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STEREOSPECIFIC INFLUENCE OF OXAZEPAM HEMISUCCINATE ON CYCLIC AMP ACCUMULATION ELICITED BY ADENOSINE IN CEREBRAL CORTICAL SLICES

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Abstract—The effects of the *d*-, *dl*- and *l*-isomers of the water-soluble benzodiazepine, oxazepam sodium hemisuccinate, on cyclic AMP levels in superfused slices of guinea-pig and rat cerebral cortex were investigated. At 100 μM these drugs decreased the accumulation of cyclic AMP elicited by adenosine in a manner closely correlated to their stereostructure and relative anticonvulsant and anxiolytic potencies *in vivo*. The drugs inhibited uptake of low concentrations of radioactive adenosine into the slices in a similar manner. Addition of theophylline or adenosine deaminase to the superfusion medium sharply decreased both cyclic AMP basal levels and levels elicited by the *d*-isomer, suggesting mediation by adenosine of the effects of benzodiazepines on cyclic AMP and hence their psychotropic action.

The actions of benzodiazepines on accumulation of cyclic AMP in guinea-pig cerebral cortex slices were studied by Schultz [1], who observed both a 100 per cent increase in basal levels and potentiation of the actions of histamine, noradrenaline and adenosine. Recently, specific high affinity binding sites for [^{3}H]diazepam, suggesting the presence of benzodiazepine receptors, have been discovered in mammalian brain [2, 3] and although some evidence points to an interaction between these receptors and GABA [4] the localisation of the binding sites does not coincide with that of GABA receptors [2] and a search for other mechanisms of action of the benzodiazepines seems justified. In the present study the effects of enantiomers of the water-soluble compound oxazepam sodium hemisuccinate, which have differing pharmacological potencies *in vivo*, on basal and adenosine-stimulated cyclic AMP levels in guinea pig brain slices have been studied.

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MATERIALS AND METHODS

Cerebral cortical slices were prepared from guinea pigs and Wistar rats, incubated and superfused in glucose-bicarbonate saline as described by Pull and McIlwain [5], except that the medium contained 2.6mM-$CaCl_2$. Incubation was for 30 min and superfusion for a further 20 min, at the end of which the slices were released into 0.32 M sucrose and homogenised in 1 ml 5% trichloroacetic acid. In uptake experiments, the slices were incubated with 1 μCi [2-^{3}H]adenosine to give a final concentration of 0.4 μM, for 20 min after superfusion before being released into sucrose.

Determination of cyclic AMP and K^+ in tissue extracts and measurement of radioactivity were as described by Newman and McIlwain [6], and lactate in collected superfusate media was measured by a fluorimetric method [5]. Cyclic [8-^{3}H]AMP (30 Ci/m-mole) and [2-^{3}H]adenosine (23 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K., and adenosine deaminase from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. The *d*-, *l*-

Table 1. Effect of different concentrations of *d*-oxazepam sodium hemisuccinate (RV 1208) and of a single concentration of the *dl*- and *l*-isomers on levels of cyclic AMP in rat cerebral cortical slices

Concentration of benzodiazepine, mM	Cyclic AMP, pmoles/mg protein			
	d-Isomer	*dl*-Isomer	*l*-Isomer	Adenosine, 0.1 mM +*d*-Isomer
0	24.0 ± 4.8 (9)			42.5 ± 10.7* (6)
0.1	30.4 ± 4.7* (7)			49.6 ± 7.5* (4)
0.15	26.5 ± 5.0 (3)	24.5 ± 4.1 (3)	17.6 ± 4.6 (3)	
0.5	39.0 ± 8.6* (4)			
1.0	45.9 ± 10.0* (4)			

Slices were incubated for 30 min and superfused for 20 min as described in Materials and Methods. Values are mean ±S.D. of the number of observations given in parentheses. * Significantly ($P < 0.025$) different from control values Student's *t* test.

