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ACTION OF OXOTREMORINE ON THE SUB-CELLULAR DISTRIBUTION OF GLYCINE IN THE RAT SPINAL CORD

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Abstract—Intraperitoneal (i.p.) injection of 250 μg/kg of oxotremorine (OT) caused a 50% decrease in the glycine content of the synaptosomal-mitochondrial fraction of spinal cord homogenates prepared from rats killed 15 min after treatment. The glycine content of the supernatant fraction was correspondingly raised. In synaptosomes isolated from the spinal cord of OT-treated rats, the decrease in glycine content was 30%. Prior administration of atropine, but not of methylatropine, abolished this effect of OT on synaptosomal glycine content. Eserine exerted a potentiating effect on the action of OT in lowering the glycine content of spinal synaptosomes. Prior administration of L-DOPA, apomorphine, haloperidol, muscarine or mecamylamine had no significant effect on the action of OT on synaptosomal glycine content. OT alone or in combination with eserine, and acetylcholine (ACh) in combination with eserine, did not alter the rate of release of glycine from spinal synaptosomes of untreated rats incubated under appropriate conditions. OT was also without effect on the rate of release of glycine from normal spinal synaptosomes subjected to electrical stimulation, as well as on the eventual glycine content of the synaptosomes. On the basis of the present findings it has been suggested that (i) glycine may be released from Renshaw cells at their synapses with motoneurones in response to the muscarinic action of OT; (ii) dopaminergic modulation may not be involved in the OT-induced glycine release from Renshaw cells; and (iii) excessive release of glycine onto motoneurones may be the causative factor of the akinesia observed in OT-induced experimental Parkinsonism.

Oxotremorine (OT)§, which is commonly used to induce Parkinson-like symptoms in experimental animals [1, 2], is a potent muscarinic agonist both in the CNS and in the periphery [3–5]. There have been many suggestions about the site of tremorogenic action of OT, but as yet no general agreement has been reached on this point [6]. In particular, there is a divergence of opinion among various groups of investigators about whether or not supraspinal structures are necessary for the development of motor disturbances caused by OT. The common belief is that the tremorogenic property of OT is central in origin [1, 2]. This suggestion, however, does not hold good in view of the observation that tremorine/OT could produce tremor below the level of transection in spinal animals [7, 8].

OT has been found to increase the level of ACh in the brain [9]. At the neuromuscular junction the agent produces a massive increase in the amount of ACh released by the motor impulse [10, 11]. Indirect evidence indicates that in decerebrate cats OT muscarinically activates the Renshaw cells by enhancing ACh release from motor axon collaterals on to the Renshaw cells [6]. This muscarinic activation of Renshaw cells by OT should result in an increased output of glycine, the inhibitory transmitter [12], from the nerve endings of the Renshaw cells onto the motoneurones.

In the present study, the effect of OT on the sub-cellular distribution of glycine in the spinal cord has been investigated. Furthermore, in view of existing evidence that cholinergic neurones are subject to dopaminergic regulation and vice versa in the CNS [13, 14], the influence of dopaminergic agents on the effect of OT on the glycine level of spinal cord synaptosomes was also investigated.

MATERIALS AND METHODS

Animals. Adult Wistar albino rats (200–250 g) of either sex from the institute's breeding stock were used throughout.

Drugs. The following drugs were used: acetylcholine chloride (E. Merck, Darmstadt, West Germany), apomorphine hydrochloride (Sandoz, East Hanover, NJ), atropine sulfate (E. Merck), L-3,4-dihydroxy phenylalanine (L-DOPA, isolated and purified from the seeds of *Mucuna pruiens* [15] in the laboratory), haloperidol B.P. (Serenace, Searle, India), methylatropine (E. Merck), mecamylamine hydrochloride (Sigma Chemical Co., St. Louis, MO), muscarine hydrochloride (Sigma), oxotremorine sesquifumarate (Aldrich Chemical Co., Milwaukee, WI) and physostigmine sulfate (eserine, E. Merck).

Isolation of the spinal cord. Stunned rats were killed and the anterior two-thirds of the spinal cord was rapidly dissected out by carefully cutting the vertebral foramen laterally at both sides using sharp and pointed scissors. The dissected tissue was kept in oxygenated normal saline at 4° until processed.

