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IN VITRO DOWN-REGULATION PREDICTS AGONIST EFFICACY AT CENTRAL MUSCARINIC CHOLINERGIC RECEPTORS

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Abstract—Agonist induced short-term down-regulation of central muscarinic cholinergic receptors in mechanically dissociated cells of the mouse brain has been shown to predict the efficacy of agonists at muscarinic receptors. Pretreatment of cells with full agonists such as carbachol or oxotremorine M resulted in a loss of available muscarinic cholinergic receptors of about 30% using [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) as radioligand, whereas a second group of agonists e.g. RS 86 were only weakly active in this regard producing a significantly smaller loss of cell surface muscarinic cholinergic receptors. The magnitude of down-regulation of muscarinic receptors induced by pretreatment with several cholinergic drugs correlates fairly well with their ability to stimulate the phosphatidylinositol turnover. It seems that the agonist induced down-regulation of muscarinic cholinergic receptors on mechanically dissociated neurons of the mouse brain is a simple screening method to test for centrally acting cholinergic agonists.

Most recent treatment strategies of geriatric memory disorders and especially of dementia of the Alzheimer's type aim to substitute the pronounced loss of central cholinergic functions of demented patients by treatment with cholinergic drugs like cholinesterase inhibitors and cholinergic agonists [1]. Research for clinically effective agonists is hampered by the lack of simple screening systems to test for centrally acting cholinergic agonists. Many cholinergic drugs characterized by peripheral test systems as agonists are only weakly active in the brain, e.g. in stimulating inositol phosphate (IP) accumulation in rat brain synaptosomes [2] or in increasing the firing rates of rat hippocampal neurons after iontophoretic application [3]. Since both methods, IP response and single cell recording, are very time consuming, better screening tests for centrally acting cholinergic agonists are needed. Lippa *et al.* [3] reported that the ability of cholinergic drugs to desensitize hippocampal muscarinic receptors in electrophysiological experiments correlates quite nicely with their ability to stimulate IP response in brain tissue. Since muscarinic cholinergic receptor desensitization can also be measured in rather simple biochemical experiments by the agonist induced receptor internalization in neuronal cells, e.g. dissociated neurons or neuroblastoma cells [4–6], we investigated if the ability of cholinergic agonist to down-regulate muscarinic cholinergic receptors on mechanically dissociated neurons of the mouse brain can be used to predict their agonist efficacy on central muscarinic cholinergic receptors as usually measured by their ability to stimulate IP accumulation.

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MATERIALS AND METHODS

Female NMRI mice (3 months old) were obtained from Interfauna (Tuttlingen, F.R.G.). Tritiated 1-[*N*-methyl-^{3}H]scopolamine methylchloride ([^{3}H]NMS, sp. act. 85 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, F.R.G.). All other chemicals were obtained from commercial suppliers.

Preparation of dissociated mouse brain cells. Mice were killed by decapitation and brains were immediately dissected on ice. After removing the cerebellum, brains were minced and suspended in ice cold medium I (NaCl 138, KCl 5.4, Na_2HPO_4 0.17, KH_2PO_4 0.22, glucose 5.5 and sucrose 58.4, all in mmol/L; pH 7.35, 340 mOsm). Tissue was dissociated by trituration through a nylon mesh (210 μm pore diameter) with a pasteur pipette. The resulting suspension was filtered by gravity through a tighter nylon mesh (102 μm pore diameter) and the dissociated cell aggregates were washed twice by centrifugation (1 min, 400 *g*, 4°) and resuspension in 20 mL ice-cold medium II (NaCl 110, KCl 5.3, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 25, sucrose 70, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) 20, all in mmol/L; pH 7.4, 340 mOsm). For down-regulation experiments dissociated brain cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum and buffered at pH 7.35 with 20 mmol/L Hepes (medium III). Final composition of cell suspension recovered by this procedure of tissue preparation consists of dispersed cells and small fragments of brain tissue of about 100 μm diameter. Viability tests performed by trypan blue exclusion method usually yielded viability values of 80–90%.

