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MODULATION OF THE NUMBER OF MUSCARINIC RECEPTORS IN MOUSE NEUROBLASTOMA CELLS BY SOMAN

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Abstract—Long-term preincubation at 37° of mouse neuroblastoma cells (clones NS-20 and N1E-115) with soman, a potent and irreversible cholinesterase inhibitor, resulted in a significant decrease in the number of [^{3}H]*N*-methylscopolamine binding sites and in the inhibition of carbamylcholine-induced cyclic GMP formation. The disappearance of surface muscarinic receptors and the desensitization of the receptor-mediated response seem to occur via accumulation of acetylcholine in the culture medium. The significance of these findings is discussed.

Most organophosphorus compounds are believed to exert their toxic effects by inhibiting the enzyme acetylcholinesterase (AChE). This presumably leads to the accumulation of excess acetylcholine (ACh) at central and peripheral synaptic sites, thus causing hyperactivity of the cholinergic functions [1, 2].

Several animal studies have shown that the symptoms of excessive cholinergic stimulation by organophosphates gradually diminish with chronic administration of these agents, and eventually lead to tolerance to their toxicity [3]. This may be explained by the low muscarinic receptor density found after repeated administration [4–6].

Neuroblastoma cells, grown in cell culture, have many properties of neurons. Some clones of C 1300 tumor [7], particularly N1E-115 (adrenergic) and NS-20 (cholinergic), possess a comparatively low content of muscarinic receptors but these are very similar to those in the brain [8]. They thus provide a suitable model for the study of adaptative changes in receptors [9, 10].

This report describes the ability of soman (pinacolyl methylphosphonofluoridate), a potent and irreversible acetylcholinesterase inhibitor, to down-regulate the muscarinic receptors in neuroblastoma cells.

MATERIALS AND METHODS

Materials. Soman was obtained from CNB (BP3 91710) Vert-le-Petit, France. [^{3}H]*N*-methylscopolamine ([^{3}H]NMS, 85 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Cells of clones N1E-115 and NS-20 of the C 1300 murine neuroblastoma were given by Dr Legault (Collège de France, Paris). All other chemicals were obtained from Sigma, Poole, U.K., and culture medium from Gibco, Uxbridge, U.K.

Cell culture conditions. Stock cultures were grown in 75-cm^2 plastic flasks (Falcon) in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential aminoacids, 50 u/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 10% (vol/vol) foetal calf serum (medium I) in a humidified atmosphere containing 7% CO_2.

About 5×10^5 cells (passage 17–25) obtained by trypsination (0.05% trypsin in medium I without serum) of a monolayer culture, were seeded into 20.0 ml of medium I in a 78-cm^2 plastic tissue-culture dish. The medium was changed on days 3 and 5, and every day thereafter by adding 10 ml of fresh medium I and removing 10 ml of medium. The cells were used on day 7 (binding experiments) or on day 10 (cyclic GMP determinations).

Binding experiments. After incubation of cells for six days, the medium was replaced with fresh medium I containing 100 nM soman. The cultures were returned to the incubator for different times. The medium was then drawn off and the cell monolayers washed twice with medium II (DMEM + 25 mM HEPES). The intact cells were incubated in medium II with [^{3}H]NMS (0.05–2 nM) and unlabelled drugs as displacers at 37° for 1 hr. Incubation was stopped by washing the cells with medium III of the following composition (mM): NaCl, 110; KCl, 5.3; $CaCl_2$, 1.8; $MgCl_2$, 1.0; Na_2HPO_4, 2.0; glucose, 25 and sucrose, 70 (pH 7.4, 340 mOsm).

Specific binding was defined as the difference between binding with or without 10^{-5} M atropine. Protein concentration was estimated as in ref. [11].

For competition experiments, binding assays were generally performed with intact cells in suspension (about 500 μg protein/tube in medium III). Each ligand concentration was assayed in triplicate in 1 ml total volume. Assay temperature was maintained at 22° except in the case of carbachol (15°). After 1 hr bound [^{3}H]-labelled ligand was separated from free ligand by vacuum filtration over Whatman GF/B filters. Each filter was then washed three times with 4 ml NaCl 9‰ at 4° to remove unbound radioactivity, and placed in a scintillation vial with 10 ml of toluene scintillator.