Preparation of tissue homogenate and sub-cellular fractions. The spinal cord tissue homogenate and synaptosomes were prepared as described by Whit-

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§ Abbreviations used: OT, oxotremorine sesquifumarate; L-DOPA, 3,4-dihydroxy-L-phenylalanine; ACh, acetylcholine.

taker and Barker [16] except that a Teflon glass homogenizer (Arthur H. Thomas, U.S.A., Size C) was used and ten complete up and down strokes were given in two minutes, keeping the homogenizer tube in ice during the course of homogenization. Crude nuclear fraction (P_1) was sedimented at $1000\,g \times 10$ min and crude mitochondrial fraction (P_2) at $17{,}000\,g \times 20$ min. Synaptosomes were prepared by the discontinuous sucrose density gradient (1.2 M, 0.8 M and 0.32 M) method in a Beckman L5-65 ultracentrifuge (spun for 70 min to a total of $5 \times 10^6\,g$ min in the Spinco SW28 rotor). Synaptosomal fraction (layer B) was separated by the method of Marchbanks [17].

Alcohol extraction of tissue homogenate and sub-cellular fractions. The method described by Rodnight [18] was followed. The alcohol was removed by drying the extract in the air-oven at 50°. The dried residue was taken up in a measured volume of water and centrifuged at $40{,}000\,g$ for 40 min. The clear supernatant was transferred carefully using a Pasteur pipette and a known portion of this aqueous extract was extracted with chloroform (4 vol.). The upper aqueous layer from the top was taken in a centrifuge tube and was centrifuged at $1000\,g$ for 10 min with some freshly added chloroform. The clear aqueous layer from the centrifuge tube was transferred using a Pasteur pipette and a known portion of it was dried in the air-oven at 50°. The resulting residue was dissolved in a measured amount of water. This aqueous solution was then passed through a column of Zeo-karb 225 (H^+ form) and treated as described by Gaitonde [19] except that the ammonia in the eluate was removed by evaporation to dryness in the air-oven at 50°. The dried residue was taken up in a measured volume of water and this aqueous extract was used for direct glycine estimation.

Glycine estimation. Glycine was determined by the fluorimetric method of Sardesai and Provido [20] in a Farrand Spectrofluorimeter, with range set at ×0.01. However, for the development of fluorescence we carried out the reaction at 37° and incubated for 1 hr as suggested by Belman [21]. For each set of experiments a standard glycine sample (5 μg) was added to a portion of one of the unknown samples prior to resin treatment. The difference of the two fluorimetric readings, i.e., unknown and mixture, was taken as the standard glycine value, corresponding to the amount of exogenous glycine present in the test aliquot.

Protein estimation. Protein was measured by the method of Lowry *et al.* [22].

In vivo *experiments with crude sub-cellular fractions.* Rats were injected with OT (250 μg/kg, i.p.), killed after 15 min and glycine levels were measured in the spinal cord homogenate and fractions P_1, P_2 and supernatant. Control animals were injected with normal saline and treated in the same way unless otherwise mentioned.

In a set of separate experiments glycine was measured in the spinal cord P_2 fraction prepared 4 hr after OT administration.

In another set of experiments the rats were injected with either atropine (1 mg/kg and 2.5 mg/kg, i.p.) or methylatropine (1 mg/kg, i.p.) 1 hr prior to OT administration. The animals were killed 15 min after administration of OT and glycine was measured in the spinal cord P_2 fraction.

In vivo *experiments with synaptosomal fraction.* Rats were injected with OT (250 μg/kg, i.p.) or eserine (1.5 mg/kg, i.p.). After 15 min the animals were killed and the glycine level was measured in the synaptosomal fraction prepared from the spinal cord. Control animals received normal saline. In separate sets of experiments atropine (2.5 mg/kg, i.p.) and methylatropine (1 mg/kg, i.p.) were injected 1 hr prior to OT and the same procedure was followed. In another set of experiments, eserine was immediately followed by OT and the animals were killed after 15 min.

Apomorphine (250 μg/kg and 2.5 mg/kg), haloperidol (2.5 mg/kg), muscarine (1 mg/kg) and mecamylamine (2.5 mg/kg) were injected intraperitoneally to separate groups of rats 30 min prior to OT, the animals killed after 15 min and the synaptosomal glycine level determined.

