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BRIEF COMMUNICATION

[^{3}H]Inositol Trisphosphate Specific Binding in Two Brain Regions of Long and Short Sleep Mice

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SMITH, T. L. *[^{3}H]Inositol trisphosphate specific binding in two brain regions of long and short sleep mice.* PHARMACOL BIOCHEM BEHAV **32**(3) 827–829, 1989.—The present investigation determined the binding characteristics of the radiolabeled second messenger, [^{3}H]In(1,4,5)P_3, in cerebral cortical and cerebellar membrane fragments from ethanol sensitive (LS) and resistant (SS) mice. The data demonstrated that in the two brain regions examined, LS and SS mouse lines do not differ with regard to their maximal receptor binding capacities or dissociation constants for [^{3}H]In(1,4,5)P_3. Moreover, [^{3}H]In(1,4,5)P_3 specific binding in cerebellum is inhibited in the presence of ethanol to the same extent in both mouse lines. It is concluded that the differential behavioral and cerebellar sensitivities to ethanol previously observed in these mouse lines do not reflect differences in [^{3}H]In(1,4,5)P_3 receptor binding characteristics of the brain regions examined.

Long sleep and short sleep mice Ethanol sensitivity Cerebral cortex Cerebellum Inositol 1,4,5-trisphosphate Radioligand binding

THE acute behavioral effects of ethanol may reflect, in part, the ability of ethanol to alter neuronal calcium disposition. In synaptosomes, for example, ethanol decreases Ca^{2+} uptake (7, 9, 12) as well as Ca^{2+} binding capacity (11). Ethanol has also been reported to increase resting and to decrease voltage-dependent intrasynaptosomal free calcium (2). In a recent study which utilized mouse lines selectively bred for their sensitivity (LS) or resistance (SS) to the acute intoxicating effects of ethanol (5), ethanol added in vitro altered resting and voltage-dependent intrasynaptosomal free calcium to the same extent in both mouse lines (3). However, the ability of these lines to mobilize synaptosomal calcium through receptor-activated pathways involving second messengers has not been documented.

Inositol-1,4,5-trisphosphate (InP_3) is a product of receptor-mediated polyphosphoinositide hydrolysis by phospholipase C and is now recognized as an important second messenger in a number of tissues (1). Nanamolar concentrations of InP_3 are able to mobilize Ca^{2+} in a wide variety of tissues including brain (4, 6, 8, 13). Specific saturable binding sites for radiolabeled InP_3 have been characterized in both rat (17) and mouse brain (14) with cerebellum containing the highest InP_3 receptor density in both species. Within the cerebellum, InP_3 specific binding is selectively associated with Purkinje cells (18). Interestingly, in several mouse strains ethanol-induced duration of loss of righting reflex and inhibition of cerebellar Purkinje neuron discharge are highly correlated (15). In addition, [^{3}H]InP_3 specific binding to cerebellar membranes is reduced following chronic ethanol consumption in C57/BL mice (14). Accordingly, it was of interest to characterize [^{3}H]InP_3 specific binding in cerebral cortex and cerebellum from LS and SS mice in an attempt to define what role, if any, these intracellular Ca^{2+} regulatory sites play in determining the differential behavioral and neuronal sensitivities previously observed in these mouse lines.

METHOD

Adult male long sleep (LS) and short sleep (SS) mice were purchased from the Institute for Behavioral Genetics, Boulder, CO. Freshly excised cerebral cortices and cerebella from two pooled mouse brains were processed in parallel. Tissues were homogenized (Polytron, setting 7, 15 sec) in 20 vol. ice-cold 50 mM Tris buffer (pH 8.3) containing: 20 mM NaCl; 100 mM KCl; 1 mM EDTA (buffer A). The tissue suspension was centrifuged at