Down-regulation experiments. Dissociated neurons were incubated at 37° in a shaking waterbath for

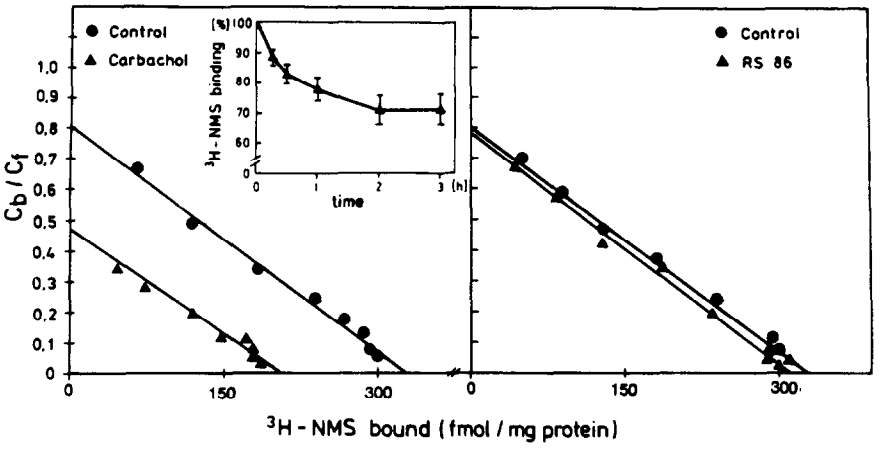

Fig. 1. Effect of preincubation of dissociated cell aggregates of mouse brain with carbachol or RS 86 on the binding properties of [^{3}H]NMS. Left: Effect of preincubation with carbachol (1 mmol/L) on the specific binding of increasing concentrations of [^{3}H]NMS. Data are given as Scatchard plot and represent a typical experiment. Insert: Time course of the effect of carbachol preincubation on the specific binding of [^{3}H]NMS. Cells were preincubated with carbachol (1 mmol/L) for the time indicated. [^{3}H]NMS binding is given as per cent of control binding. Right: Effect of preincubation with RS 86 (1 mmol/L) on the specific binding of increasing concentrations of [^{3}H]NMS. Data are given as Scatchard plot and represent a typical experiment.

2 hr in a final volume of 4 mL with or without the agonist (1 mmol/L). At the end of incubation period the suspension of cells was centrifuged at 400 *g* for 1 min at 4° and washed twice with 20 mL of ice-cold medium II. Washed cells were suspended in cold medium II and used for the receptor binding experiments.

Binding assay. Cells were incubated in quadruplicate for 90 min at 15° with [^{3}H]NMS (2 nM) in a final volume of 0.3 mL in absence and in presence of 2 μM atropine as blank. The incubation mixture was filtered through Whatman GF/B glass fiber filters. The filters were washed three times with 5 mL of ice-cold 0.9% NaCl solution and placed in minivials. After extraction into 4 mL Quick-szint 402 (Zinsser, Frankfurt, F.R.G.) radioactivity was measured in a Beckman scintillation counter at 40% counting efficiency.

RESULTS

In agreement with experiments on neuroblastoma cells [5, 6] and dissociated neurons from rat brain [5] pretreatment of mechanically dissociated neurons from mouse brain with carbachol resulted in a reduction of the available number of muscarinic cholinergic receptors by about 30% as determined by the specific binding of the hydrophilic antagonist [^{3}H]NMS (Fig. 1). Carbachol induced muscarinic receptor short-term down-regulation [4–6] was maximal at a carbachol concentration of 1 mmol/L (data not shown) and was fully pronounced after 2 hr (Fig. 1). Pretreatment with carbachol did not alter the dissociation constant of the radioligand (Fig. 1) strongly indicating that the washing procedure was sufficient to completely remove the unlabelled carbachol. When dissociated neurons were pretreated with the cholinergic agonist RS 86 under similar experimental conditions, muscarinic cholinergic receptor density as determined by the specific binding of [^{3}H]NMS was not changed (Fig. 1). There was no difference in the reduction of [^{3}H]NMS binding sites derived either from Scatchard analysis or from single point determination using a saturating concentration of [^{3}H]NMS (2 nM). The eleven cholinergic agonists tested showed considerable differences in their ability to induce a down-regulation of muscarinic receptors on dissociated neurons (Table 1), e.g. agonists like muscarine, acetylcholine or oxotremorine M were nearly as potent as carbachol, while all other agonists were considerably weaker than carbachol in down-regulating muscarinic cholinergic receptors and only reached 10–50% of the effect of carbachol (Fig. 2). Very interestingly, the ability of all agonists to induce down-regulation of muscarinic cholinergic receptors showed excellent correlations with their ability to stimulate phosphatidylinositol turnover in the brain (Fig. 2). Due to the high concentration used (1 mmol/L) receptor occupation can be assumed to be nearly 100% for the agonists investigated [9].