L-DOPA in two different doses (50 and 100 mg/kg) was given orally at 0 hr, 24 hr and 36 hr intervals. 6 hr after the last dose, OT was injected and the animals were killed after 15 min.

In vitro *effects of drugs on the release of glycine from isolated spinal cord synaptosomes.* Rat spinal cord synaptosomes were suspended in 3 ml of Krebs–Ringer phosphate saline [23] containing 10 mM glucose and 0.5 mM L-glutamine [24] and incubated at 37° with oxygenation; 25 min after pre-incubation in presence or in absence of eserine (5 μg/ml), OT (1 μg/ml) or ACh (1 μg and 10 μg/ml) was added and incubation continued for another 15 min. At the end of 40 min, the suspension was rapidly cooled and synaptosomes separated by centrifugation at $30{,}000\,g \times 10$ min at 4°. The supernatant was decanted and glycine was estimated both in the supernatant and in the residue after deproteinization with ethanol.

Electrical stimulation and effect of incubation. Three millilitres of synaptosome suspensions were incubated for 25 min and OT was added (1 μg/ml). After 5 min of incubation electrical pulses (square wave pulses of 15 V at 100 Hz; 0.4 ms duration) were applied for 10 min. Glycine was measured both in the medium and in the tissue after further incubation for 30 min.

RESULTS

Effect of drugs on the glycine levels of crude fractions of spinal cord homogenate. OT (250 μg/kg, i.p.) did not change the endogenous glycine level in the spinal cord homogenate. However, the glycine levels of both P_1 and P_2 fractions were reduced by OT and the glycine concentration in the supernatant fraction was correspondingly raised. There was about 50% reduction of the glycine level in the P_2 fraction whereas the change in the P_1 fraction was about 27% only (Table 1).

Prior administration of atropine (1 mg/kg) partially counteracted the effect of OT on the glycine level of spinal cord P_2 fraction and a higher dose (2.5 mg/kg) of atropine practically abolished it. However, prior injection of methylatropine failed to counteract the effect of OT. Four hours after OT

Table 1. Effect of oxotremorine on the glycine levels in sub-cellular fractions of rat spinal cord

	Control	Oxotremorine treated
Homogenate	347.49 ± 7.10 (4)	344.40 ± 4.08 (4)
P_1 Fraction	48.09 ± 1.36 (4)	35.14 ± 1.31 (4)*
P_2 Fraction	52.49 ± 2.50 (4)	28.61 ± 1.87 (4)*
Supernatant	239.73 ± 5.18 (4)	283.66 ± 5.24 (4)*

Oxotremorine (250 μg/kg, i.p.) was injected 15 min before killing the animals. Control animals were injected with normal saline. Glycine was measured as described in the text [20].

Values are expressed in μg of glycine/g wet tissue. Results are mean ± S.D. Numbers in parentheses indicate number of experiments.

* Significant difference from control ($P < 0.01$).

administration, the glycine content in the spinal P_2 fraction was found to have risen as compared to 15 min after OT (Table 2).

In vivo *effect of drugs on the glycine content of spinal cord synaptosomes.* OT (250 μg/kg) caused a reduction of the glycine content of spinal cord synaptosomes by about 30% as compared to the control. Prior administration of atropine (2.5 mg/kg) but not methylatropine (1 mg/kg) counteracted this effect of OT (Fig. 1). Eserine (1.5 mg/kg) also caused a decrease (about 10%) in the glycine level of spinal cord synaptosomes and potentiated the effect of OT on the same (Fig. 1).

Prior administration of L-DOPA, apomorphine, haloperidol, muscarine and mecamylamine did not significantly alter the effect of OT on the spinal synaptosomal glycine level.

In vitro *effect of drugs on the spinal cord synaptosomal glycine level.* When spinal cord synaptosomes were incubated in the presence of OT alone, OT and eserine, or ACh and eserine, there was no significant increase in the release of glycine into the incubation medium.

Effect of OT on electrically evoked release of glycine from isolated synaptosomes. The control total (tissue plus medium) glycine level and that in the medium after electrical stimulation were found to be 5.68 ± 0.36 ($n = 4$) and 2.26 ± 0.12 ($n = 4$) μg/mg protein respectively. OT incubation failed to significantly alter the amount of total glycine as well as that released into the medium after electrical stimulation. The total glycine level and that in the medium after OT were 5.64 ± 0.34 ($n = 4$) and 2.31 ± 0.15 ($n = 4$) μg/mg protein respectively.

