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SHORT COMMUNICATIONS

Effects of slow cooling *in situ* and freeze–thaw procedures on [^{3}H]hemicholinium-3 binding in rat cerebral cortical plasma membranes

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Hemicholinium-3 (HC-3) is one of the most potent inhibitors of high-affinity choline transport known [1]. Recently, this compound has been prepared in a tritiated form with high specific activity and has been used to estimate the number of neuronal choline-transport sites [2–4]. Since high-affinity choline transport is found predominantly associated with cholinergic nerve terminals and appears to be rate limiting for acetylcholine (ACh) synthesis in the brain, [^{3}H]HC-3 appears to be a potentially useful marker for functional cholinergic innervation in this organ [1, 5]. Such a marker would be particularly useful for the post-mortem diagnoses of pathological conditions associated with reduction in presynaptic cholinergic activity such as Alzheimer's disease. We therefore investigated whether [^{3}H]HC-3 binding is sensitive to conditions that human brains are often exposed to post-mortem, e.g. slow cooling to room temperature and freeze–thaw procedures.

Methods

Tissue preparation. Adult Sprague–Dawley male albino rats (150–250 g) were decapitated, and their cerebral cortices were rapidly removed and placed in 15 ml of cold (4°) 10 mM glycylglycine (GG) buffer, pH 7.4 [2, 6]. Homogenization of the samples was performed using a Janke and Kunkel Ultra-Turrax polytron at 25% maximum speed for two 15-sec intervals. Tissue samples were then centrifuged (40,000 *g* for 10 min) and the supernatant fraction was discarded. The membrane pellet was resuspended with the polytron in 35 ml of GG buffer and then collected by centrifugation (40,000 *g* for 10 min). Discarding the supernatant fraction, the membrane pellet was resuspended with the polytron in 35 ml of the same buffer. Membranes were then resuspended in 2 ml of GG buffer after a final centrifugation (40,000 *g* for 10 min). For the freeze–thaw studies, some samples were frozen in dry ice and acetone and thawed in water (22°) before performing binding assays.

For slow cooling studies, intact rat brains were rapidly removed and placed in tubes containing non-oxygenated Krebs–Ringer (KR) buffer (pH 7.4) prewarmed to 37°. These tubes were placed in a water bath at 37° and allowed to cool slowly at a rate similar to that of the human brain tissue post-mortem, as described previously [7].

[^{3}H]*HC-3 binding assay.* The specific binding of [^{3}H]HC-3 (120 Ci/mmole; New England Nuclear, Boston, MA) was measured essentially as described previously [2]. Fifteen microliters of 150 mM NaCl, 35 μl GG buffer and 25 μl of nonradioactive HC-3 (12 nM to 60 μM, as specified) were added to each sample. Next, 25 μl of [^{3}H]HC-3 (6 nM) was added to each sample to give a final HC-3 concentration range of 1 nM to 10 μM. Membranes (50 μl) were added next to give a protein range of 0.034 to 0.036 mg/tube. Each sample was vortexed for 5 sec at 30% maximum speed and incubated at 37° in a water bath for 30 min. Blanks were treated similarly except that GG buffer was used to replace the tissue. Tissues were filtered through Whatman GF/B filters using a Millipore vacuum manifold. These GF/B filters had been pre-soaked in 0.1% (v/v) polyethylenimine in water for 30 min to reduce non-specific filter-binding. Each sample was washed with three 3-ml washes of ice-cold GG buffer containing 150 mM NaCl. After allowing the filters to air-dry, they were counted via liquid scintillation spectrophotometry in 10 ml of Liquiscint (National Diagnostics, NJ). Specific binding was determined as described previously [2], which involves first subtracting [^{3}H]-binding to filter blanks, followed by subtracting the 10 μM [^{3}H]HC-3 binding values that were due to tissue. The equality of means was determined with the one-way analysis of variance [8]. Each experiment was performed several times in duplicate, and all values are expressed as means ± SEM. Scatchard analysis were performed with the Ligand Program on an Apple IIe computer. Protein levels were estimated using the Bio-rad assay, which involves Coomassie blue staining of peptides.

Results and discussion

[^{3}H]HC-3 bound to rat cerebral cortical plasma membranes in a specific manner with a K_D of 7 nM and a B_{max} of 35 fmoles/mg protein (Fig. 1). Specific binding was dependent on protein concentrations between 0.03 and 0.36 mg/150 μl ($R^2 = 0.85$, $P < 0.01$, for protein vs saturable binding at 2 nM HC-3) and was blocked by choline (IC_{50} of 40–50 μM). When rat brains were removed and allowed to cool in KR buffer at a rate similar to the human brain cooling rate, specific [^{3}H]HC-3 binding was not affected for up to 5 hr (Fig. 2). One cycle of freezing and thawing inhibited specific [^{3}H]HC-3 binding (2 nM) by 6 ± 3% and a second cycle inhibited specific binding by 43 ± 5%.