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Receptor-mediated cyclic GMP formation. Control and soman-treated cells were harvested as described for ACh and resuspended at a density of approximately 10^6 cells/ml in medium III. The cell suspension was then distributed in plastic tubes in 900-μl aliquots and preincubated for 15 min at 37° in a shaker bath. Intact cells were stimulated by addition of 100 μl carbamylcholine (CBC) to give a final concentration of 1 mM. The reaction was terminated by addition of 100 μl 50% (w/v) trichloroacetic acid (TCA). The cells were then collected by high-speed centrifugation (20,000 *g*). Thereafter, TCA of the deproteinized sample was removed by water-saturated ethyl ether extraction (four times with 2 vol). The aqueous phase was lyophilized overnight. The dried residue was resuspended in distilled water and assayed for radioimmunoassay of cyclic GMP, using an assay kit from Institut Pasteur Production (Lyon, France). The sensitivity threshold was 0.01 pmol/ml of extract solution. Precipitable protein in TCA extracts was determined by the method of Lowry *et al.* [11].

Acetylcholine assay. After incubation with soman, medium I was drawn off, the cells were washed with medium III, suspended in TCA 5% (w/v), homogenized (Polytron, set 5, 20 s) and centrifugated at 10,000 *g* for 20 min. The pellet was discarded and the TCA in the supernatant was extracted by ethyl ether. Acetylcholine content (ACh) was measured in the aqueous phase as described by Israel *et al.* [12].

Other biochemical measurements. Choline acetyltransferase activity (ChAT) was measured as described by Fonnum [15]. Acetylcholinesterase (AChE): sedimented culture cells were suspended in 0.04 M Tris–buffer plus 1% Triton X-100 and sonically disrupted at low temperature. After centrifugation at 12,000 *g* for 15 min, the supernatant was assayed for AChE activity by the method of Ellman *et al.* [14].

RESULTS

Characterization of [^{3}H]*NMS binding on intact NS-20 cells*

Since [^{3}H]NMS is a hydrophilic ligand, this muscarinic receptor probe has been used to monitor changes in cell surface receptors.

The binding of [^{3}H]NMS to differentiated neuroblastoma NS-20 cells was specific, reversible and saturable. A Scatchard plot of the specific binding data was linear, indicating that the ligand recognizes a single class of noninteracting binding sites. When the binding was performed directly in multiwell plates, the dissociation constant of the complex formed at 37° between [^{3}H]NMS and its receptor was 0.26 ± 0.065 nM (passage 22, 4 separate isotherms) and the average B_{max} was 26 ± 1.6 fmol/mg protein. If muscarinic receptor binding was determined with intact cells in suspension in PBS (medium III), the K_D value was 0.83 ± 0.18 nM (passage 21, 6 separate isotherms), and the maximal binding capacity was 19 ± 0.9 fmol/mg protein.

The affinity of binding sites for various cholinergic compounds was measured using equilibrium competition experiments. The data clearly show that

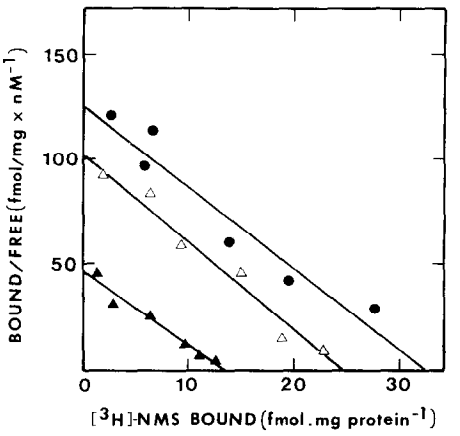

Fig. 1. Effect of soman and carbamylcholine on [^{3}H]NMS binding to intact NS-20 neuroblastoma cells (subculture 22; seven days after subculture). ● Control culture, K_D = 0.26 nM, B_{max} = 32.0 fmol/mg protein; ▲ culture treated with 1 mM CBC for 24 hr, K_D = 0.28 nM, B_{max} = 13.4 fmol/mg protein; △ culture treated with 100 nM soman for 24 hr, K_D = 0.24 nM, B_{max} = 24.3 fmol/mg protein. Each point is the average of four determinations.

[^{3}H]NMS binding sites had the pharmacological specificity of muscarinic acetylcholine receptors since [^{3}H]NMS binding (0.8 nM) was readily displaced by several muscarinic antagonists and agonists. Potent muscarinic antagonists, atropine and scopolamine, blocked binding with half-maximal inhibition at 40 and 3 nM, respectively. Carbamylcholine and oxotremorine, activators of muscarinic ACh receptors, blocked binding half-maximally at 90 and 30 μM. The nicotinic ligand, hexamethonium, was unable to displace radioligand from receptors ($IC_{50} > 100$ μM). Saturation binding isotherms for [^{3}H]NMS to intact NS-20 cells are depicted in Fig. 1.