DISCUSSION

It has been demonstrated that OT leads to increased levels of ACh and decreased ACh turnover in the brain [9]. Schuberth *et al.* [25] have suggested that this effect of OT might be mediated through activation of muscarinic receptors by OT. In the present study OT did not affect the total glycine content in the spinal cord homogenate. However, the level of glycine in the P_1 and P_2 fractions was significantly decreased and the glycine level in the super-

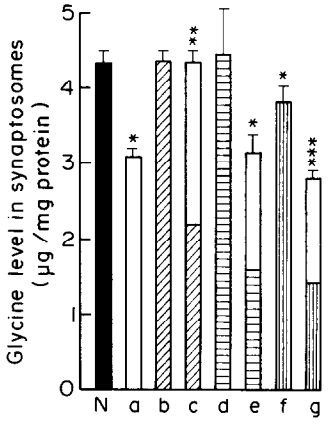

Fig. 1. Histogram showing (a) reduction of glycine level in synaptosomes of rat spinal cord after oxotremorine (250 μg/kg i.p.) administration; (b) failure of atropine (2.5 mg/kg i.p.) to alter glycine level; (c) reversal of (a) in presence of (b); (d) effect of methylatropine (1 mg/kg i.p.); (e), (a) in presence of (d); (f) effect of eserine (1.5 mg/kg i.p.); and (g), (a) in presence of (f). All values represent mean of four experiments ± S.D. except in (a), where number of experiments is six. *Significant reduction from N ($P < 0.01$). ** Significant increase from (a) ($P < 0.01$). *** Significant reduction from (a) ($P < 0.01$).

Table 2. Effect of oxotremorine, atropine and methylatropine on the glycine content of rat spinal cord P_2 fraction

	Control	Glycine (μg/g wet tissue) in the P_2 fraction	Experimental
Normal saline	57.55 ± 2.49	oxotremorine (15 min after injection) (250 μg/kg) (4 hr after injection)	29.35 ± 1.92* 44.82 ± 2.36†
Atropine (1 mg/kg)	56.11 ± 2.90	atropine + oxotremorine (1 mg/kg)	38.98 ± 2.46†
Atropine (2.5 mg/kg)	55.97 ± 2.77	atropine + oxotremorine (2.5 mg/kg)	58.09 ± 2.34†
Methylatropine (1 mg/kg)	54.26 ± 3.15	methylatropine + oxotremorine (1 mg/kg)	30.17 ± 2.06*

Values are mean ± S.D. of 4 experiments.

All drugs were injected i.p.; see text for conditions of experiments.

* Denotes significant reduction from control ($P < 0.01$).

† Denotes significant increase from oxotremorine (15 min) ($P < 0.01$).

natant was correspondingly increased after OT. These observations indicate the possibility that one of the effects of OT may be to induce excess release of glycine from its stores in the spinal inhibitory synapses.

Since majority of the nerve ending particles are present in the P_2 fraction, emphasis was given to this fraction in the present investigation. The studies with the P_2 fraction indicate that the glycine releasing effect of OT is mediated through muscarinic cholinergic action of the drug since prior administration of atropine antagonized this effect. Experiments with synaptosomes prepared from spinal cord P_2 fraction of OT-treated animals showed a similar glycine releasing effect of OT. The central origin of this muscarinic effect of OT is established by the fact that the peripherally acting drug methylatropine, which does not penetrate into the CNS to any significant degree, failed to counteract the decrease in spinal cord synaptosomal glycine content induced by OT. On the other hand atropine, which freely penetrates into the CNS, abolished the effect of OT. Similar interactions of OT, atropine and methylatropine on the brain ACh level have been reported [3].

In vivo involvement of ACh in decreasing spinal synaptosomal glycine level is evident from the observation that administration of eserine, the anti-cholinesterase agent, slightly reduced the glycine level and injection of eserine together with OT potentiated the action of OT on the synaptosomal glycine level (Fig. 1).

