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High affinity choline uptake and acetylcholine release by guinea pig neocortex synaptosomes: inhibition by adenosine derivatives

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An increasing number of reports indicate that adenosine or related compounds may function as modulators in the central nervous system. Thus, it has been shown that adenosine produces a marked depressive effect on synaptically evoked responses in studies using *in vitro* slices of rat olfactory cortex [1] and hippocampal formation [2]. Iontophoretic studies of the actions of purine and pyrimidine nucleotides have led to the discovery that adenosine and various adenine nucleotides are potent depressants of the spontaneous firing of rat cerebral cortical neurons [3]; this depressant effect has been attributed to a presynaptic action of adenosine, including a reduction in transmitter output [4].

Indeed, adenosine inhibited the release of acetylcholine, dopamine and 5-hydroxytryptamine from slices of rat corpus striatum [5], noradrenaline, γ-aminobutyric acid from slices of rat cerebral cortex [6, 7] and dopamine from rat striatal synaptosomes [8]. In all these instances release of labelled transmitter was induced by potassium — depolarisation and the effect of adenosine was generally small. The release of adenosine and its derivatives has been shown to occur from cerebral cortex preparations [9–12], where cholinergic transmission has been reliably identified [13]. On the other hand it has been suggested that the activity of cerebral cortical neurons [4], as well as cholinergic transmission [14], are subjected to modulation by locally released purines. It seems likely that purine compounds can modulate the cholinergic transmission by acting presynaptically, either on the release of acetylcholine or on the high affinity choline uptake or on both.

Since, so far, there has not been any *in vitro* study of the presynaptic effect of adenosine and its derivatives on the mechanisms related to cholinergic transmission using cerebral synaptosome preparations, we have examined the action of adenosine and related compounds on the high affinity [^{14}C]choline uptake and the release of [^{14}C]acetylcholine from guinea pig neocortex synaptosomes.

Synaptosomes derived from the neocortex of guinea pigs were prepared as previously described [12]. The uptake experiments were carried out with a synaptosome suspension (0.6 mg protein) incubated at room temperature in a glucose–bicarbonate medium containing [Me-^{14}C]choline chloride (0.78 μM, 1 μCi) (Radiochemical Centre, Amersham, U.K.) for 40 min. Separation of tissue-bound ^{14}C from that remaining in the incubation medium was achieved by rapid filtration through a cellulose filter (Sartorius, 0.2 μm pore size) followed by washing with 25 ml of glucose–bicarbonate saline. The radioactivity retained on the filters was counted in 10 ml of liquid scintillant (toluene–Triton–X 100, 2:1, v/v; PPO 0.55%, w/v; POPOP 0.1%, w/v) in a Beckman spectrometer LS 150 type.

The release experiments were carried out with synaptosome beds prepared as previously described [15]. Four beds were separately incubated at 37°, for 30 min, in 5 ml of glucose–bicarbonate saline containing [Me-^{14}C]choline chloride (4 μM, 1.2 μCi). The standard glucose–bicarbonate medium used for incubation and superfusion contained 120 mM NaCl, 3.5 mM KCl, 1.25 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose, equilibrated with a gas mixture of 95% O_2/5% CO_2. After a 20 min washout period, 13 successive 2-min fractions were collected. When used, test agents were present in the superfusion fluid of 2 beds after 6 min of collections. Depolarization induced release was performed for 10 min by superfusion with glucose–bicarbonate medium containing 33 mM K^+, after 16 min of collections. After superfusion, synaptosome beds were removed from their holders, and promptly transferred from the fluid to 10% (w/v) trichloroacetic acid in a test-tube homogeniser. Thereafter, the release of radioactivity, in excess of spontaneous release, resulting from 33 mM K^+-depolarization was calculated as percentage of released radioactivity at the onset of stimulation.

Protein was determined according to the method of Miller [16].