Scatchard analysis of the [^{3}H]NMS binding data collected using intact N1E-115 cells indicated an average K_D of 0.03 nM with an average B_{max} of 32 fmol/mg of protein.

Effect of soman on [^{3}H]*NMS binding to neuroblastoma cells*

After the exposure of NS-20 or N1E-115 cells to 100 nM soman for 1 or 24 hr we obtained complete and irreversible inhibition of AChE (activity of control between 80–90 nmol substrate/min/mg protein).

As shown in Table 1, no change in [^{3}H]NMS binding was observed within 1 hr. On the contrary, sustained exposure to soman for 24 hr showed a significant 25% decrease ($P < 0.001$, Student's t) for NS-20 cells and 9% decrease for N1E-115 cells ($P < 0.01$). Since a loss in NMS binding could be due to a decrease in either receptor number or binding affinity, Scatchard analyses of NMS binding to control cells and 24-hr soman-treated NS-20 cells were carried out (Fig. 1). These analyses revealed that the decrease in [^{3}H]NMS binding by soman treatment was the result of a reduction in the density of muscarinic receptors (B_{max} decreased from 32.0 to 24.3 fmol/mg protein). No change in the dissociation constant was observed.

Table 1. Effect of soman on [^{3}H]NMS binding in neuroblastoma cells (N1E-115 and NS-20)

Cell line	[^{3}H]NMS specifically bound (fmol/mg protein) 1 hr	24 hr
NS-20 (p 25)		
Control	12.2 ± 0.6	12.3 ± 0.5
Soman (100 nM)	12.0 ± 0.9*	9.2 ± 0.4**
CBC (1 mM)	8.6 ± 0.8**	4.7 ± 0.2**
N1E-115 (p 29)		
Control	17.1 ± 0.6	16.0 ± 0.4
Soman (100 nM)	16.4 ± 0.8*	14.5 ± 0.5***
CBC (1 mM)	12.7 ± 0.7**	8.0 ± 0.6**

NS-20 cells (subculture 25; seven days after subculture) or N1E-115 cells (subculture 29; seven days after subculture) were incubated for 1 or 24 hr with soman at a final concentration of 100 nM and then assayed for [^{3}H]NMS binding (1 nM). Results are expressed as mean ± S.E.M. (N = 4).

* Not significant.

** $P < 0.001$.

*** $0.001 < P < 0.01$.

Under the same conditions, the CBC (1 mM) caused a 60% decrease in the number of [^{3}H]NMS binding.

Binding assays were carried out with 0.1 μM soman in the wells. This resulted in 100% inhibition of AChE activity, but did not produce any change of [^{3}H]NMS binding, as compared with controls.

The muscarinic antagonist, atropine, blocked the binding loss induced by soman (Table 2). NS-20 cells receiving 100 nM soman for 24 hr exhibited a 25% decrease in the [^{3}H]NMS binding, whereas those treated with atropine and soman exhibited no change in binding. Atropine alone had no effect.

Furthermore, the loss of NMS binding was partly reversible. For example, one group of dishes exhibited a 25% decrease in binding after 24 hr with soman, while a second group of dishes, assayed after a further 24-hr recovery period (without soman), still showed a significant 6% decrease.

Table 2. Antagonism of soman-induced loss of [^{3}H]NMS binding by atropine in neuroblastoma cells NS-20

Addition	[^{3}H]NMS bound (fmol/mg protein ± S.E.M.)	
Control†	15.4 ± 0.5	N = 10
Soman†	11.6 ± 0.9***	N = 10
Atropine†	15.6 ± 0.7*	N = 8
Soman + atropine†	15.2 ± 0.8*	N = 10
Soman‡	14.5 ± 0.9**	N = 8

† Cultures (subculture 26; seven days after subculture) were incubated in the presence and the absence of 100 nM soman or 10 nM atropine for 24 hr. The cells were harvested, washed and assayed for [^{3}H]NMS binding (N = number of dishes).

‡ Cultures (subculture 26; seven days after subculture) were first treated with 100 nM soman. After 24 hr, the cells were washed three times in medium and incubated for an additional time of 24 hr. The concentration of [^{3}H]NMS used was 1 nM.

* Not significant.

