The effect of sodium channel activators on muscarinic receptors of neuroblastoma cells

Graeme Milligan and Philip G. Strange

Department of Biochemistry, The Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, England

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Incubation of neuroblastoma N1E 115 cells with veratrine leads to an apparent reduction in the number of muscarinic acetylcholine receptors assayed by [^{3}H]scopolamine methyl chloride binding. No true down-regulation of the receptors occurs but a component of veratrine with muscarinic receptor affinity, which is not veratridine, enters the intracellular water space during the incubation period and competes with [^{3}H]scopolamine methyl chloride for the muscarinic binding sites in subsequent ligand binding assays unless it is carefully washed away. Treatment of cells with the agonist carbamoylcholine does, however, lead to a true downregulation of muscarinic receptors.

Muscarinic receptor *Neuroblastoma cell* *Veratridine* *Veratrine*

1. INTRODUCTION

The alkaloid toxin veratridine is a lipid-soluble polycyclic compound which depolarizes excitable cells by causing persistent sodium channel activation [1]. Veratrine, a commercially available product containing a mixture of *Veratrum* alkaloids has been used as a source of veratridine [2,3] and has been reported to cause a loss of muscarinic acetylcholine receptor sites in synaptosomes from rat cerebral cortex [2]. This veratrine-stimulated loss of muscarinic receptor sites could be prevented by the inclusion of tetrodotoxin in the incubation medium. The adrenergic neuroblastoma clone N1E 115 [4] has been widely used to study aspects of neuronal function [5–7] and is known to possess inhibitory muscarinic receptors [8]. Here we present results showing that an apparent loss of muscarinic receptor binding sites in cells of N1E 115 induced by veratrine is due to a component of veratrine possessing muscarinic receptor antagonist activity.

2. MATERIALS AND METHODS

[^{3}H]Scopolamine methyl chloride ([^{3}H]NMS) (53.5 Ci/mmol) was obtained from New England Nuclear (Southampton). Veratrine, veratridine, tetrodotoxin and atropine sulphate were obtained from Sigma (London) Chemicals (Poole). All other chemicals were of the highest purity available and were obtained from commercial sources.

[^{3}H]NMS binding to a broken cell preparation of N1E 115 was assayed by a rapid filtration method. [^{3}H]NMS ($\sim$500 pM) was incubated with the broken cell preparation (0.5 mg protein/ml) at 25°C for 60 min with the addition of competing drugs where appropriate in 1 ml final vol. of a Hepes–phosphate–saline buffer (pH 7.4) [9]. Incubation was terminated by addition of 2 ml ice-cold buffer and the mixture was filtered rapidly under reduced pressure through a Whatman GF/B glass fibre filter presoaked in buffer. The filter was then quickly washed with 3 portions (2 ml) of ice-cold buffer and transferred to 3 ml Fisofluor I scintillant (Fisons Ltd., Loughborough) containing 0.2 ml water for determination of bound radioactivity. Specific [^{3}H]NMS binding was defined as that displaceable by 20 μM ($\pm$)atropine and under these conditions the specific binding was $\sim$70% of the total binding.

N1E 115 cells (P 20-30) were grown in the tissue culture in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) foetal calf serum (Gibco Biocult, Paisley) without antibiotics at 37°C in a humidified atmosphere containing 10%

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CO_2. The medium was changed on days 3, 5 and 6 after subculture and the cells harvested on day 7.

The effect of veratrine on muscarinic receptor binding site number was assessed by adding 8 ml of veratrine solution (100–500 μM) to plates (55 cm^2) of N1E 115 cells from which the medium had been removed and the cell monolayer washed with 2 × 5 ml portions of buffer. (The concentration of the veratrine solution was calculated assuming an av. M_r = 600 for veratrine. Such an assumption is necessary in order to enable experiments with veratrine to be carried out quantitatively and in order to estimate the affinity of any component of veratrine for muscarinic receptors. The actual M_r-value of any receptor-active substance is unimportant for the determination of pseudo Hill coefficients from displacement binding curves.) The plates were then incubated for 6 h at 37°C. Incubation was terminated by removal of the incubation medium and washing the cell monolayer with 3 × 5 ml portions of ice-cold buffer before the cells were removed mechanically from the plate using a stream of buffer (total vol. 2 ml) and frozen at −80°C until the muscarinic binding sites were assayed. Studies on agonist-mediated down-regulation were performed as above except that carbamoylcholine (1 mM) replaced veratrine. A modified procedure including extended washing was adopted in some experiments (fig.3).

