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[^{3}H]-*myo*-INOSITOL UPTAKE IN RAT CORTICAL SLICES

IDENTIFICATION OF Na^+-DEPENDENT AND Na^+-INDEPENDENT SYSTEMS*

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Abstract—[^{3}H]-*myo*-Inositol (MI) uptake was measured *in vitro* using chopped rat cerebral cortical tissue. The uptake and accumulation of MI were linearly proportional to the amount of protein (0.1 to 4.0 mg) in the incubation medium. The uptake was also linear vs time for the first 20 min of incubation. When the uptake was observed at various substrate concentrations, it was found to be unsaturable up to a concentration of 0.78 M. Decreasing the concentration of NaCl or increasing the concentration of KCl in the incubation medium resulted in inhibition of the uptake and accumulation of MI. Inhibition of MI uptake was also produced by veratrine, ouabain and A23187 which alter the ionic gradients across the neuronal membranes. Inhibition of oxidative metabolism with dinitrophenol did not alter MI uptake. Sodium-independent uptake appeared to be the same as that which occurred at 0°. Sodium-independent uptake was still present in water-lysed homogenates and was inhibited by relatively high concentrations of ethanol. Thus, it appears that approximately one-half of the [^{3}H]inositol uptake and accumulation in chopped rat cerebral cortex occurs by a sodium-dependent mechanism that can be altered by drugs which change the sodium gradient and the remaining occurs by a sodium-independent mechanism that can be altered by ethanol which is known to change membrane fluidity of neuronal membranes.

We have recently obtained a clearer understanding of the role of membrane phosphoinositides in the transduction of receptor-mediated cellular responses involving the mobilization of calcium ions [1, 2]. The phosphoinositide response to receptor activation involves the synthesis of newly formed phosphoinositides from a cellular pool of inositol. It has been reported that approximately half of brain inositol comes from plasma [3] and enters brain circulation via a saturable energy-dependent uptake system in the choroid plexus [4, 5]. However, before *myo*-inositol is useful in neurotransmitter transduction, it first must be delivered to the extraneuronal environment for transport across the neuronal membrane.

The availability of *myo*-inositol to the CNS appears to be determined by a combination of the following five processes: (1) *myo*-inositol synthesis in the gastrointestinal tract and liver, (2) absorption of newly synthesized and dietary *myo*-inositol from the gastrointestinal tract, (3) uptake of plasma *myo*-inositol by choroid plexus, (4) uptake of *myo*-inositol from the CSF across the neuronal membrane to the cytosol, and (5) synthesis of *myo*-inositol from glucose in neuronal tissue. The uptake of *myo*-inositol into neuronal elements is of additional importance in studies involving the measurement of the phosphoinositide response since [^{3}H]-*myo*-inositol (MI) is used to label the cytoplasmic *myo*-inositol pool [6]. A nonsaturable MI transport system has been described in rat brain synaptosomes [7]. This uptake system does not require metabolic energy and is not affected by inhibition of Na^+,K^+-ATPase by ouabain. On the other hand, uptake of MI in cerebral cortical slices of rabbit has been shown to be saturable between 0.1 and 0.3 mM MI with a maximal uptake rate of 23 nmol/g/30 min and half-maximal velocity at 0.055 mM [8]. This system is at least partially energy dependent, and tissue to medium ratios greater than unity could be attained. It is not clear whether the differences between these two studies are due to species (rat vs rabbit) or type of preparation (synaptosomes vs slices). The purpose of the current study is to characterize MI uptake into chopped rat brain tissue with the emphasis on the sodium ion requirement of the uptake process.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (250–275 g) were obtained from Sasco Inc. (Omaha, NE). The rats were maintained in the University of Kansas Animal Care Facility on a 12:12 light–dark cycle (7:00 a.m. on and 7:00 p.m. off) with food and water *ad lib.*

Tissue preparation. Cerebral cortex was isolated and chopped according to the following procedure. Following decapitation, the brain was quickly removed and rinsed with ice-cold Krebs' bicarbonate buffer. The buffer had the following composition (concentrations in mM): NaCl, 108; KCl, 4.7;

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$MgSO_4$, 1.18; $CaCl_2$, 2.54; EDTA, 0.05; KH_2PO_4, 1.2; glucose, 10; $NaHCO_3$, 25. A cortical hemisphere was dissected from the underlying subcortical regions and trimmed of white matter and meninges. The cerebral cortex was then chopped (0.3 × 0.3 mm) twice bidirectionally on a McIlwain tissue chopper and suspended in 3.0 ml of ice-cold oxygenated buffer. After gentle centrifugation (3000 rpm, 1200 *g*, 5 min) at 4°, the chopped tissue was resuspended in 3.0 ml of fresh buffer.

Uptake of MI. An aliquot of the suspension of tissue slices (215 μl) was added to each sample (approximately 1 to 1.5 mg, dry weight). Samples were then aerated (95% O_2/5% CO_2), capped, and preincubated for 15 min. MI (1.0 μCi, 25 μl) and 10 μl of vehicle, drug or KCl were then added to the tissue suspension. In the sodium experiments, choline chloride was substituted for NaCl to maintain osmolality throughout the entire experiment. Atropine (10^{-5} M) was present in these experiments to avoid cholinergically-mediated effects on MI uptake.

