Effect of Ethanol and DSIP on the $^{36}\text{Cl}^-$ Flux in Synaptosomal Vesicles

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ABSTRACT

Delta-sleep-inducing peptide (DSIP) is an endogenous substance that regulates the response of the organism to stress. It was found that DSIP, like diazepam and ethanol, activates muscimol-stimulated $^{36}\text{Cl}^-$ uptake in the rat brain cortex and partially counteracts the stimulatory action of ethanol on this process. The effect of peptide disappears at the lowering of the incubation temperature. We propose that DSIP is a concordant regulator partly mediating its action through the membrane phospholipids.

Index Entries: Delta-sleep-inducing peptide; muscimol stimulated $^{36}\text{Cl}^-$ uptake; ethanol; stress; diazepam.

INTRODUCTION

Delta-sleep-inducing peptide (DSIP) is an endogenous substance known as a stress regulator. DSIP elicits a wide spectrum of effects (for review, *see* Graf and Kastin, 1986). The detailed mechanism of its action has not been sufficiently investigated. A lot of data indicate the interactions between DSIP and ethanol. Ethanol injection leads to an increase in DSIP level in the brain. The animals predisposed to voluntary ethanol consumption have a lower DSIP concentration as compared with non-predisposed ones (Burov et al., 1982). Ethanol and DSIP relieve the stress

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reaction, but the mechanisms of their action are opposite, e.g., ethanol increases the level of corticosterone in the blood plasma, whereas DSIP decreases its level under stress conditions (Yukhananov et al., 1990). Probably, DSIP affects stress reaction changing the level of corticosterone through the CRF release (Graf and Kastin, 1986). Like benzodiazepines, DSIP has anxiolytic properties in some tests (e.g., competition for safety place on the electrode floor), but DSIP has not expressed any activity in Vogel's test, and has not possessed any myorelaxation and sedation (Burov et al., 1986). The nature of DSIP actions in stress conditions (emotional, cold, and other) may be the same as its anxiolytic one.

Neurochemical mechanism of DSIP action is not clear, but there are numerous data that show that DSIP affects monoamines level, increases activity of MAO B (Dovedova et al., 1983), and changes the concentration of 5-HT and its metabolites (Maisky et al., 1990).

There are some evidences that ethanol mediates part of its effects through GABA/Benzodiazepine receptor Cl^- ionophore complex (Allan and Harris, 1987; Morrow et al., 1988). DSIP, as well as ethanol in low concentration, does not interact with recognition sites of benzodiazepine receptors. But it is not clear whether DSIP influences on Cl^- transport are mediated by GABA. In this work, the direct action of DSIP and ethanol on $^{36}\text{Cl}^-$ transport in synaptosomal vesicles was studied.

MATERIALS AND METHODS

The rats were obtained from the animal farm Stolbovaya, in the Moscow region, and were kept in the animal house for 8–10 d before experiments. The synaptosomal vesicles were prepared from the rat brain cortex. The cortex immediately after rat decapitation was placed in ice-cold Krebs buffer (NaCl , 145 mM; KCl , 5 mM; MgSO_4 , 1 mM; HEPES (Sigma, USA) 10 mM, pH 7.4 at 20°C) 1 g tissue:15 mL buffer and was homogenized by hand (10–15 strokes) in a glass-Teflon homogenizer. The homogenate was filtered by gravity through four layers of nylon nets with pore size 300, 99, 60, and 27 μm . The filtered homogenate was then centrifuged at 1800 g for 10 min in K-23 (DDR). The pellet was resuspended in the same buffer and centrifuged once more, after second centrifugation the pellet was dissolved in Krebs buffer containing 10 mM D-glucose to give the final concentration approx 15–20 mg of protein/mL. The aliquots (0.1 mL) of synaptosomal vesicles were preincubated for 10 min at 30°C (unless otherwise indicated) and then 0.5 mCi of Na^{36}Cl (5.8 MBq/mmol, Isotop, USSR) in Krebs solution in which part of NaCl was substituted by Na^{36}Cl at final concentration 5 $\mu\text{Ci/mL}$ was added to the synaptosomal vesicles. Drugs were usually added simultaneously with Na^{36}Cl . Uptake of Cl^- was terminated after 5 s by the dilution of half of incubation mixture to 400 times by cold Krebs buffer with 100 μM picrotoxin (Sigma, St. Louis, MO) and immediately filtered through GF/C filters (Whatman, UK) and then washed with 4 mL of the same solution.

The filters radioactivity was counted in toluene scintillators on 1219 Racbeta (LKB, Sweden) with 95% efficiency. The protein was measured by the modified method of Lowry (Lees and Paxman, 1972).