DISCUSSION

Short-term exposure of neuronal cells (mechanically dissociated neurons, neuroblastoma cells) to cholinergic agonists has been repeatedly demonstrated to down-regulate between 30 and 50% of muscarinic receptors located on the cell surface by sequestration into intracellular structures [4–6, 8]. Agonist induced receptor internalization seems to be paralleled by desensitization of muscarinic cholinergic receptor function as measured by agonist induced accumulation of cyclic GMP or of inositol phosphates in mouse neuroblastoma cells [5, 6], and has been suggested as a specific response of the M_1

Table 1. Agonist induced down-regulation of [^{3}H]NMS binding sites on mechanically dissociated cells of the mouse brain

Compound	Specific [^{3}H]NMS binding (% of control)
McN-A 343	98.5 ± 6.5
RS 86	93.5 ± 9.0
Bethanechol	89.6 ± 10.8
Pilocarpine	87.4 ± 3.6
Oxotremorine	85.7 ± 3.7
Arecoline	83.2 ± 5.1
Acetylcholine	73.1 ± 6.1
Methacholine	72.9 ± 5.2
Muscarine	71.4 ± 1.9
Carbachol	67.2 ± 6.5
Oxotremorine M	65.2 ± 6.1

Effect of 11 muscarinic receptor agonists on subsequent [^{3}H]NMS binding in intact mouse brain cells. Dissociated mouse brain cells were preincubated with or without the various muscarinic agonists in a concentration of 1 mM for 2 hr at 37°. Diisopropylfluorophosphate (10 μM; no effect on [^{3}H]NMS binding itself) was added to cells 30 min before the addition of acetylcholine and methacholine to inhibit acetylcholinesterase activity. After preincubation with the agonists cells were centrifuged and washed. The cell pellet was resuspended in medium II and binding assay was performed as described under Methods. Values are represented as the mean ± SEM from four to six independent experiments.

subtype [5]. However, agonist-induced down-regulation has also been observed with human SK-N-SH neuroblastoma cells, which possess high levels of high molecular weight muscarinic cholinergic receptors of the M_3 subtype [7]. Thus, involvement of M_3 receptors in agonist-induced down-regulation cannot be excluded. The biochemical mechanisms leading to down-regulation of receptor density and function are not yet well understood. Recent work by Lai *et al.* [9] suggests that products of the phosphoinositide hydrolysis are probably not involved in agonist-induced down-regulation of receptor density.

On the other hand, agonist induced muscarinic cholinergic receptor down-regulation and agonist induced muscarinic receptor response as measured by cyclic GMP accumulation in neuroblastoma cells seem to require similar agonist efficacy for maximal response [5, 6]. This possible relationship between the ability of cholinergic agonists to internalize as well as to stimulate muscarinic receptors as also described previously [7, 8] opens the possibility to use agonist induced muscarinic cholinergic receptor down-regulation as a relatively simple *in vitro* model to investigate agonist efficacy of cholinergic drugs on central muscarinic receptors. In agreement with this assumption we could demonstrate in the present communication that cholinergic agonists with

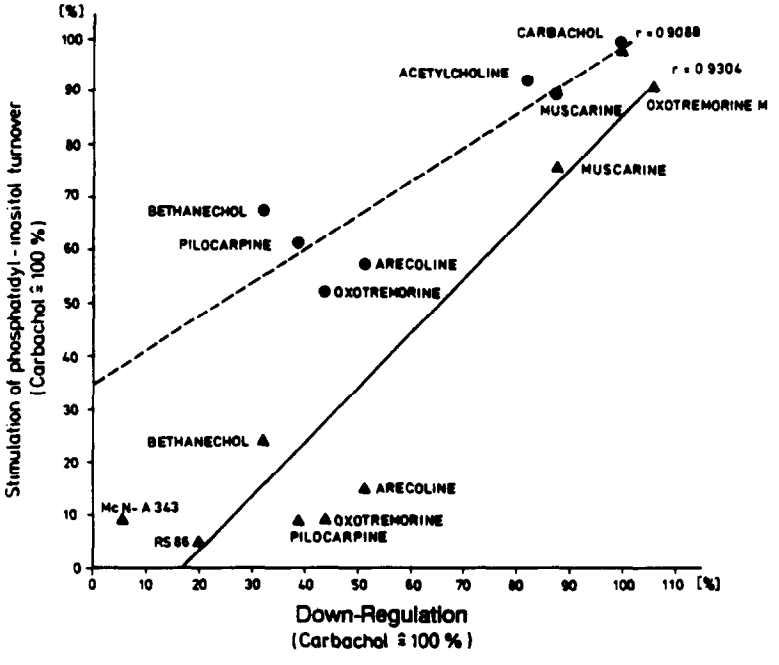

Fig. 2. The relationship between the abilities of various cholinergic agonists to downregulate muscarinic cholinergic receptors on dissociated mouse brain neurons and to stimulate PI turnover in the brain. Data for PI turnover were taken from Fisher *et al.* [2] (●) or from Freedman *et al.* [8] (▲). Data for the down-regulation of specific [^{3}H]NMS binding by agonists (1 mmol/L) represent means of four to six individual experiments as described in Materials and Methods.