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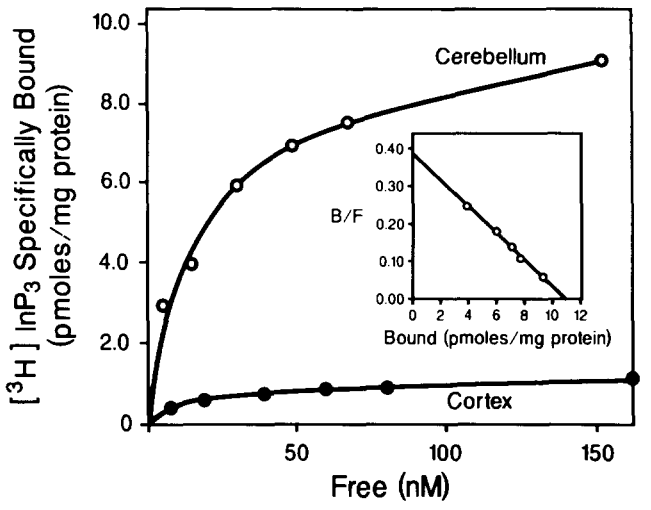

FIG. 1. [^{3}H]In(1,4,5)P_3 specific binding in cerebellar and cerebral cortical membrane fragments from SS mice. Data represent typical saturation isotherm experiments performed in triplicate. A Scatchard plot of the cerebellar data (insert) gave K_D and B_{max} values of 27 nM and 10.80 pmoles/mg protein, respectively.

35,000 × g for 10 min and the pellet resuspended in the same buffer (17).

The assay conditions for determining [^{3}H]In(1,4,5)P_3 specific binding were as follows: 200 μl (200–400 μg protein) of membrane suspension was added in triplicate to glass tubes containing 10 to 160 nM [^{3}H]In(1,4,5)P_3 (20 Ci/mmol, NEN) in buffer A plus ethanol when indicated to give a total volume of 500 μl. Nonspecific binding was determined in the presence of 10 μM unlabeled InP_3. After incubation on ice (4°) for 10 min, the samples were centrifuged in microfuge tubes at 10,000 × g for 10 min and the pellet washed once with 500 μl buffer A. Solubilization of the pellet was achieved by the addition of 100 μl tissue solubilizer (NCS) and further incubation with shaking for 30 min at 45°C. Samples were transferred to scintillation vials and counted after 24 hours by liquid scintillation spectroscopy with a counting efficiency of 40%. Preliminary experiments established that binding equilibrium was reached within one minute and remained the same at ten minutes under the present conditions. Specific binding was linear within the tissue protein range of 90–400 μg/0.5 ml. When K_D and B_{max} values were determined, Scatchard analyses of the binding data were performed. The slope and intercept of the straight line were generated by least squares analyses. Independent *t*-tests were used to compare K_D and B_{max} values of LS and SS mice, while paired *t*-tests were used for within group comparisons, i.e., effect of added ethanol on [^{3}H]In(1,4,5)P_3 specific binding within a given tissue preparation.

RESULTS

The results of typical saturation isotherm experiments in which cerebral cortical and cerebellar membrane fragments from SS mice were incubated with varying amounts of [^{3}H]In(1,4,5)P_3 are illustrated in Fig. 1. Specific binding approached maximal values in cortex and cerebellum at approximately 40 nM and 160 nM, respectively. Nonspecific binding accounted for less than 10% of the total binding. Similarly prepared tissues form LS mice exhibited virtually identical saturation binding profiles. A summary of B_{max} and K_D values as generated by Scatchard analyses of LS and SS binding data is given in Table 1. The data reveal no statistically

TABLE 1

[^{3}H]In(1,4,5)P_3 SPECIFIC BINDING IN TWO BRAIN REGIONS OF "LONG" AND "SHORT" SLEEP MICE

	B_{max} (pmoles/mg protein)	K_D (nM)
LS Mice		
cerebral cortex	1.00 ± 0.16	32 ± 6
cerebellum	9.33 ± 1.38	17 ± 5
SS Mice		
cerebral cortex	1.19 ± 0.32	26 ± 5
cerebellum	11.16 ± 1.23	21 ± 6

Results were obtained from Scatchard analyses of saturation isotherms for [^{3}H]Ins(1,4,5)P_3 specific binding to membrane fragments prepared from selected brain regions. Values represent the mean ± S.D. of 3–4 individual experiments performed in triplicate in which tissues from two pooled mouse brains were used.

significant differences between LS and SS mouse lines in either their maximum binding capacities (B_{max}) for [^{3}H]In(1,4,5)P_3 or their dissociation constants (K_D) in either the cerebral cortex or the cerebellum. However, B_{max} values for cerebellum in both mouse lines were 9–10 times higher than those found in the cerebral cortex. The regional heterogeneity of specific binding observed in the present investigation is in accord with earlier reports in rat (17) and in C57/BL mice from this laboratory in which a filtration assay for [^{3}H]In(1,4,5)P_3 specific binding was employed (14). K_D values were similar for both brain regions of the SS mice. In the LS mice the K_D in cerebellum was approximately half of that observed in the cerebral cortex of the same mice.