Muscimol-dependent $^{36}\text{Cl}^-$ uptake was calculated by subtracting $^{36}\text{Cl}^-$ uptake observed in the absence of muscimol (basal uptake) from that observed in the presence of muscimol. Percent of activation represents the percent increase of uptake when compared with muscimol-dependent Cl^- uptake without drugs.

The dose-response curve for effect of muscimol on Cl^- uptake was considered as hyperbola and for calculating of its characteristics we used the following equation:

$$A = \frac{k^* * [\text{M}]}{\mu + [\text{M}]} * \Delta t * [\text{Cl}^-] \quad (1)$$

where A is muscimol-dependent $^{36}\text{Cl}^-$ uptake, μ is the constant depending on the affinity of muscimol to GABA receptors and its ability to induce interconversion from closed to open state the Cl^- channel, $[\text{M}]$ is the muscimol concentration, k^* is in our experiments the characteristic of conductance of Cl^- channel, Δt is the time of incubation, $[\text{Cl}^-]$ is the concentration of Cl^- in the incubation medium (*see appendix* for further details).

DSIP was synthesized in Inst. of Biorg. Chem. Academy of Sci. USSR (Moscow, USSR). Drug effects were evaluated statistically by one-way ANOVA followed with Sheffe's test. The curves characteristics were calculated by nonlinear fitting according to the Marquardt algorithm (Marquardt, 1963) and the comparisons of characteristics by one way ANOVA followed by Kruskal-Wallis test. The calculations were made with the "Logstat" program.

RESULTS

Muscimol stimulates the $^{36}\text{Cl}^-$ uptake in dose-dependent manner (Fig. 1) with $\text{ED}_{50} = 5.99 \mu\text{M}$. The maximal effect of muscimol stimulation was already reached at $50 \mu\text{M}$.

We tried to determine the effect of diazepam on muscimol-dependent $^{36}\text{Cl}^-$ uptake at the concentration of muscimol ($5 \mu\text{M}$), which induced a half maximal effect on the chloride transport (Fig. 2). ED_{50} for the action of diazepam is approx $0.6 \mu\text{M}$. In our experiment, ethanol possessed a biphasic effect on muscimol-dependent Cl^- transport at 30°C (Fig. 3). The stimulation of muscimol transport was observed at the range of 1–50 mM. The maximal ethanol activation (28.58%) was observed at 25 mM, whereas the higher ethanol concentrations (200–500 mM) decrease in Cl^- uptake up to 82.09% at the concentration of 500 mM (see Table 1).

DSIP potentiates the muscimol-dependent uptake and also exhibits the bell-shaped concentration-response relationship (Fig. 4). We found,

Fig. 1. Effect of muscimol on the $^{36}\text{Cl}^-$ uptake at 30°C . Data are the means $\pm$ s.e.m. from five experiments in quadruplicate. The basal uptake of muscimol was 12.48 ± 1.14 nmol/mg of protein. The temperature of incubation was 30°C . The ordinate represents $^{36}\text{Cl}^-$ uptake nmol/mg of protein, the abscissa represents the muscimol concentration in micromolars. For the means of μ and k^* see Table 2.

Fig. 2. Effect of diazepam on muscimol-stimulated $^{36}\text{Cl}^-$ uptake. Data are the means $\pm$ s.e.m., $n = 8$. The temperature of incubation was 30°C . The muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 22.4 ± 2.20 nmol/mg of protein. Muscimol concentration is $5 \mu\text{M}$. The ordinate represents the changes (%) of $^{36}\text{Cl}^-$ uptake to the one without drugs. The abscissa represents the diazepam concentration in micromolars. $p < 0.05$, as compared to basal uptake.

Fig. 3. Effect of ethanol on muscimol-stimulated $^{36}\text{Cl}^-$ uptake. Data are the means $\pm$ s.e.m., $n = 8$. The temperature of incubation was 30°C . The muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 24.5 ± 3.45 nmol/mg of protein. Muscimol concentration is 5 mM. The ordinate is shown in Fig. 2. The abscissa represents the ethanol concentration in millimolars. * $p < 0.05$, as compared to basal uptake.

that DSIP only slightly increases the basal, muscimol-independent uptake of Cl^- (data not shown).

The temperature lowering to 0°C practically does not change the effect of muscimol on chloride uptake (Fig. 5), μ and k^* are similar to the ones at 30°C (Table 2). But the stimulatory effect of DSIP and ethanol on muscimol-dependent Cl^- uptake practically completely abolished at 0°C (Table 2). The short preincubation (5–15 min) of the vesicles with DSIP and ethanol do not affect the negative results (data not shown). We perform the analysis of muscimol action on Cl^- uptake in the presence of effective concentration of ethanol, DSIP, and diazepam (Figs. 6–8). The characteristics of muscimol-stimulated chloride uptake were calculated according to Eq. (1).