Preliminary experiments showed that synaptosomes accumulated radioactivity in a linear fashion when incubated with 0.78 μM [^{14}C]choline for 40 min at room temperature. This accumulation amounted to 5.1 nmol of [^{14}C]choline per 100 mg protein. This uptake of [^{14}C]choline by synaptosomes was reduced when Na^+ ions were replaced by Li^+ ions in the incubation fluid, but it was not altered when Ca^{2+} ions were absent (Table 1). Hemicholinium-3 inhibited severely the uptake of choline which was almost abolished at low temperature (Table 1). These results indicated that we were actually measuring the high affinity choline uptake by the synaptosomes. ATP (10 mM) caused a marked inhibition of the uptake of [^{14}C]choline (Table 2) but at a lower concentration it was ineffective. AMP–PNP, a non hydrolysable ATP analog [17], when used at a concentration of 1 mM, caused a strong inhibition of the rate of choline uptake. GTP (10 mM) also was a potent

Table 1. High affinity [^{14}C]choline uptake by guinea pig neocortex synaptosomes in various conditions of incubation

Incubation condition	[^{14}C]choline uptake (per cent of control) (mean ± S.D.)	(*n*)
No Na^+	46.1 ± 4.3	(4)*
No Ca^{2+}	97.9 ± 3.6	(3)
1 μM Hemicholinium-3	23.9 ± 2.2	(4)*
10 μM Hemicholinium-3	15.8 ± 1.4	(4)*
0°	10.4	(1)

n = number of experiments.

* Significantly different from control; $P \leqq 0.0005$ (Student's *t*-test for paired observations)

The uptake of [^{14}C]choline (0.78 μM) into metabolising synaptosomes was estimated at room temperature as described in the text. When absent, Na^+ ions were replaced by Li^+ ions.

Table 2. Effect of adenosine and related agents on high affinity [^{14}C]choline uptake by guinea pig neocortex synaptosomes

Test agent	Concentration (mM)	Concentration (μM)	[^{14}C]choline uptake per cent of control (mean ± S.D.)	(n)
ATP	10		62.8 ± 5.0	(7)*
ATP	1		109.0 ± 5.9	(3)
ATP		10	108.8 ± 10.3	(9)
ATP		0.01	107.1 ± 0.2	(2)
ADP	10		80.5 ± 1.9	(3)
AMP	10		103.9 ± 2.3	(3)
Adenosine	10		103.2 ± 7.5	(2)
Adenosine	0.1		105.0 ± 10.1	(3)
Adenosine		1	105.4 ± 5.8	(2)
AMP – PNP	1		67.6 ± 10.9	(4)*
GTP	10		47.3 ± 3.7	(6)*
2′-Deoxyadenosine	10		85.3 ± 7.7	(4)*
Theophylline	10		64.2 ± 7.2	(4)*
ATP + theophylline	10		39.1 ± 13.1	(3)

n = number of experiments.
* Significantly different from control; $P < 0.005$ (Student's t-test for paired observations).

inhibitor of the choline uptake. ADP also caused an inhibition of the choline uptake, in contrast with AMP and adenosine which had no effect. The more phosphorylated adenine nucleotides were apparently the more potent. 2′-Deoxyadenosine, a non competitive inhibitor of adenosine [18], appeared to be effective, with a potency similar to that of ADP. Theophylline, at a low dose (0.1 mM), had no action on the ATP-induced decrease in choline uptake (not shown). At a higher dose (10 mM), theophylline by itself caused an inhibition which was partially additive to the inhibitory effect of ATP on choline uptake (Table 2).

The release of [^{14}C]acetylcholine from synaptosome beds evoked by K^+-depolarisation was evaluated by estimating the ^{14}C output, since choline is said not to be released by K^+-depolarisation of [^{14}C]choline-prelabelled synaptosomes [19]. The increases over basal ^{14}C efflux caused by depolarization with 33 mM K^+-glucose–bicarbonate saline reached 150%. Eserine sulphate (50 μM) an inhibitor of acetylcholinesterase (EC 3.1.1.7), added into the superfusion medium, did not affect the K^+-stimulated release, nor did adenosine deaminase (EC 3.5.4.4) (10 μg/ml). This suggests that the uptake of [^{14}C]choline did not interfere with the estimation of ^{14}C output and that endogenous adenosine did not control the release of acetylcholine from neocortex synaptosomes. The subsequent experiments were then performed without eserine and adenosine deaminase.