In other experiments, the effects of ethanol added in vitro on the specific binding of 25 nM [^{3}H]In(1,4,5)P_3 specific binding were determined in cerebellar tissues from LS and SS mice. As illustrated in Fig. 2, ethanol produced a dose-dependent inhibition

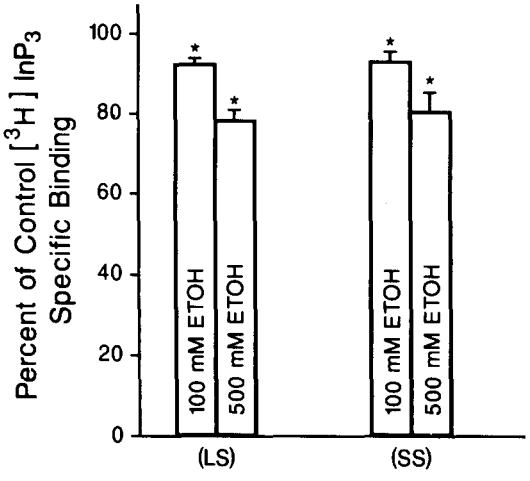

FIG. 2. Effects of ethanol on [^{3}H]In(1,4,5)P_3 specific binding in cerebella of LS and SS mice. Each tissue sample was incubated in the presence of either 0, 100, or 500 mM ethanol plus 25 nM [^{3}H]In(1,4,5)P_3 at 4° for 10 min as described in the Method section. Values are expressed as percent of control and represent the mean ± S.D. for four individual experiments performed in triplicate. *Indicates $p<0.001$ (paired *t*-test).

of specific binding which was of the same magnitude in both mouse lines. Although the inhibitory effects of ethanol were modest (10–20%), they were highly significant at the concentrations of ethanol tested.

DISCUSSION

The present investigation was designed to demonstrate whether or not [^{3}H]In(1,4,5)P_3 specific binding in brain tissue from LS and SS mice could provide a possible biochemical mechanism or explanation for the differential electrophysiological and behavioral effects of ethanol previously observed in these mouse lines. The B_{max} values obtained in the present investigation are somewhat higher than those recently reported in C57/BL mice in this laboratory in which a filtration assay for [^{3}H]In(1,4,5)P_3 specific binding was employed (14). Recent findings suggest that this discrepancy is likely due to the higher efficiency of the centrifugation technique which accommodates the rapid dissociation rate of bound [^{3}H]In(1,4,5)P_3 (19). Thus, the centrifugation technique used in the present investigation is a superior method for the accurate quantitation of [^{3}H]InP_3 specific binding. The centrifugation data demonstrate that in the two brain regions examined, LS and SS mice do not differ with regard to their B_{max} or K_D values. Moreover, [^{3}H]In(1,4,5)P_3 specific binding in cerebellum is inhibited in the presence of ethanol to the same extent in both mouse lines. However, the degree of inhibition is minimal at 100 mM ethanol and may have little functional relevance to the behavioral effect of ethanol in vivo. Therefore, the present findings strongly suggest that the differential electrophysiological and behavioral sensitivities to ethanol previously documented in LS and SS mice do not reflect line differences in [^{3}H]In(1,4,5)P_3 receptor densities or dissociation constants. Our findings do not rule out the possibility, however, that for a given quantity of InP_3 produced by cell-surface receptor activation, line differences in the magnitude of calcium mobilization may exist. Alternatively, neurotransmitter enzymes such as tyrosine hydroxylase and tryptophan hydroxylase, which are regulated by Ca^{2+} through calmodulin dependent protein kinase (16), may be differentially activated in LS and SS mouse lines in the presence of similar Ca^{2+} concentrations. In this regard, previous findings suggest that line differences in ethanol-induced sleep time may be a direct consequence of differences in catecholaminergic neurotransmission (10). Further investigations will be required to distinguish between these possibilities.

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