** $0.02 < P < 0.05$.

*** $P < 0.001$.

Effect on ACh levels and ChAT activity

Because of the total inhibition of AChE activity, soman produced a marked increase of ACh level both intracellularly and in culture medium (Table 3). The increase in ACh in cells attained 500% after 24 hr. Levels of ACh in the culture medium also increased (65-fold after 24 hr incubation). The enzyme involved in the synthesis of ACh, ChAT, was not significantly affected in NS-20 cells after 24-hr soman treatment.

Effect of soman on muscarinic receptor-mediated cyclic GMP formation

Figure 2 shows the time course of changes in cyclic GMP concentration in response to CBC added to the incubation medium. The agonist (1 mM) produced a 10-fold increase in cyclic GMP. In all cases the maxi-

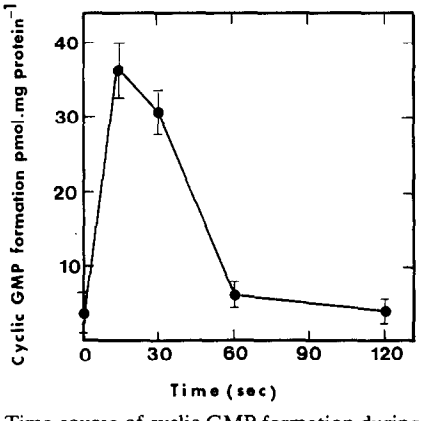

Fig. 2. Time course of cyclic GMP formation during stimulation with 1 mM carbamylcholine in NS-20 cells (subculture 22, 10 days after subculture). Maximum stimulation in the presence of CBC was ~10-fold over basal. These data represent mean ± S.E.M. of three similar experiments.

Table 3. Comparison of intracellular to extracellular ACh-content in neuroblastoma clone NS-20 (subculture 28; seven days after subculture) under incubation with 100 nM soman

Time after start of experiment	Cholineacetyltransferase (pmol/mg protein/min)	Acetylcholine Amount (ng/mg protein) Intracellular	Culture medium
1 hr			
Control	396 ± 25	93.8 ± 18.2	18.2 ± 2.3
Soman	392 ± 23	327.6 ± 25.4**	26.4 ± 4.1*
24 hr			
Control	378 ± 32	127.2 ± 17.3	11.7 ± 3.2
Soman	330 ± 28	793.6 ± 41.1**	797.2 ± 37.6**

Soman was added to the culture medium at time zero. One and 24 hr later, medium was taken and assayed for ACh. At the same time, cells were harvested and assayed for intracellular ACh content after washing twice with PBS solution. Each value represents the mean ± S.E.M. of four determinations. Significant differences from control values.
*$P < 0.05$.
** $P < 0.001$.

mal response was reached within 30 sec after the addition of CBC and declined rapidly thereafter. CBC increased cyclic GMP formation 33-fold over basal levels in NS-20 cells and 3-fold over basal levels in N1E-115 cells.

Soman dramatically reduced CBC-stimulated cyclic GMP formation in neuroblastoma cells (above all in NS-20 cells) treated for 24 hr. Moreover, soman slightly increased basal levels only in NS-20 cells (see Table 4).

DISCUSSION

Preincubation of NS-20 cells (cholinergic) and N1E-115 cells (non-cholinergic) with 100 nM soman, a potent irreversible inhibitor of cholinesterase, resulted in a rapid and total inhibition of AChE activity in both cell lines. During a 24-hr incubation period, the concentration of soman was high enough to inhibit even newly synthesized enzyme. A slow, moderate (10–25%) but significant, reduction of [^{3}H]NMS was observed in the two clones exposed to soman. The phenomenon was a time-dependent process, partly reversible and blocked by atropine. Moreover, Scatchard analysis of saturation experiments revealed a decrease in the density of surface receptors with no change in affinity.

One possibility that should be considered to explain the disappearance of surface muscarinic receptors is that it may be due to the accumulation of acetylcholine in culture medium. Indeed, since AChE activity is completely destroyed and since activity of ChAT is not significantly influenced by the toxic, intracellularly accumulated ACh in the cells might be released into the surrounding medium. In fact, we observed an increase in the amount of ACh in both the culture medium and within the NS-20 cells after soman treatment. The amplitude of elevation of intracellular ACh reached 500% for a period of 24 hr, and large amounts were released into the medium probably by diffusion.

The difference in disappearance of receptors observed between NS-20 and N1E-115 could very probably be attributed to the fact that N1E-115 cells are almost devoid of ChAT activity. Szutowicz *et al.* [15] reported that ChAT activity in clone N1E-115 is 40 times lower than in clone NS-20 (0.009 ± 0.001 and 0.35 ± 0.01 nmole/min/mg protein, respectively).