Table 2. Effect of *d*-, *dl*- and *l*-isomers (RV 1208, RV 1206, RV 1210) of oxazepam sodium hemisuccinate on levels of cyclic AMP in cerebral cortical slices from guinea pig in the presence and absence of 0.1 mM adenosine

Drugs (0.1 mM)	cyclic-AMP, pmoles/mg protein	
	No adenosine	Adenosine (0.1 mM)
None	13.5 ± 5.1 (10)	229.8 ± 57 (8)
d-Isomer	28.2 ± 4.2* (3)	201.9 ± 34.1 (6)
dl-Isomer	15.4 ± 0.3 (3)	145.0 ± 21.6† (5)
l-Isomer	14.2 ± 1.5 (4)	107.8 ± 41.6† (3)

Slices were incubated for 30 min and superfused for 20 min as described in Materials and Methods. Values are mean ± S.D. of the numbers of samples given in parentheses. * Significantly ($P < 0.0025$) different from control values by Student's *t* test. † Significantly ($P < 0.01$) different from control and *d*-isomer values obtained in the presence of adenosine.

and *dl*-isomers of oxazepam sodium hemisuccinate (RV 1208, RV 1210 and RV 1206 respectively) were kindly supplied by Professor P. Fresia, Ravizza, Milan and solutions were made up fresh daily in glucose–bicarbonate saline. Stability of the compounds was assessed by measuring the absorption of 25 μM solutions at 235 nm, using a Unicam SP 800 spectrophotometer. There was no change in absorption of any of the compounds after 20 min incubation, whereas in the presence of 0.1 M NaOH, absorption was decreased by 12 per cent in 2 hr.

RESULTS

Basal levels of cyclic AMP in incubated slices were increased by superfusion with the *d*-isomer of oxazepam sodium hemisuccinate (0.1 mM) in both rats and guinea-pigs, but were unaltered by the *l*- or *dl*-isomers (Tables 1 and 2). The effect of the *d*-isomer in rats was concentration-dependent. When 0.1 mM adenosine was included in the superfusion medium, cyclic AMP levels were approximately doubled in rat slices and increased 17-fold in guinea-pig slices. These increases were maintained in the presence of the *d*-isomer (studied in rat and guinea-pig slices) but reduced in the presence of the other two isomers (studied in guinea-pig slices only, Table 2). Under the same conditions neither the K^+ contents of the tissues nor their lactate outputs were altered. K^+ was 50.5 ± 6.1 μeq/g in control incubations (mean ± S.D. of 4 values) and 50.9 ± 0.5 μeq/g after superfusion with 0.1 mM *l*-isomer whereas in the presence of 0.1 mM adenosine and 0.1 mM *d*-isomer levels were 56.7 ± 3.6 μeq/g as compared to 60.1 ± 2.5 μeq/g in the presence of adenosine alone. Lactate output was 30.3 ± 5.6 μmoles/g/hr in control incubations and 29.1 ± 6.6 μmoles/g/hr in the presence of *l*-isomer (mean ± S.D. of 6 values). All three isomers inhibited uptake of [^{3}H]adenosine into the tissues (Table 3), the order of potency corresponding to the cyclic AMP levels obtained with the compounds in the presence of adenosine.

Table 3. Effect of *d*-, *dl*- and *l*-isomers (RV 1208, RV 1206, RV 1210) of oxazepam sodium hemisuccinate on uptake of low concentration of [^{3}H]adenosine in guinea-pig cerebral cortical slices

Drugs (0.1 mM)	nCi/g tissue	% inhibition
None	1658.8 ± 48.2 (4)	
d-Isomer	1367.2 ± 73.9* (4)	17.6%
dl-Isomer	1435.6 ± 172.5† (4)	13.5%
l-Isomer	1530.3 ± 164 (4)	7.7%

Slices were incubated for 30 min and superfused for 20 min before addition of 1μ Ci [2-^{3}H]adenosine containing 2 nmoles in a volume of 120 μl, giving a final concentration of 0.4 μM. The slices were incubated for a further 20 min before being homogenised as described in Materials and Methods and 0.2 ml of the supernatant counted in duplicate. Values are mean ± S.D. of the numbers of samples given in parentheses.
* Significantly ($P < 0.0005$) different from control values by Student's *t* test. † Significantly ($P < 0.05$) different from control values by Student's *t* test.