3. RESULTS AND DISCUSSION

In initial experiments, incubation of plates of N1E 115 with veratrine (100–500 μM) for 6 h followed by washing of the plates with 3 × 5 ml portions of buffer led to a concentration-dependent reduction of specific [^{3}H]NMS binding to broken cell preparations in comparison to those which had been incubated without veratrine (not shown), in agreement with [2]. However, the loss of specific binding of [^{3}H]NMS was not prevented by inclusion of the sodium channel blocker tetrodotoxin at concentrations up to 20 μM in the incubation medium (fig.1). This indicates that the observed reduction of specific [^{3}H]NMS binding after treatment of the cells with veratrine was not caused by a depolarization of the plasma membrane mediated by veratridine.

When veratrine at varying concentrations was added to a muscarinic receptor binding assay of a broken cell preparation which had not been exposed to veratrine previously, specific [^{3}H]NMS binding was completely displaced with an IC_{50} (concentration of substance giving 50% inhibition of binding) of 0.32 μM (corrected for receptor occupancy) (fig.2) the displacement having a pseudo Hill coefficient of 0.93. This value for the corrected IC_{50} of the receptor-active component of veratrine must necessarily be only an estimate of the true value owing to the assumed M_r-value of veratrine, but this assumption does not affect the value of the pseudo Hill coefficient obtained. For brain muscarinic receptors, classical muscarinic antagonists produce pseudo Hill coefficients close to 1.0 while muscarinic agonists produce pseudo Hill coefficients significantly less than 1.0 [11]. These data suggest, therefore, that the component of veratrine which interacts with muscarinic receptors may be an antagonist. The inhibition of [^{3}H]NMS binding is competitive as shown by saturation binding analyses performed in the presence and absence of veratrine (in preparation). Veratridine (90–95%) was unable to displace >18% of the specific [^{3}H]NMS binding at up to 100 μM so that the receptor-active component of veratrine is not veratridine. Because of the ability of veratrine to displace [^{3}H]NMS binding we examined the possibility that the observed reduction in binding in N1E 115 upon chronic exposure was related to the washing procedure or to the uptake into the cells during the incubation of a component of veratrine which inhibits muscarinic receptor binding. Using an extended washing procedure (fig.3) we have shown that this is indeed the case. In a typical experiment pellets P_2 and P_3 (extensively washed membrane fractions) gave 102.3% and 107.7%, respectively, of the control specific binding in comparison to 51.7% observed without the extended washing procedures (table 1). That the extracellular fluid after the standard washing procedure (S_1 supernatant) still contained a component capable of displacing specific [^{3}H]NMS binding was shown by the fact that the standard veratrine incubation showed reduced specific binding in comparison to a veratrine incubation in which the S_1 supernatant was removed and replaced with fresh buffer prior to freezing the cells (P_1). The S_1 supernatant also inhibited specific [^{3}H]NMS binding when added to broken cell preparations which had not previously been exposed to veratrine (table 1). The S_2 super-

