; (▽) physostigmine 3×10^{-5} M and 20 Hz; (□) physostigmine 3×10^{-5} M and 10 Hz; (○) atropine 1.5×10^{-6} M and 20 Hz; (◐) 10 Hz; (◒) 20 Hz; (◪) atropine 1.5×10^{-6} M and 10 Hz; (◩) atropine + physostigmine and 20 Hz; (◑) atropine + physostigmine and 10 Hz.

conduction block, the latter drug through its local anaesthetic properties (De Elio, 1948). This could explain the decrease in HACU rate at 20 Hz in the brain slices perfused with atropine 1.5×10^{-6} M and physostigmine 3×10^{-5} M alone or together.

The addition of atropine 1.5×10^{-8} M or physostigmine 3×10^{-5} M to the incubation medium respectively enhanced or decreased the effect of the electrical stimulation on HACU rate.

It has been shown that at similar concentrations, atropine stimulates (Szerb, Hadhazy & Dudar, 1977) and physostigmine inhibits (Szerb & Somogyi, 1973) ACh output from electrically or K^+-stimulated (Polak, 1971) brain slices by removing or enhancing respectively an inhibitory control on ACh output exerted by extracellular ACh on muscarinic presynaptic receptors (Polak, 1971; Hadhazy & Szerb, 1977; Rospar, Lefresne, Beaujouan & Glowinsky, 1977).

Atropine 1.5×10^{-6} M removed the inhibitory effect of physostigmine on HACU rate and the superfusion of the slices with physostigmine and atropine brought about an increase in HACU rate greater than with atropine only. However, even under these conditions HACU rate was lower at 20 Hz than at 10 Hz stimulation frequency as expected from a partial impairment of nerve conduction. Bourdois, Mitchell, Somogyi & Szerb (1974) reported that labelled ACh output from stimulated cortical slices was larger in the presence of physostigmine and atropine than of atropine alone. Furthermore, by inhibiting cholinesterase, physostigmine could reduce the availability of extracellular choline of endogenous origin thus enhancing the uptake of radioactive choline.

In conclusion, from our experiments HACU rate appears to be directly coupled to the impulse flow through the cholinergic nerve endings and a good indicator of the ensuing changes in ACh utilization. HACU rate is modulated by cholinergic agonists and antagonists probably through presynaptic muscarinic receptors as shown for ACh release (Polak, 1971; Hadhazy & Szerb, 1977; Rospar *et al.*, 1977). From the experiments in which ACh content and HACU rate were measured in the slices and their synaptosomes it appears that within given experimental conditions a linear relationship exists between HACU rate and the reciprocal of ACh content, corroborating the finding that the concentration of synaptosomal cytoplasmic ACh regulates HACU rate (Whittaker & Dowdall, 1975).

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