undoubtedly a matter that deserves close attention. The ability of these fractions to bind calcium has been demonstrated recently [1, 7]. Meanwhile it has been shown that high doses of ethimizole increase the total calcium concentration in brain tissue [9]. Since ethimizole is largely antagonistic in its effects to caffeine, which inhibits Ca^{++} uptake by intracellular membranes, the possibility cannot be ruled out that it may have a specific influence on the calcium metabolism of particular cell structures, which is closely bound with the cyclic nucleotide system and modified when there is a change in state of the excitable cell membrane [1, 11].

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EFFECT OF ANTIOXIDANTS ON [^{3}H]SEROTONIN RELEASE BY RAT BRAIN SYNAPTOSOMES

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KEY WORDS: synaptosomes; serotonin; peroxidation; antioxidants.

Release of neurotransmitters mediating Ca^{++}-dependent mechanisms is a key stage in the chain of events lying at the basis of synaptic transmission [1]. It has recently been established that certain lipids and, in particular, peroxidation products of free fatty acids and glycerophosphatides are highly efficient Ca^{++} ionophores [6]. Hence it follows that compounds which can affect free-radical lipid peroxidation (LPO) and, consequently, which can change the content of Ca^{++} ionophores in the synaptic membrane, may be regulators of neurotransmitter secretion.

The object of this investigation was to study the effect of LPO inhibitors of different chemical nature on release and reuptake of [^{3}H]serotonin by the synaptosomal fraction of rat cerebral cortex.

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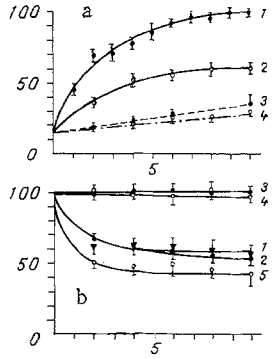

Fig. 1

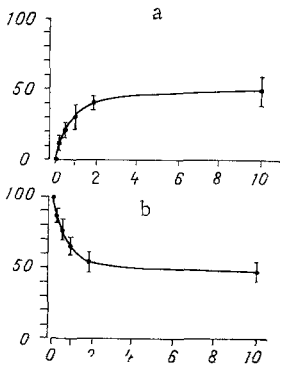

Fig. 2

Fig. 1. Kinetics of [^{3}H]serotonin uptake (a) and release (b) by rat brain synaptosomes. 1, 3) Control at 37 and 4°C respectively; 2, 4) 10^{-5} M 4-methyl-2,6-di-tert-butylphenol at 37 and 4°C respectively; 5) K^+-induced release in presence of antioxidant. Abscissa, incubation time (in min); ordinate, effectiveness of uptake or release (in % of maximal [^{3}H]serotonin concentration).

Fig. 2. Dependence of action of 4-methyl-2,6-di-tert-butylphenol on uptake (a) and release (b) of [^{3}H]serotonin by brain synaptosomes on concentration. Abscissa, concentration of antioxidant (in M × 10^{-5}); ordinate, effectiveness of uptake or release (in % of maximal [^{3}H]serotonin concentration).

EXPERIMENTAL METHOD

The fraction of unpurified rat cerebral cortical synaptosomes was obtained by a slightly modified method [4]. Synaptosomes were incubated and washed in Krebs–Ringer solution of the following composition: 120 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 0.75 mM $CaCl_2$, 20 mM Tris-HCl, 10 mM glucose, and 10 μM pargyline, pH 7.4 (20°C).

The synaptosomes were suspended up to the working concentration (1 mg protein/ml) and incubated at 37°C with constant stirring. [^{3}H]Serotonin (specific radioactivity 13.2 mCi/mmole, from Amersham Corporation, England) was added in a volume of 100 μl to a final concentration of 20 nM.