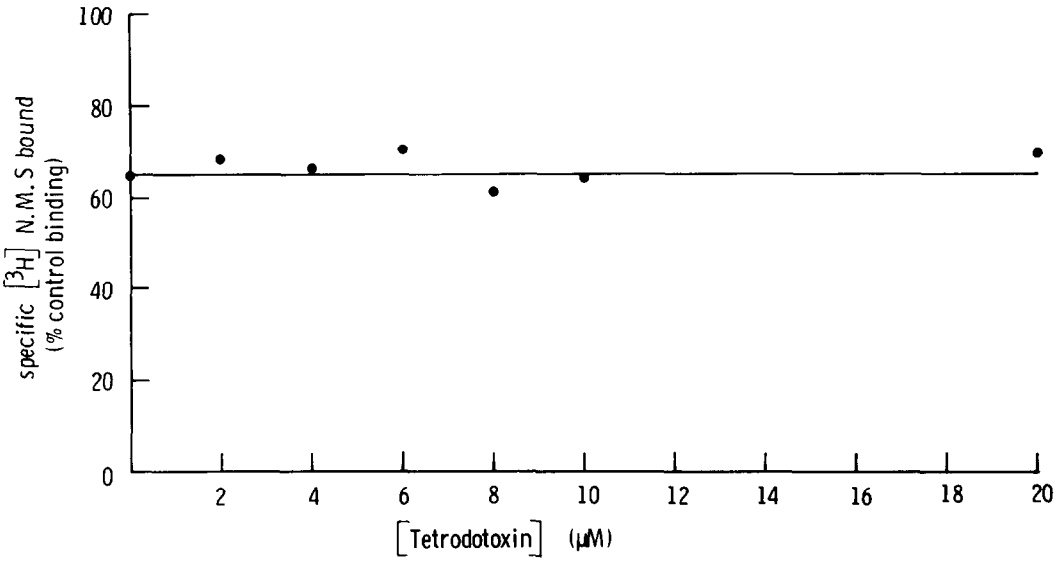

Fig.1. The effect of tetrodotoxin on the veratrine-induced loss of specific [^{3}H]NMS binding in broken cell preparations of N1E 115. Plates of N1E 115 were incubated for 6 h at 37°C in the presence of 300 μM veratrine and increasing concentrations of tetrodotoxin. Cells were harvested and prepared as described and the binding of [^{3}H]NMS (~500 pM) was determined. Each point is the mean of quadruplicate assays. 100% binding is that observed in incubations in the absence of veratrine.

natant, which was derived from intracellular fluid (fig.3), was also able to inhibit specific [^{3}H]NMS binding to control broken cell preparations and the S_3 supernatant (equivalent to S_1 + S_2) inhibited specific binding of [^{3}H]NMS to an extent equiv-

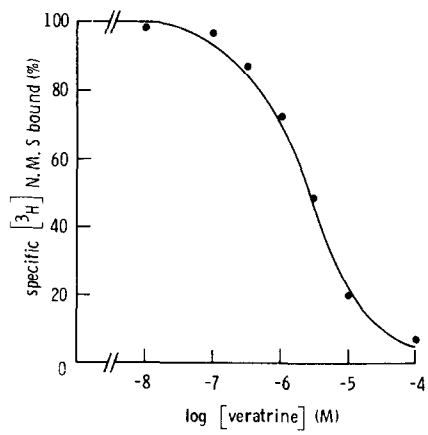

Fig.2. Displacement of specific [^{3}H]NMS (500 pM) binding by veratrine. Increasing concentrations of veratrine were included in the standard assay and points were determined in quadruplicate. An av. M_r = 600 was assumed for veratrine (see text).

Table 1

Veratrine treatment and muscarinic acetylcholine receptor binding in N1E 115 cells

Condition	Standard procedure	Extended washing procedure		
		P_1	P_2	P_3
	Veratrine-treated cells			
% control binding	51.7	77.8	102.3	107.7
Condition	+ S_1	+ S_2	+ S_3	(S_1+S_2)
	Untreated cells			
% control binding	64.3	67.4	52.5	49.0

Cells were treated with veratrine (300 μM, 37°C, 6 h) using the standard procedure or the extended washing procedure (fig.3) and muscarinic receptors assayed as described (0.5 mg protein/assay). In addition supernatant fractions (S_1, S_2, S_3) (volume equivalent to 0.5 mg protein from the fraction from which it was isolated) were added to membrane fragments from cells previously untreated with veratrine and muscarinic receptors assayed. The results are from a typical experiment each point being determined in quadruplicate