[^{3}H]NMS is a competitive antagonist, and as such,

Table 4. Effect of soman on the muscarinic receptor-mediated cyclic GMP response in clones NS-20 and N1E-115

Cell line	Cyclic GMP (pmol/mg protein) Control	Contact with soman 100 nM 1 hr	24 hr
NS-20 (p 20)			
CBC ⊖	2.55 ± 0.29	3.08 ± 0.28	3.86 ± 0.18†
CBC ⊕	86.26 ± 10.70‡	98.32 ± 7.86‡	14.27 ± 1.23‡
N1E-115 (p 24)			
CBC ⊖	2.47 ± 0.53	2.06 ± 0.09	1.58 ± 0.16
CBC ⊕	8.21 ± 0.34‡	6.23 ± 0.27‡	3.89 ± 0.22‡

Values are expressed as pmol/mg protein and are means ± S.E.M. (N = 5).
† Significantly different from control-carbamylcholine ⊖, $P < 0.01$.
‡ Significantly different from corresponding value carbamylcholine ⊕, $P < 0.001$.

its binding can be displaced by other compounds capable of attaching to the [^{3}H]NMS binding site. Therefore, excess ACh not removed by the assay wash procedure could reduce the specific binding of [^{3}H]NMS. In this case, the competition between [^{3}H]NMS and ACh would result in reduced affinity of the labelled ligand for the muscarinic receptors, but without significant decrease in the number of binding sites. This possibility can be excluded since the K_D values are similar in control and treated cells. Alternatively, it is conceivable that soman might compete for the [^{3}H]NMS binding site. But, we observed that high doses of soman, enough to inhibit AChE, did not result in binding decrease. Decreases in muscarinic receptor binding produced by soman are specific since preliminary studies have demonstrated that 100 nM soman did not affect energy metabolism or protein synthesis in NS-20 cells for 24 hr [16]. It is therefore reasonable to assume that changes in muscarinic receptor number produced by soman appear secondary to ACh accumulation.

Long exposure of neuroblastoma cells to soman also resulted in desensitization of the receptor-mediated increase in cyclic GMP levels, an effect interpreted as a decrease in the efficiency of receptor-effector coupling. The soman-induced decrease in cell surface muscarinic receptors correlates closely with desensitization of receptor-mediated cyclic GMP formation but we observed that the cyclic GMP response falls faster than the loss of muscarinic binding sites. This observation suggests that the decrease in cyclic GMP response might be related to a preferential disappearance of low-affinity sites of agonist mediating cyclic GMP formation [8]. On the other hand, lipophilic soman may also lead to uncoupling of muscarinic receptors from their effectors or from other membrane components that might modulate the receptor conformation. Indeed, AChE inhibitor may exert noncholinergic effects through a nonspecific change in membrane fluidity [17].

A brief comparison of these results with reports concerning effects of muscarinic agonists on neural cells may be of interest. Recently, a rapid (30 min) and reversible agonist-induced disappearance of muscarinic cell surface receptors was observed in N1E-115 and hybrid cells when the binding was carried out in intact cells using [^{3}H]-labelled methylscopolamine [18–20]. Brief exposure of the receptors lead to desensitization of the cellular response to agonists [9, 20, 21]. This phase probably corresponds to an internalization process without degradation of the receptors. Moreover, studies with neuroblastoma membrane cells using [^{3}H]quinuclidinyl benzilate also demonstrated that long-term incubation (several hours) with muscarinic agonists cause a dose and time-dependent loss of muscarinic sites [10, 22]. This "true" disappearance of receptor sites is accompanied by a reduction in the physiological responsiveness to further cholinergic stimulation [23]. Therefore, these data and our results support the idea that acetylcholine is responsible for the muscarinic receptor down-regulation.

The changes in binding that we observed *in vitro*, using mouse neuroblastoma NS-20 cells, bear real similarities to those observed *in vivo* in tissues from organophosphate-treated animals. Indeed, it has been demonstrated that chronic AChE inhibition afforded by organophosphorus insecticides is associated with a decrease in the number of muscarinic receptors [3, 13]. This mechanism has been suggested to mediate, at least partially, the tolerance to the toxicity of anticholinesterase compounds [3]. In the case of soman, the tolerance appears to exist [24] and the down-regulation of receptors has recently been reported in the brain [25].

Since neuroblastoma cells have the ability to respond to a direct or an indirect agonistic muscarinic stimulation, they may be considered as a suitable tool for studying biochemical mechanisms of the tolerance to AChE inhibitors.

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