The synaptosomes were incubated with [^{3}H]serotonin for 10 min at 37°C, after which uptake of the label was stopped by cooling and the synaptosomes were washed twice with cold Krebs–Ringer solution. Serotonin release was induced by 50 mM KCl. At the end of incubation the samples were filtered, washed 3 times with cold Tris-buffer, and the filter was placed in a flask containing scintillation fluid for measurement of radioactivity.

The antioxidants [α-tocopherol, 4-methyl-2,6-di-tert-butylphenol (from Serva, West Germany), 7-hydroxychlorpromazine (from Regis), chlorpromazine (from Reakhim, USSR), paginol, and 2-methyl-6-ethyl-3-hydroxypyridine (synthesized at the Institute of Chemical Physics, Academy of Sciences of the USSR)] were added to the suspension of synaptosomes as aqueous or alcoholic solutions (5 μl/ml), with a corresponding alcohol control.

LPO products were recorded spectrophotometrically by determination of TBA-active products and spectrofluorometrically by determination of fluorescent products [2].

EXPERIMENTAL RESULTS

Curves showing the kinetics of uptake and K^+-stimulated release of [^{3}H]serotonin from synaptosomes are given in Fig. 1. Both processes are temperature-dependent and do not take place at 4°C. In the presence of antioxidant (4-methyl-2,6-di-tert-butylphenol) a marked decrease was observed in the efficiency of [^{3}H]serotonin uptake by the synaptosomes (Fig. 1a) and release of the mediator also was stimulated (Fig. 1b).

After combined addition of antioxidant and 50 mM KCl a very slight additive effect was observed. It will be clear from Fig. 1 that the action of the antioxidant was an energy-dependent active process that did not take place at 4°C.

TABLE 1. Action of Antioxidants on [^{3}H]-Serotonin Uptake and Release

Substance, 5×10^{-5} M	Decrease of uptake, %	Stimulation of release, %
Alcohol	8,1±2,2	14,8±3,6
4-Methyl-2,6-di-tert-butylphenol	39,7±3,0	32,8±3,4
Paginol	30,0±4,2	26,0±3,0
2-Methyl-6-ethyl-3-hydroxypyridine	30,0±6,3	26,5±3,0
α-Tocopherol	11,0±3,1	15,8±4,0
Chlorpromazine	74,0±5,6	34,5±5,5
7-Hydroxychlorpromazine	83,5±13,4	46,0±5,0
Amitriptyline	80,0±4,2	27,0±10,7
KCl	43,6±8,0	42,5±4,6

Legend. Paginol is an antioxidant belonging to the screened phenol class.

These results are in good agreement with those of experiments which showed that the effects of antioxidants on [^{3}H]serotonin uptake and release are mediated by Ca^+-dependent mechanisms, for when a calcium-free medium was used or after the addition of EGTA, forming complexes with Ca^{++} ions, inhibition of uptake and stimulation of release of the mediator by the antioxidant were virtually not observed (they were reduced to 5 ± 3% and 7 ± 4%, respectively).

The results of a study of dependence of the action of 4-methyl-2,6-di-tert-butylphenol on uptake and release of [^{3}H]serotonin on antioxidant concentration are given in Fig. 2. It will be clear from Fig. 2 that inhibition of uptake and stimulation of release rose steadily with an increase in antioxidant concentration; half the maximal effect was reached with antioxidant concentrations of about $5 \cdot 10^{-4}$ M.

In the next series of experiments the action of antioxidants of different chemical structure on uptake and release of [^{3}H]serotonin was studied. All the compounds tested were found to be inhibitors of serotonin uptake and their activity fell in the following order: 7-hydroxychloropromazine > chlorpromazine > 4-methyl-2,6-di-tert-butylphenol > paginol = 2-methyl-6-ethyl-3-hydroxypyridine > α-tocopherol. The last compound had only a slight inhibitory action, whereas the first two were equivalent in their effectiveness to the action of the well-known uptake inhibitor amitriptyline.

