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ORTHOVANADATE INDUCES LOSS OF MUSCARINIC CHOLINERGIC BINDING SITES

Erik Danielsson, Anders Undén, and Tamas Bartfai

Department of Biochemistry, Arrhenius Laboratory,
University of Stockholm, S-106 91 Stockholm, Sweden

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In homogenates of corpus striatum from rats, incubated in the presence of adenosine triphosphate (2 mM) (45 min, 37°C), a loss (approx. 50%) of muscarinic cholinergic binding sites could be dectected using a ^{3}H-3-Quinuclidinyl benzilate (^{3}H-3-QNB) binding assay. We found that only vanadium-contaminated ATP induced loss of muscarinic binding sites. Orthovanadate (1 mM) alone in the absence of ATP induced loss (approx. 60%) of binding sites just as "vanadium-ATP" did.

One mode of regulation of the sensitivity of the postsynaptic cells to neurotransmitters involves changes in the availability (number) and affinity in neurotransmitter receptors (1, 2). Exposure to agonists induces short term (min-hrs) loss of responsivness: "desensitization" (3). Chronic exposure to agonists for weeks or longer periods leads to development of "subsensitivity" (4). Desensitization of muscarinic receptors has been demonstrated in the muscarnic system in mouse neuroblastoma clone cells N1E 115 (5-7), in embryonic chicken cerebrum cells (8), and in chick heart embryo (9). Exposure of neuroblastoma cells to muscarinic agonists lead to loss of binding sites without any apparent change in receptor affinity (10). Electrically stimulated synaptosomes show a loss of bindings sites, an effect that disappears if voltage dependent sodium-channels are blocked selectively by tetrodotoxin (11). Membranes prepared from rat brain cortex show loss of muscarinic recep-

Please address correspondence to Dr. E. Danielsson at the above address.

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tors when incubated under "phosphorylating" conditions (12, 13). We attempted to study this effect in detail. During these experiments anomalous results appeared indicating that loss of ^{3}H-3-QNB binding sites may be caused by orthovanadate rather than by ATP.

MATERIALS AND METHODS

Materials

^{3}H-3-QNB (33.1 Ci/mmol) was purchased from New England Nuclear, Boston, Mass., USA. The following lots of 5' ATP were used: from Sigma & Co. St. Louis, Mo., USA; lot numbers A-5394 and A-6144 (formerly lot number: A-3127) and from Boehringer Mannheim, FRG (Prod. no. 127523). Analytical grade $Na_3VO_4 \cdot 14\ H_2O$ was obtained from BDH Chemicals Ltd., Poole, England; lot number 6025320. 8-Bromo cyclic AMP, 8-Bromo cyclic GMP, cyclic AMP, and cyclic GMP were purchased from Sigma & Co. Lumagel was purchased from Lumag AG, Basel. All other reagents were of analytical grade and obtained from commercial sources.

Incubation of striatal homogenate for study of "internalization" of receptors

Corpus striatum was dissected from white male Sprague-Dawley rats weighing 160-200 grams. A 10% (w/v) homogenate was prepared with loose-fitting glass-teflon homogenizer (15 strokes, 695 rpm) in ice-cold 0.32 M sucrose containing phosphate-buffer (5 mM, pH 7.4). The striatal homogenate was diluted 4 times with ice-cold Krebs-Ringer's buffer (14) (NaCl 138 mM, KCl 5.3 mM, $MgCl_2$ 1 mM, NaH_2PO_4 1 mM, $NaHCO_3$ 11 mM, glucose 10 mM, $CaCl_2$ 1.9 mM, and CH_3 COONa 1 mM).

The incubation mixture consisted of 1.5 ml of the diluted homogenate and 3 ml of buffer containing various agents such as ATP, 8-Br cyclic nucleotides and orthovanadate (table 1). Incubation was carried out for 45 min under continuous bubbling with a 95% O_2-5% CO_2 gas mixture. The incubation was terminated by placing the test tubes on an ice bath. The membrane preparation was lysed by freezing and by resuspension in hypotonic buffer and washed free from added agents (ATP, 8-Br cyclic nucleotides, orthovanadate) before the number of receptors was determined as described below.