Both theophylline and adenosine deaminase diminished basal cyclic AMP levels in control tissues (Table 4). When *d*-isomer was added to the superfusion medium, cyclic AMP levels obtained with theophylline were significantly increased, while with adenosine deaminase there was no change.

DISCUSSION

The stimulant actions of benzodiazepines on cyclic AMP levels in brain slices have been explained by inhibition of phosphodiesterases and inhibition of uptake of low concentrations of adenosine [1, 7]. The unexpected decrease in adenosine-stimulated cyclic AMP levels brought about by the three stereoisomers observed in the present work is probably connected with their water solubility. Some degree of lipophilicity is necessary for a compound to be an effective phosphodiesterase inhibitor [1], as shown also by the failure of theophylline to elevate cyclic AMP levels (Table 4), due to its inability to penetrate cell membranes and reach the phosphodiesterase sites. Similarly in rat brain, the water-soluble compound chlordiazepoxide was only one-third as active as diazepam as a phosphodiesterase inhibitor [8]. The relative decreases in cyclic AMP accumulation elicited by adenosine brought about by the three isomers paralleled their stereostructure, indicating a stereospecific interaction with benzodiazepine receptors. The *d*- and *l*-isomers have been shown to have different potencies in displacing [^{3}H]diazepam from crude synaptosomal membranes of rat brain, the *d*-isomer being 10 times as effective as the *l*-isomer in producing 50 per cent inhibition of binding

Table 4. Effects of theophylline and adenosine deaminase on cyclic AMP levels elicited by *d*-oxazepam sodium hemisuccinate (RV 1208) in guinea-pig cerebral cortex slices

Drugs	Cyclic AMP, pmoles/mg protein	
	Theophylline, 0.5 mM	Adenosine deaminase, 10 μg/ml
None	3.8 ± 0.3 (4)	4.7 ± 0.8 (4)
d-Isomer, 0.1 mM	6.6 ± 0.9* (4)	5.6 ± 0.8 (3)

Slices were incubated for 30 min and superfused for 20 min as described in Materials and Methods. Control incubations without any addition to the media under the same conditions gave cyclic AMP values of 13.5 ± 5.1 pmol/mg protein (see Table 1). Values are mean ± S.D. of the numbers of samples given in parentheses. * Significantly ($P < 0.0025$) different from control values by Student's *t* test.

[9]. The different activities of the oxazepam hemisuccinate isomers on both basal, as determined on rat and guinea-pig slices, and on adenosine-stimulated cyclic AMP levels, are also consistent with their reported differential anticonvulsant activities, potentiation of narcosis, spontaneous motor activities and muscle relaxant activities in animals [10], and anxiolytic activities in man [11]. The difference between the relative potencies of the isomers *in vitro* and *in vivo* may be due to some degree of hydrolysis by a soluble stereospecific esterase which renders the compounds optically inactive [12], but under the conditions of the present study there was no change in the stability of the compounds. The three isomers also had differential effects in inhibiting the uptake of low concentrations of adenosine, the order of potency of the compounds following their affinities for the receptor. Binding of benzodiazepines could therefore lead to changes at the cell membrane and result in different availabilities of extracellular adenosine, which is responsible for activation of the adenosine-sensitive adenylate cyclase [7, 13]. The *d*-isomer, which is the most potent inhibitor of adenosine uptake and also produced highest cyclic AMP concentrations both with and without added adenosine, was completely ineffective in the presence of adenosine deaminase. In the presence of theophylline, which is an antagonist of the adenosine-sensitive adenylate cyclase but does not inhibit adenosine uptake [7], the level of cyclic AMP produced by the *d*-isomer was significantly less than that obtained in basal conditions, but was nevertheless greater than produced by theophylline alone. The present results therefore suggest that interaction of benzodiazepines with their stereospecific receptor sites may be coupled to changes in activity of the adenosine-sensitive adenylate cyclase system, and adenosine may be the endogenous molecule mediating at least in part the actions of these drugs.

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