It was shown that diazepam practically has not affected the k^* . But we found the significant decrease of μ (Fig. 6, Table 2). The ethanol and DSIP after the adding to the incubation medium have changed both μ and k^* (see Figs. 7 and 8, Table 2). DSIP has a more pronounced effect and decreased k^* at 33 %, and μ at 72 %. Ethanol also decreases both parameters, but slighter (Table 2).

In further experiments, we tried to study the interaction between ethanol and DSIP *in vitro*. After preincubation of synaptosomal vesicles with $2 \mu\text{M}$ of DSIP for 5 min, the ethanol (5–10 mM) does not increase the DSIP effect, but abolishes the DSIP enhancement of $^{36}\text{Cl}^-$ uptake and even decreases the muscimol stimulation (Fig. 9). Thus the ethanol and DSIP have similar effects itself, but antagonize each other after combined incubation.

Table 1
Effect of the Ethanol and DSIP on the Muscimol-Stimulated Cl^- Uptake at Different Temperature

	Drug conc.	Temp. of incubation ($^{\circ}\text{C}$)	Change of uptake (%)
Ethanol	100 mM	30	93.79 ± 5.33^a
	200 mM	30	87.24 ± 6.13^a
	500 mM	30	82.09 ± 14.83^a
	10 mM	0	98.31 ± 4.44^b
	25 mM	0	102.31 ± 1.95^b
	50 mM	0	108.45 ± 7.33^b
DSIP	0.5 μM	0	110.2 ± 6.78^b
	1 μM	0	98.3 ± 4.21^b
	2 μM	0	94.31 ± 7.61^b
	5 μM	0	92.60 ± 10.53^b

Data are the means $\pm$ s.e.m. of the change of uptake compared to the one without ethanol or DSIP, $n = 4$.

^athe muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 19.5 ± 1.88 nmol/mg of protein.

^bthe muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 20.78 ± 1.95 nmol/mg of protein. Muscimol concentration is 5 μM .

Fig. 4. Effect of DSIP on muscimol-stimulated $^{36}\text{Cl}^-$ uptake. Data are the means $\pm$ s.e.m., $n = 8$. The temperature of incubation 30°C . The muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 24.51 ± 3.45 nmol/mg of protein. Muscimol concentration is 5 μM . The ordinate is shown in Fig. 2. The abscissa DSIP concentration in micromolars. * $p < 0.05$, as compared to uptake without drugs. DSIP was added in the incubation mixture with muscimol.

Fig. 5. Effect of muscimol on the $^{36}\text{Cl}^-$ uptake at 0°C . Data are the means $\pm$ s.e.m. from two experiments in quadruplicate. The basal uptake of muscimol was 10.4 ± 3.46 nmol/mg of protein. The temperature of incubation was 0°C . The coordinates are shown in Fig. 1. The means of μ and k^* are shown in Table 2.

Table 2
Effect of Different Drugs on the Characteristics of $^{36}\text{Cl}^-$ Uptake

Drugs	μ [μM]	$k^* \cdot 10^{-9}$ [l/sec*mg of protein]
muscimol (at 30°C)	5.21 ± 0.67	50.28 ± 1.65
muscimol + diazepam(1 μM)	3.09 ± 1.05	47.03 ± 3.95
muscimol + ethanol(25 mM)	$3.40 \pm 0.72^*$	43.51 ± 2.59
muscimol + DSIP(1 μM)	$1.48 \pm 0.61^*$	38.74 ± 2.70
muscimol (at 0°C)	$4.29 \pm 0.23^*$	50.64 ± 0.85

The table presents means $\pm$ s.e.m., which are calculated from the curves presented on Figs. 1 and 5–8. μ = complex constant, depending from the affinity of GABA receptors and interaction of GABA receptors with Cl^- ionophore.

k^* = constant, which depends from concentration of Cl^- ionophore and its conductance, but in our experiment this is a characteristic of Cl^- gate conductance (for further details, see appendix).

* $p < 0.05$, as compared to muscimol alone.

Fig. 6. Effect of muscimol on the $^{36}\text{Cl}^-$ uptake at 30°C in the presence of $1\ \mu\text{M}$ diazepam. Data are the means $\pm$ s.e.m. from three experiments in quadruplicate. The basal uptake of muscimol was 12.25 ± 2.62 nmol/mg of protein. The temperature of incubation is 30°C . The coordinates are shown in Fig. 1. The means of μ and k^* are shown in Table 2. --- (muscimol); • (muscimol + $1\ \mu\text{M}$ diazepam.)