Determination of ^{3}H-3-QNB binding sites

After the incubation with ATP, 8-Br cyclic nucleotides, or orthovanadate the striatal homogenate (4.5 ml) was diluted with 10 ml ice-cold phosphate buffer (5 mM, pH 7.4) and homogenized with a Polytron PTA 7 at setting 5 for 10 s. The lysate was centrifuged at 30,000 x g for 20 min in a Beckmann ultrcentrifuge. The pellet was resuspended in ice-cold phosphate buffer (5 mM, pH 7.4), and recentrifuged at 30,000 x g for 20 min. This pellet was resuspended in a Krebs-Ringer's buffer containing HEPES (5 mM, pH 7.4) and assayed for ^{3}H-QNB binding sites. 0.1 to 0.4 mg proteins was used in 1 ml incubation volume. The incubation was performed at a saturating ^{3}H-3-QNB concentration (1 nM) in a HEPES buffered Krebs-Ringer's solution (NaCl 137 mM, KCl 2.68 mM, $MgCl_2$ 1.05 mM, $CaCl_2$ 1.8 mM, HEPES 5 mM (pH 7.4), and glucose 1 mg/ml) at 37°C for 60 min. The samples

were filtered on Whatman GF/C filters and washed twice with 10 ml ice-cold Krebs-Ringer's buffer. The bound ^{3}H-3-QNB on the filters was counted in a liquid scintillation spectrometer using Lumagel as the scintillation cocktail. Non-specific binding was defined as binding in the presence of 10 µM atropine. The non-specific binding was less than 5% of total binding. Specific binding was defined as the difference between total binding and non-specific binding.

Protein concentration was determined according to the method of Lowry et al. (16), with bovine serum albumin dissolved in distilled water as standard (30-250 µg/ml).

RESULTS AND DISCUSSION

It was examined whether the reported receptor loss which occurs under "phosphorylating" conditions (12, 13) (i.e. in the presence of ATP), is influenced by the presence of muscarinic agonists or cyclic nucleotides which may act as "second messengers" (17). Table I summarizes our data on incubation of membrane vesicles (prepared isotonically) with ATP (Sigma A-6144), muscarinic agonists, cyclic nucleotides and their analogues. Approximately 49 ± 4% (n = 27) loss of ^{3}H-3-QNB

T A B L E I

Influence of various agents on ^{3}H-3-QNB binding sites in rat striatial homogenates

Conditions	% muscarinic receptors observed	(q)	n
No addition	100 ± 5	(2)	27
ATP (lot A 6144) 2 mM	51 ± 4**	(7)	27
Acetylcholine, 1 mM	99 ± 5	(3)	9
Carbamylcholine, 1 mM	98 ± 7	(3)	9
cAMP, 0.1 mM	91 ± 8	(3)	9
cGMP, 0.1 mM	96 ± 7	(3)	9
8-Br-cAMP, 0.1 mM	102 ± 2	(3)	13
8-Br-cGMP, 0.1 mM	98 ± 4	(3)	9
ATP (lot A 6144) 2 mM + 8-Br-cAMP, 0.1 mM	54 ± 4**	(7)	27
ATP (lot A 6144) 2 mM + 8-Br-cGMP, 0.1 mM	54 ± 2**	(3)	9

Specific ^{3}H-3-QNB binding: 100% corresponds to 1.7 picomoles/mg protein. Statistical significance was tested using Students t-test.

**corresponds to significant difference on the 0.01 level

q is the number of incubations of striatal homogenates with the various agents (i.e. the number of independent experiments)

n is the total number of experimental points; each incubation mixture was assayed in triplicates or quadruplicates.

receptors was found in the presence of ATP. This loss was not affected by the muscarinic agonists carbamylcholine (1 mM) or acetylcholine (1 mM). Acetylcholine was added in the presence of 10 µM eserine. Cyclic AMP, cyclic GMP, 8-Br-cyclic AMP, and 8-Br-cyclic GMP each in 0.1 mM concentration had no effect on the number of receptors found in membrane preparation neither in the presence or absence of ATP. Cyclic nucleotides were added in the presence of 3-isobutyl-1-methyl-xanthine (1 mM), a 3'5'-cyclic nucleotide phosphodiesterase inhibitor.

The only agent in the first series of experiments which induced loss of receptors was ATP (2 mM, Sigma A-6144). When we switched to another lot of ATP, which was prepared to be essentially free of vanadine (Sigma A-5394), not even ATP (2 mM) caused loss of receptors although the incubation were carried out under identical conditions as with lot A-6144. Similar results were obtained with vanadate-free ATP from Boehringer (lot 127523)(Fig. 1).

The product information department of Sigma informed us that lot A-6144 according to atomic absorbance analysis contains 5-40 ppm vanadium.