The failure of muscarine to affect the glycine content of spinal synaptosomes is probably due to the fact that muscarine does not cross the blood–brain barrier freely [26] and the concentration of this drug in the CNS does not reach a level adequate to cause a significant effect like that of OT on the spinal synaptosomal glycine content. The difference in *in vivo* action of these two drugs may be dependent upon the concentration but may also be due to qualitative differences in action between OT and muscarine [26].

Using intercollicular decerbrate and spinal cats Ganguly *et al.* [6] have demonstrated that OT muscarinically enhanced the transmission (ACh release) from motor axon collaterals to the Renshaw cells and that ACh in its turn excited the Renshaw cells also by muscarinic action. Possibly the Renshaw cells are also excited by direct muscarinic action of OT [6]. The present study indicates that this combined muscarinic activation of the Renshaw cells in the presence of OT causes excess release of the inhibitory transmitter glycine from their axon terminals onto the motoneurones thus curbing the motoneurone activity. In this way OT may produce a disbalance between motor ouput and recurrent inhibition in the spinal cord.

The failure of OT to reduce the glycine content in isolated spinal synaptosomes in our experiments indicates that the inhibitory interneurones of the spinal cord which release glycine from their axon terminals onto the motoneurones have muscarinic cholinergic receptors on their cell bodies but not on any other sites. Thus in isolated spinal synaptosomal preparations, muscarinic activation of the Renshaw cells by OT or ACh is not possible because such isolated preparations do not contain any intact neuronal cell body; besides neuronal connections between motoneurones and Renshaw cells are absent in such isolated preparations.

Though the presence of nicotinic cholinergic receptors in spinal inhibitory interneurones has been demonstrated [27], in the present study mecamylamine, the nicotinic antagonist, failed to affect the spinal synaptosomal glycine level as well as the action of OT on the same. This observation may be explained in either of the following two ways: (a) the release of glycine from Renshaw cells at the synapses with motoneurones is associated with the muscarinic cholinergic activation of the Renshaw cells and not with the nicotinic activation; and (b) it has been suggested that the muscarinic cholinergic receptors on the Renshaw cells may be functionally more important than the usually more easily revealed nicotinic receptors [27] and so it may be assumed that possibly *in vivo* glycine is released from the nerve terminals of inhibitory interneurones by the activation of functionally important muscarinic receptors associated with these cells and the nicotinic cholinergic receptors may not be involved in the release of glycine from inhibitory interneurones.

That OT does not interfere with the equilibrium between spontaneous glycine release and uptake by synaptosomes is evident from the experiments wherein OT failed to change the glycine levels in the medium and in the tissue during *in vitro* incubation of spinal synaptosomes after electrically evoked release of glycine as compared to control. Therefore, inhibition of motoneurone activity by OT action may not be due to inhibition of glycine uptake by the presynaptic nerve ending from which glycine was previously released by the action of the drug on the Renshaw cells.

The presence of dopaminergic neurones and dopamine receptors in the spinal cord has been demonstrated [28–31] and it has been reported that both dopamine and L-DOPA affect spinal reflexes and motoneurone function [32]. Recently Ganguly and Das [11] have reported the existence of dopaminoceptive muscarinic receptors on motor nerve endings. In the present study, the results of *in vivo* experiments with OT, L-DOPA, apomorphine and haloperidol militate against any pre-synaptic dopaminergic modulation at motor axon collaterals during the OT induced glycine release from the Renshaw cells. However, the possibility of such modulation cannot be entirely ruled out by our experiments. Indeed, pre-treatment with L-DOPA has been found to protect OT induced tremor to some extent [33] and to inhibit the antidromically activated spike discharges of single Renshaw cells [34].

One of the most striking features of the motor disability induced by OT in experimental animals is bradykinesia. In Parkinsonian patients also, different aspects of bradykinesia have been documented by various authors [35–37]; however, the most characteristic feature is the slowness of movement or hypokinesia [38]. From the present study it appears that the net effect of the Parkinsonimimetic drug, OT, on the spinal cord glycine level is the excess release of glycine from the nerve terminals of Renshaw cells at the synapses with motoneurones due

to muscarinic cholinergic action. On the basis of the data presented in this paper, it may be suggested that this excess release of glycine onto the motoneurones could explain the hypokinesia, one of the cardinal symptoms of OT-induced Parkinsonism in experimental animals.

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