Fig. 7. Effect of muscimol on the $^{36}\text{Cl}^-$ uptake at 30°C in the presence of $25\ \text{mM}$ ethanol. Data are the means $\pm$ s.e.m. from three experiments in quadruplicate. The basal uptake of muscimol was 13.72 ± 1.46 nmol/mg of protein. The temperature of incubation is 30°C . The coordinates are shown in Fig. 1. The means of μ and k^* are shown in Table 2. --- (muscimol); • (muscimol + $25\ \text{mM}$ ethanol).

Fig. 8. Effect of muscimol on the $^{36}\text{Cl}^-$ uptake at 30°C in the presence of $1\ \mu\text{M}$ DSIP. Data are the means $\pm$ s.e.m. from three experiments in quadruplicate. The basal uptake of muscimol was 15.64 ± 3.11 nmol/mg of protein. The temperature of incubation is 30°C . The coordinates are shown in Fig. 1. The means of μ and k^* are shown in Table 2. --- (muscimol); $\square$ (muscimol + $1\ \mu\text{M}$ DSIP).

Fig. 9. Effect of ethanol and DSIP on muscimol-stimulated Cl^- uptake. Data are the means $\pm$ s.e.m., $n = 8$. The temperature of incubation is 30°C . The muscimol-dependent $^{36}\text{Cl}^-$ uptake without drugs was 23.24 ± 3.23 nmol/mg of protein. Muscimol concentration is $5\ \mu\text{M}$, DSIP concentration is $2\ \mu\text{M}$. The coordinates are shown in Fig. 3. * $p < 0.05$, as compared to uptake without ethanol and DSIP.

DISCUSSION

The study of Cl^- uptake is a suitable method for investigation of drugs effects on the GABA-benzodiazepine receptor Cl^- ionophore complex in vitro. In our experiment we confirmed the stimulating effect of muscimol on the chloride transport. The presented data are in agreement with other reports (Harris and Allan, 1985; Suzdak, 1986). We received ED_{50} for diazepam action on muscimol-stimulated Cl^- uptake is approx $0.6 \mu\text{M}$, the similar was reported by Morrow et al. (1988). That is comparable to its affinity to benzodiazepine receptors. In present experiments we found that ethanol and DSIP similarly activate the chloride uptake at 30°C , but do not affect muscimol-stimulated chloride uptake at 0°C , as it was shown for ethanol elsewhere (Mcquilkin and Harris, 1990). It was proposed (Goldstein, 1981) that a lot of the ethanol effects are mediated through fluidization of the neuronal membranes. The ethanol likely affects the interaction GABA/Benzodiazepine receptor Cl^- ionophore complex with phospholipids microenvironment and its action depends on the phase transition of lipid bilayer and weakened at low temperature. The absence of the changes of the stimulating effect of muscimol at 0°C probably indicates the phase state of the lipid does not affect the interaction of muscimol receptor and chloride ionophore. The unexpected result was the absence of DSIP influence on muscimol-stimulated Cl^- uptake at 0°C . Based on this fact, we propose that in the realization of DSIP action could play a role the lipids microenvironment of GABA/Benzodiazepine receptor Cl^- ionophore too, whereas the diazepam action probably does not depend on the membrane lipids phase transition.

We find that diazepam practically does not affect the k^* , but induces a significant decrease of μ . The latter characterizes the affinity of GABA-A receptors to muscimol and the interaction of GABA-receptors with Cl^- ionophore (see appendix)

$$\text{Let } \mu = K_d * \mu^* \quad (2)$$

where μ^* is a characteristic for the interaction of Cl^- gate with GABA receptors, and K_d is affinity of the latter. It is well known that diazepam increases the affinity (decreases K_d) of GABA receptors (Olsen, 1982). Perhaps it is a direct changing the GABA receptors conformations and therefore its effect was practically independent from the temperature.

In spite of the similarities in the effects of ethanol, DSIP, and diazepam, they seem to have different mechanisms of action. Both ethanol and DSIP after adding to incubation medium change μ and k^* (Table 2). These ones practically do not change the affinity GABA-receptor at low concentration (unpublished data) and their action could be mediated through Cl^- ionophore. Probably the biphasic effects of the ethanol and DSIP on the chloride uptake are connected with opposite action on μ and k^* .

The ethanol and DSIP enhance the muscimol-dependent Cl^- uptake

in familiar manner, and may have a common site of action. So one could expect that DSIP would have to increase or not affect the action of ethanol on Cl^- uptake. But the coincubation of ethanol and DSIP blocks the enhancement of Cl^- uptake induced by DSIP itself and moreover decreased the muscimol stimulation (Fig. 9).