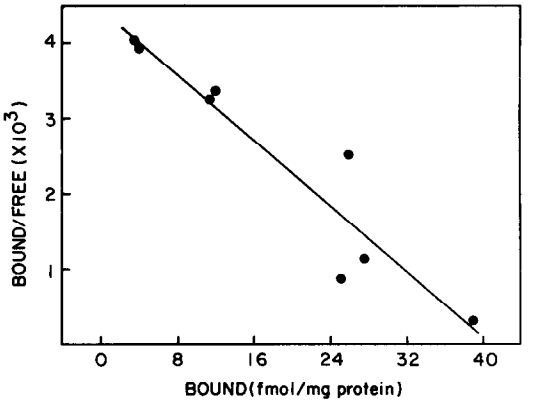

Fig. 1. Scatchard analysis of cerebral cortical [^{3}H]HC-3 binding. Rat cerebral cortical plasma membranes were incubated for 30 min at 37° with specified [^{3}H]HC-3 concentrations (1–50 nM), with or without 10 μM unlabeled HC-3 to determine specific binding as described in the text, which is expressed as fmoles/mg protein. Statistical analysis was performed by least-squares analysis ($r^2 = 0.65$). Each value is the mean of three values/concentration.

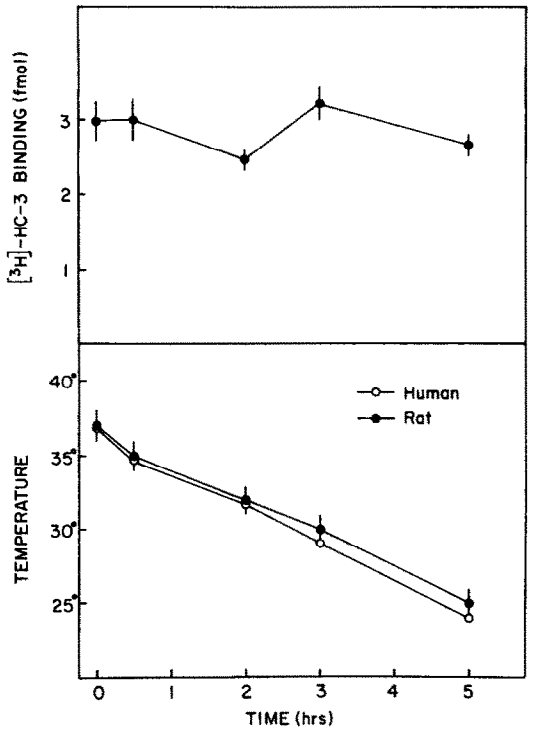

Fig. 2. Effects of slow cooling on specific [^{3}H]HC-3 binding to rat cerebral cortical plasma membranes. Rat brains were placed in non-oxygenated KR at 37° and slowly cooled to room temperature in a water bath incubator. At specified intervals, the temperature of the KR bathing the brains was measured, and then the cerebral cortices were dissected out from these brains to prepare plasma membranes as described in the text. The plasma membranes were then incubated with 2 nM or 10 μM [^{3}H]HC-3, for 30 min at 37° to determine specific binding as described in Methods. Binding values are expressed as mean ± SEM dpm of specific [^{3}H]HC-3 binding. Protein values were consistently between 0.034 and 0.036 mg in these tubes. The human post-mortem brain temperature data were taken from Ref. 7.

Alzheimer's disease and other dementias are associated with profound reductions in cerebral cortical and hippocampal cholinergic function, as measured by changes in choline acetyltransferase activity [9], high-affinity choline uptake [10], presynaptic M_2 muscarinic receptor-binding [11, 12], and ACh levels [9]. We therefore investigated whether [^{3}H]HC-3 binding to high-affinity choline transport sites on plasma membranes may be useful for post-mortem estimation of presynaptic cholinergic activity. Any reduction in the number of transport sites should reflect a loss of cholinergic synthetic capacity [1, 5]. [^{3}H]HC-3 binds to high-affinity choline transport sites on rat brain plasma membranes in a saturable, sodium-dependent, and choline-sensitive manner [2–4]. Published K_D values for specific binding to different brain regions are less than 10 nM, compatible with our results and known IC_{50} values for transport inhibition [2–4].

Using the rat cerebral cortex, we obtained control values from fresh tissue that would be very difficult with humans. Our results suggest that post-mortem clinical analyses of this cerebral cortical [^{3}H]HC-3 binding is feasible, since tissue can slowly cool to room temperature and be frozen as a homogenate without affecting specific binding capacity. Thus it appears that [^{3}H]HC-3 binding may be useful for the post-mortem diagnosis of cortical cholinergic function.

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Cromolyn inhibition of protein kinase C activity

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The mode of action of the antiallergic cromolyn drugs on the mast cell is unknown [1–3], and the possibility occurred to us that this might be studied more satisfactorily in the reptilian pigment cell in which the biopharmacological control of melanosome movement is more easily analysed kinetically [4, 5] than release of histamine and other vasoactive agents from mast cell granules. The method used was developed in the lizard *Anolis carolinensis* [6] and the pharmacology upon which our analysis depends is as follows (Fig. 1). In the melanophores of *Anolis carolinensis* as in other lower vertebrates [7, 8] α-MSH induces pigment dispersion mediated by cAMP [9, 10] following interaction with receptors linked to adenylate cyclase [11, 12]. The α_2 adrenoreceptor agonists inhibit the α-MSH response [1^{21}