The action of the antioxidants in stimulating release (Table 1) also was found to be directly proportional to the degree of inhibition of uptake (coefficient of correlation $r = 0.868$, $P < 0.01$). This suggests that antioxidants are not true inhibitors of uptake, like amitriptyline, but their action is mediated through stimulation of release, i.e., by a shift of dynamic equilibrium.

The question arises: What is the mechanism of action of antioxidants on [^{3}H]serotonin release? Free-radical oxidation of unsaturated fatty-acid residues is known to take place both directly in the composition of phospholipids, leading to the formation of hydroperoxides of the phospholipids as primary molecular products, but also after preliminary "chipping out" of aliphatic acyl groups from the phospholipid molecules by phospholipases [5]. In the last case the primary molecular products of the reaction are endoperoxides of prostaglandins (cyclo-oxygenase pathway). In principle the inhibitors of free-radical oxidation used in the investigation can inhibit either of the reactions mentioned [3]. Consequently it seemed important to determine to what extent the effects of the inhibitors used on mediator uptake and release are connected with peroxidation of membrane phospholipids or are due to their action on enzymic metabolism of unsaturated fatty acids (by a cyclo-oxygenase or lipo-oxygenase mechanism).

The possibility of realization of the first of these two possible pathways was examined in this investigation. A direct measurement was made of the concentration of phospholipid peroxidation products during uptake and release of mediators (Table 2). The results showed that K^+-stimulated release is not accompanied by accumulation of LPO products. Meanwhile it follows from the data that activation of LPO leads to release of mediator, evidently on account of total damage to the synaptosomal membranes.

TABLE 2. Accumulation of LPO Products and [^{3}H]Serotonin Release under the Influence of Different Procedures (M ± m)

Type of procedure	MDA, nmoles/mg protein	Fluorescent products, % of control	Change in release, % of control
Control	4,93±0,45	100±15	0
Stimulation of LPO (0.5 mM ascorbate + 10 μM Fe^{++})	18,2±0,56	—	95±3
Stimulation of release (KCl)	4,57±0,49	95±15	43,3±5
Inhibition of LPO (4-methyl-2,6-di-tert-butylphenol)	4,90±0,45	93±12	33±3

Legend. Duration of exposure 10 min.

Meanwhile the antioxidant stimulates mediator release and at the same time completely blocks LPO in the synaptosomal membranes. In other words, the action of LPO activators and of the antioxidant on mediator uptake and release is similar in direction.

The results in Table 2 are thus evidence of absence of correlation between changes in the content of phospholipid peroxidation products in synaptosomal membranes and the efficiency of serotonin release, and taken as a whole they suggest that the effects of antioxidants thus revealed are not mediated through mechanisms of inhibition of phospholipid peroxidation.

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ROLE OF THE OPIOIDERGIC SYSTEM IN RESPIRATION CONTROL

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KEY WORDS: morphine; enkephalin analog; respiration; electromyogram; TSH releasing factor; naloxone; bradykinin; opiate receptors.

One of us (V. M. B. [1]) showed in experiments on rats that certain peptides (bradykinin, TSH releasing factor, $ACTH_{4-7}$ fragment, melanostatin analog), when injected intravenously, weaken the analgesic effect of morphine and of enkephalin analog Tyr-D-Ala-Gly-Phe(NO_2)NH_2, which is equal to morphine in its analgesic activity. Fragment $ACTH_{4-7}$ and melanostatin analog have no marked effect on respiration parameters in waking rabbits and do not abolish respiratory depression due to morphine and enkephalin analog in animals. TSH releasing factor (TSHRF) completely abolishes respiratory depression caused by morphine and enkephalin analogs in doses not changing morphine analgesia [1].

The object of this investigation was to compare the effect of the opiate antagonist naloxone and of TSHRF and bradykinin on respiratory depression caused by morphine and enkephalin analog Tyr-D-Ala-Gly-Phe(NO_2)NH_2.

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