In order to explain somehow these results, we proposed that DSIP augments the sensitivity of chloride channel to muscimol (decreases μ) and decreases the apparent number of Cl^- channel or their conductance (decreases k^*), whereas the ethanol probably decreases the stability of the DSIP-induced conformation of Cl^- channel and thus blocks the action of DSIP. The ethanol itself can stimulate muscimol-dependent Cl^- transport, increasing the interaction the ionophore and receptor (decreasing μ).

Thus we suggest that several effects of DSIP are tied with its action on Cl^- uptake. DSIP induces secretion of enkephalins (Nakamura et al., 1989) and the similar effect was shown for diazepam (Wuster et al., 1980). DSIP, like benzodiazepines and ethanol, exhibits some anxiolytic activity (see Introduction), it relieves the ethanol abstinence (Dick et al., 1984). However, DSIP antagonizes the toxicity of ethanol. The LD50 for ethanol in our experiment increases after injection of DSIP (0.1 mg/kg ip) from 10.55 g/kg to 11.80 g/kg. The peptide administration decreased the ethanol consumption by mice and rat in free choice situation (Yukhananov and Maisky, 1989). We studied the effects of DSIP and ethanol on the 5-HT metabolism in the brain of rat with different reaction to stress. It was found that ethanol decreased the metabolism of 5-HT in *n. accumbens* of rat with higher sensitivity to stress and DSIP injected previously reversed this action of ethanol (Maisky et al., 1990). These and some other effects of DSIP may be mediated through changes of Cl^- uptake. We assume that DSIP is endogenous-peptide-related to the system of concordant regulation, which controls the organism reactions in extreme situations. Probably DSIP causes its action through the phospholipid microenvironment of the receptors, especially the lipid microenvironment of the GABA-benzodiazepine receptor Cl^- ionophore complex.

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APPENDIX

To understand and to explain the peculiarities in the effects of drugs on the muscimol-stimulated Cl^- uptake and find the suitable interpretation of μ and k^* , we based on the following assumptions.

First, the muscimol induced the transition of Cl^- ionophore from

closed (X_c) to open (X_o) state. Secondly, in the experimental condition, we measure the initial phase of Cl^- transport and therefore velocity of chloride uptake (F_x) would be dependent only on concentration of Cl^- and Cl^- ionophore:

$$d\text{Cl}/dt = F_x k_1 * X * [\text{Cl}^-] + k_2 * X_c * [\text{Cl}^-] \quad (\text{A1})$$

where X is concentration of ionophore in the open (o) and closed (c) states, $[\text{Cl}^-]$ is a concentration of Cl^- outside of synaptosomal vesicles, k_1 and k_2 are the constants characterizing the conductance of the ionophore in the open and closed state correspondingly. And the muscimol-dependent uptake of Cl^- :

$$F_x^* = k_1 * X * [\text{Cl}^-] \quad (\text{A2})$$

The concentration of X_o depends on the concentration of X_c and muscimol. For initial rate of interaction we can write:

$$X = 1/\mu * X_o * [\text{M}] \quad (\text{A3})$$

where the μ is the constant depending on the affinity of muscimol to GABA receptors and its ability to induce interconversion $X_o \rightleftharpoons X_c$

$$\text{then } X_o = \frac{X_t * [\text{M}]}{\mu + [\text{M}]} \quad (\text{A4})$$

where X_t is the total concentration of X .

Muscimol-dependent uptake of Cl^- (A_x) is $F_x * \Delta t$, then

$$A = \frac{k_1 * X_t * [\text{M}]}{\mu + [\text{M}]} * \Delta t * [\text{Cl}^-] \quad (\text{A5})$$

and if we signify,

$$k^* = k_1 * X_t \quad (\text{A6})$$

we receive the Eq. (1), based on it we are able to estimate k^* and μ . Thus we can evaluate the interconnection of GABA receptors with chloride-ionophore (μ) and the conductance of ionophore (k_1). We can estimate k_1 because the total concentration of GABA/Benzodiazepine receptor Cl^- ionophore complex in our experimental conditions is practically constant. Really we can receive only some approximation of k_1 , because muscimol induces the rapid desensitization of receptors (Cash and Subbarao, 1987). The k_1 is only quasi constant, its dependence from time reflects the velocity of desensitization. In our experiment, the time of incubation was *constant*, therefore we are able partly to distinguish between the mechanism of action of different drugs on the muscimol-stimulated Cl^- uptake, measuring their action on these parameters.

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