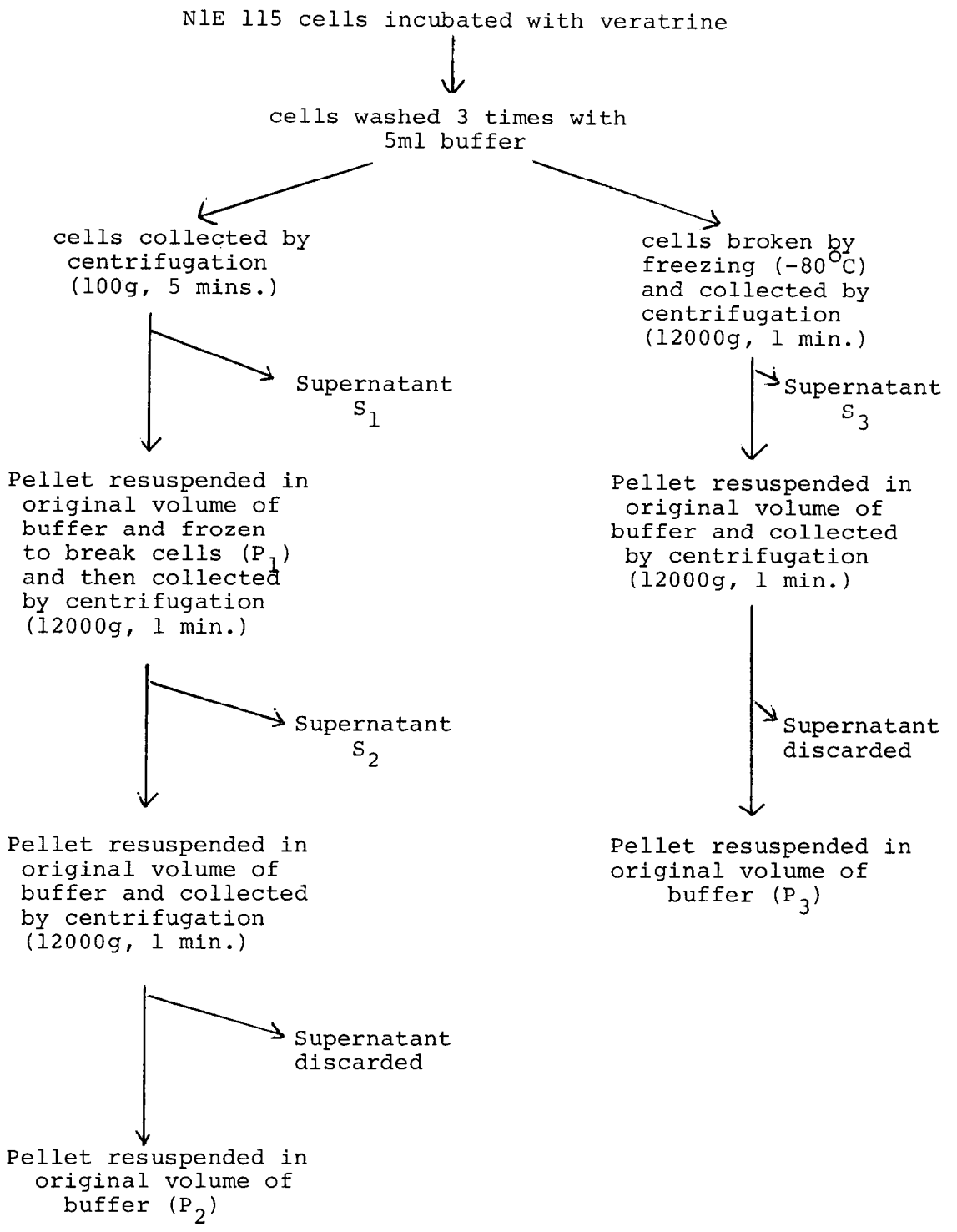

Fig.3. Extended washing procedure for removal of veratrine associated with cells.

Table 2

Carbamoylcholine treatment and muscarinic acetylcholine receptor binding in N1E 115 cells

Condition	Standard procedure	Extended washing procedure	
		P_1	P_2
	Carbamoylcholine-treated cells		
% control binding	41.4	39.9	57.4
Condition	+ S_1	+ S_2	+ S_3
	Untreated cells		
% control binding	95.4	84.4	80.0

Cells were treated with carbamoylcholine (1 mM, 37°C, 6 h) using the standard procedure or the extended washing procedure (fig.3) and muscarinic receptors assayed as described (0.5 mg protein/assay). In addition supernatant fractions (S_1, S_2, S_3) (volume equivalent to 0.5 mg protein from the fraction from which it was isolated) were added to membrane fragments from cells previously untreated with carbamoylcholine and muscarinic receptors assayed. The results are from a typical experiment each point being determined in quadruplicate

alent to that expected for the combined supernatants S_1 and S_2 (table 1).

As a comparison we examined whether agonist-stimulated downregulation of muscarinic receptor binding sites would be affected by the extended washing procedure. N1E 115 cells were incubated for 6 h with carbamoylcholine (1 mM) (table 2). The carbamoylcholine treated cells showed 41.4% of the specific [^{3}H]NMS binding of control incubations and the extended washing procedure increased this to 57.4% of control binding. Addition of the S_1 supernatant to cells previously untreated with carbamoylcholine had little effect on binding but supernatants S_2 and S_3 both reduced specific [^{3}H]NMS binding by 15–20% suggesting that over the incubation period a small amount of carbamoylcholine enters or becomes associated with the cells.

These results show that veratrine contains a component whose actions are consistent with it being a muscarinic antagonist and that veratrine produces no true loss of muscarinic receptor binding sites in cells of N1E 115 grown in tissue culture while the cholinergic agonist carbamoylcholine stimulates a true loss of binding sites.

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