Orthovanadate was tested as the possible agent responsible for the receptor loss observed with the ATP lot containing vanadium, as it is known that orthovanadate (VO_4^{3-}) contamination of ATP is responsible for inhibition of Na^+/K^+ ATPase and of other enzymes (18). Fig. 2 shows that VO_4^{3-} (1 mM) alone induces a 40 ± 10% loss of muscarinic receptor sites. Addition of vanadium-contaminated ATP (2 mM) does not increase the receptor loss significantly. Our experiments tend to suggest that the observed receptor loss is dependent upon the oxidizing properties of VO_4^{3-}. Orthovanadate is known to oxidize sulfhydryl groups and catechols (19). It has also been previously

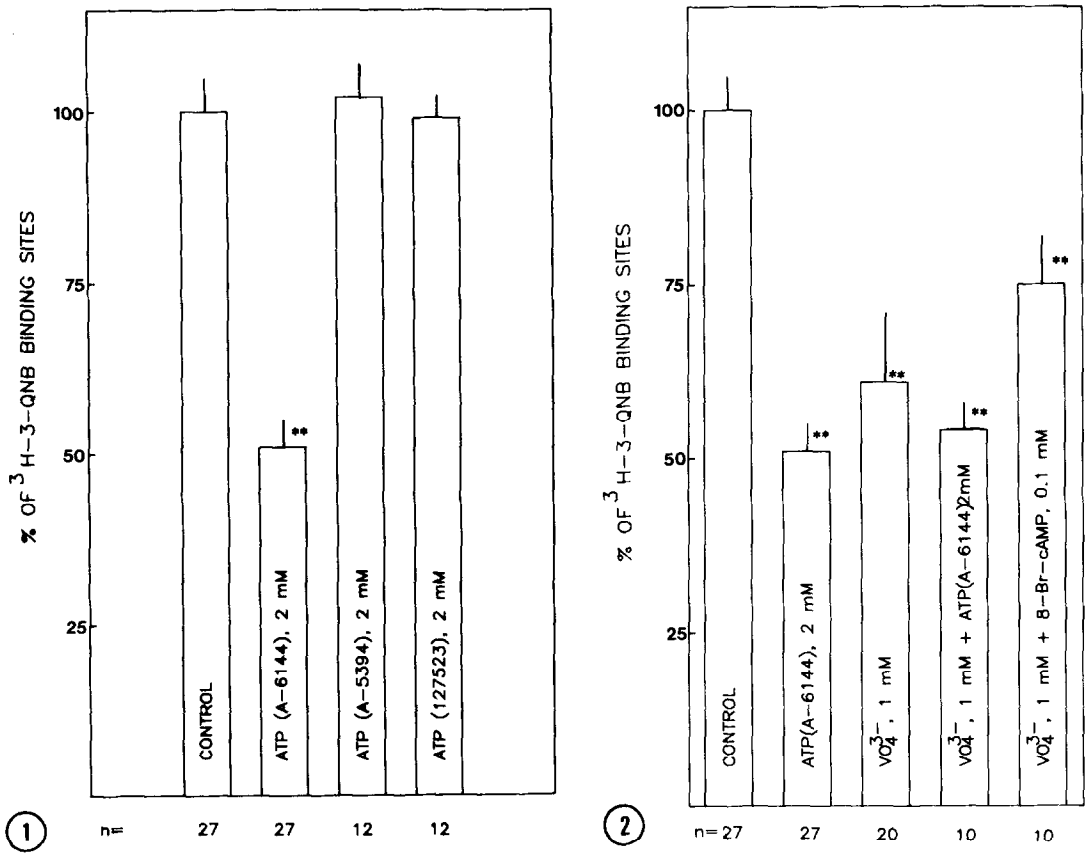

Fig. 1. Loss of ^{3}H-3-QNB binding sites using different commercially available ATP batches. 1.7 picomoles of specific ^{3}H-3-QNB binding sites per mg protein correspond to 100%.

Fig. 2. Loss of binding sites when Na_3VO_4 was included in the presence and absence of ATP. 100% corresponds to 1.8 picomoles specific ^{3}H-3-QNB binding sites per mg protein.

demonstrated that the thiolreagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (1 mM) causes an about 50% loss of muscarinic receptor sites (20).

Previous studies (12, 13) have nevertheless indicated a loss of muscarinic receptors in membrane vesicles in the presence of "vanadium-free" ATP. (Dr. Burgoyne, personal communication). One possible reason for the discrepancy between the results in references 12 and 13 and this report is the different media used for the incubations; we used a physiological medium

(Krebs-Ringer's buffer) instead of the phosphate buffer used in the previous studies.

In conclusion, our experiments clearly show that VO_4^{3-} (1 mM) can produce loss of receptor sites without the presence of ATP.

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