different agonist efficacies for central muscarinic receptors [2] desensitize central muscarinic cholinergic receptors to different degrees. Moreover, the abilities of these agonists to down-regulate central muscarinic receptors show an excellent correlation with their abilities to stimulate the accumulation of inositol phosphates, the most important transducing mechanism of central muscarinic receptors [2, 6].

Thus, agonist induced muscarinic receptor loss on dissociated neurons of the mouse brain represents a very simple model to investigate the agonist efficacy of cholinergic agonists on central muscarinic receptors. This model has the advantage compared to the estimation of the potency ratio for the inhibition of antagonist and agonist binding [10, 11] that it seems to be much more closely related to the cellular response. It requires intact cells, physiological temperature and physiological buffer [4, 7] and it seems to differentiate agonists with low efficacy much better than the ratio of the inhibition constants for antagonists and agonist binding [10, 11]. It is much less time consuming as a screening system than measuring the agonist induced accumulation of inositol phosphates, but it uses the same cellular model. This offers the possibility that agonist response as measured by the intracellular accumulation of inositol phosphates can be investigated in the same cellular system* as a second step in the screening procedure. The limitations of the model are due to the possible involvement of M_1 and M_3 receptors [7]. Both subtypes might be coupled to PI turnover with different degrees of efficacy and might be down-regulated at different rates. This might cause problems in discriminating agonists of very low efficacy (Fig. 2).

* Stoll L, Schubert T and Müller WE, Age related deficits of m-cholinoceptor function in the mouse. Partial restoration by chronic piracetam treatment. Submitted for publication.

REFERENCES

1. Bartus RT, Dean RL, Beer B and Lippa AS, The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**: 408–417, 1982.
2. Fisher SK, Klinger PD and Agranoff BW, Muscarinic agonist binding and phospholipid turnover in brain. *J Biol Chem* **258**: 7358–7363, 1983.
3. Lippa AS, Critchett DJ and Joseph JA, Desensitization of muscarinic acetylcholine receptors: possible relation to receptor heterogenity and phosphoinositides. *Brain Res* **366**: 98–105, 1986.
4. El-Fakahany EE and Lee JH, Agonist induced muscarinic acetylcholine receptor down-regulation in intact rat brain cells. *Eur J Pharmacol* **132**: 21–30, 1986.
5. Cioffi CL and El-Fakahany EE, Short-term desensitization of muscarinic cholinergic receptors in mouse neuroblastoma cells: selective loss of agonist low-affinity and pirenzepine high-affinity binding sites. *J Pharmacol Exp Ther* **238**: 916–923, 1986.
6. Cioffi CL and El-Fakahany EE, Differential sensitivity of phosphoinositide and cyclic GMP responses to short-term regulation by a muscarinic agonist in mouse neuroblastoma cells. *Biochem Pharmacol* **38**: 1827–1834, 1989.
7. Thompson AK and Fisher SK, Relationship between agonist-induced muscarinic receptors loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: Methodological considerations. *J Pharmacol Exp Ther* **252**: 744–752, 1990.
8. Harden TK, Petch LA, Traynelis SF and Waldo GL, Agonist induced alteration in the membrane form of muscarinic cholinergic receptors. *J Biol Chem* **260**: 13060–13066, 1985.
9. Lai WS, Rogers TB and El-Fakahany EE, Protein Kinase C is involved in desensitization of muscarinic receptors induced by phorbol esters but not by receptor agonists. *Biochem J* **267**: 23–29, 1990.
10. Freedman SB, Harley EA and Iversen LL, Relative affinities of drugs acting at cholinoceptors in displacing agonist and antagonist radioligands: the NMS/Oxo-M ratio as an index of efficacy at cortical muscarinic receptors. *Br J Pharmacol* **93**: 437–445, 1988.
11. Moret C, Pastrie I and Briley M, Potency ratio for the inhibition of [^{3}H]Cis-Methyldioxolane binding predicts agonist or antagonist activity on muscarinic receptors. *Meth Find Exptl Clin Pharmacol* **10**: 619–621, 1988.