The K^+-induced release of ^{14}C radioactivity was not affected by adenosine (10 mM and 10 μM) and adenosine (10 mM) plus dipyridamole (20 μM) (Table 3). AMP at a concentration of 10 mM also appeared to be ineffective, in contrast with ADP and ATP which strongly inhibited the K^+-induced release but not the spontaneous release of [^{14}C]acetylcholine. ATP (10 μM) did not significantly affect the release. In the presence of 5 mM Ca^{2+}, the inhibitory effect of ATP was still largely significant, while GTP was without effect. 2′-Deoxyadenosine (10 mM) caused a strong inhibition of the evoked release, with a potency similar to that of ADP, while 2′-deoxyadenosine (10 mM) + ATP (1 mM) completely blocked the stimulation of release by K^+ (Table 3).

In this study, we have not been able to demonstrate a modulatory effect of adenosine on the release of acetylcholine from guinea pig neocortex synaptosomes, in contrast with previous results obtained with rat striatal slices [5]. We were not able to explain these differences although it should be recalled that a synaptosomal suspension or bed consists of individually small entities not connected as in a tissue. However our results are in keeping with those obtained by Vizi and Knoll [23] with isolated cortex slices of the rat. ATP seems to play a role both on the uptake of choline and the release of acetylcholine, but only at high concentrations. The inhibitory effect of this nucleotide on the evoked release of acetylcholine does not seem to be due only to Ca^{2+} chelation since it was still significant in the presence of 5 mM Ca^{2+}. Moreover, GTP, in the presence of 5 mM Ca^{2+}, had no effect on the release of acetylcholine, while ADP and 2′-deoxyadenosine in the presence of 0.75 mM Ca^{2+}, were as efficient as ATP plus 5 mM Ca^{2+} (Table 3). These high concentrations of purine should be compared with those applied in some electrophysiological studies [4], and in the peripheral nervous system [20].

The present findings do not explain the action of ATP on cholinergic neurotransmission in the brain. Externally applied ATP in relatively high concentrations might inhibit the Na^+–K^+ ATPase. Such an inhibition of ATPase by ouabain resulted in an increased release of acetylcholine and an inhibition of choline uptake (unpublished results). This suggests that the inhibition of [^{14}C]choline uptake and [^{14}C]acetylcholine release by high concentration (10 mM) of ATP was not due to an inhibition of Na^+–K^+ ATPase. ATP has been shown markedly to reduce the high affinity transport of glutamate but not that of aspartate and GABA by rat brain synaptosomes [21, 22]. In addition, the incubation of rat cortical synaptosomes in the presence of 1 mM ATP markedly reduced Ca^{2+} dependent K^+-induced release of preloaded [^{3}H]GABA [21]. These effects of ATP have been attributed to a phosphorylation of presynaptic membrane proteins.

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Table 3. Effect of adenosine and related agents on 33 mM K^+-induced release of [^{14}C]acetylcholine from guinea pig neocortex synaptosome beds

Test agent	Concentration (mM)	[^{14}C]acetylcholine release per cent of control (mean ± S.D.)	(*n*)
ATP	10	25.0 ± 9.2	(6)*
ATP	0.01	116.4 ± 48.1	(6)
ADP	10	47.6 ± 7.0	(4)*
AMP	10	102.2 ± 24.9	(4)
Adenosine	10	113.6 ± 21.6	(5)
Adenosine	0.01	100.0 ± 15.1	(3)
ATP + Ca^{2+}	10 5	54.7 ± 8.2	(4)*
GTP + Ca^{2+}	10 5	102.9 ± 11.0	(4)
2′-Deoxyadenosine	10	41.1 ± 6.6	(4)*
ATP + 2′-Deoxyadenosine	10 10	8.5 ± 5.8	(4)*

The release of ^{14}C into medium containing 4.75 mM K^+ was approximately 800 dpm/2 min/mg protein. Increasing the K^+ concentration produced an increase of ^{14}C output which reached about 2300 dpm/2min/mg protein.

n = number of experiments.

* Significantly different from control; $P < 0.001$ (Student's *t*-test for paired observations).

† Significantly different from the effect of 10 mM ATP; $P < 0.005$ (Student's *t*-test for paired observations).

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