After 10 min of incubation, the uptake was terminated by the addition of 1.0 ml of ice-cold buffer. The incubation tubes were then placed on ice. After gentle centrifugation (see above), the tissue samples were washed individually five times with 1.0 ml of ice-cold buffer. The slices were then resuspended in 1.5 ml of ice-cold distilled water and homogenized using a glass/glass Wheaton 2.0 ml homogenizer. After centrifugation (13,500 *g*, 10 min) the supernatant fraction was added to a Dowex AG-X8 (formate form) ion exchange column (0.5 × 2.0 cm). The MI was eluted using 10 ml of distilled water as previously described [6]. An aliquot of the eluate was assayed by liquid scintillation spectrometry (Beckman LS100), and data are expressed as dpm/mg protein. Protein was estimated using the biuret method as described by Layne [9]. Modifications of the procedure necessary for individual experiments are described in the figure legends.

Statistics. All of the dose–response data were evaluated initially by Analysis of Variance and Multiple Comparisons post-hoc testing via Tukey B (SPSS) [10].

Materials. MI (14 Ci/mmol) was purchased from the American Radiolabeled Co., St. Louis, MO. All drugs were purchased from Sigma.

RESULTS

Assay validation. Initial studies were performed to ascertain conditions under which the intracellular accumulation of MI could be measured. It required 10 × 1 ml washes after the 60-min incubation in order to extrapolate the curve through the origin (Fig. 1A). Figure 1B illustrates the time course for MI uptake ($F = 76.0$, df 5/17, $P < 0.001$) after 5 × 1 ml washes followed by subtraction of an ice control blank (a measure of non-specific extracellular binding). The ice control blanks at each of the time points were not sufficiently different and were thus averaged together for a value of 2.38 ± 0.22 dpm/mg protein. Comparison of the time course curve in Fig. 1B (at 60 min) with the 10 × 1 ml wash curve in Fig. 1A (at 1.0 to 1.5 mg) shows comparable uptake under the two conditions. Thus, it appears as if 10 × 1 ml

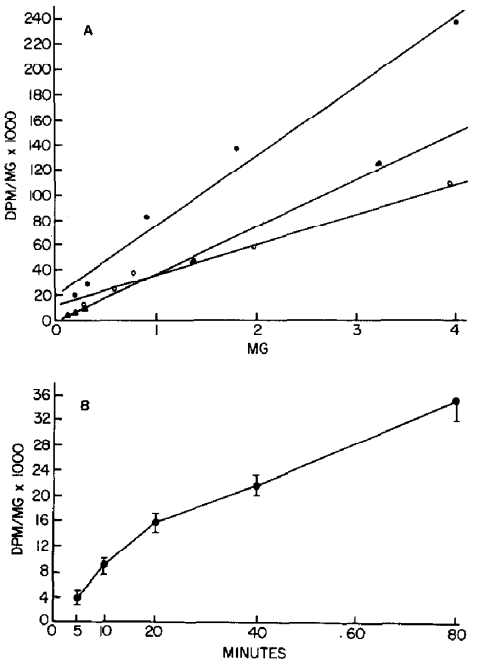

Fig. 1. Protein dependence and time course of MI uptake. Chopped cortical tissue was incubated for 60 min at 37° with 5 μCi MI. Values represent accumulation of MI in the tissue expressed as dpm/mg protein × 1000. Panel A: Effect of 2 (●), 5 (○) or 10 (▲) 1 ml washes on protein linearity. The ice control blank was not subtracted. Panel B: Time course of MI uptake after subtraction of ice control blank (N = 4).

washes eliminate a significant contribution of extracellular binding and accumulation in the interstitial space. That which does occur with less frequent washes can be accounted for by subtraction of an ice control blank. A 10-min time period was chosen for the following uptake experiments since it is in the linear portion of the MI uptake curve. When the chopped tissue was preincubated for 15 min in water, the suspension centrifuged and then incubated for 10 min in Kreb's bicarbonate buffer, the lysis of the cells resulted in a 40% decrease in MI uptake. In these experiments the values were: buffer preincubation/uptake at 37°, 8400 ± 460 dpm/mg protein; water preincubation/uptake at 37°, 5140 ± 130 dpm/mg protein; buffer preincubation/uptake at 4°, 2040 ± 100 dpm/mg protein. The values are the means ± SEM of four determinations.

Kinetics of MI uptake. A study of MI uptake kinetics into rat cerebral cortical slices was initiated to determine affinities and maximal velocities. However, as the data in Fig. 2 show, MI uptake into cortical slices was linear over a wide range of concentrations (eight orders of magnitude). This relationship existed from 0.1 μM to 0.78 M; the latter concentration is the solubility limit for MI. Therefore, MI uptake in rat cortical slices appears to be a non-saturable system.

Sodium and potassium dependence of MI uptake. The data presented in Fig. 3 illustrate the effect of altering the sodium or potassium concentrations on

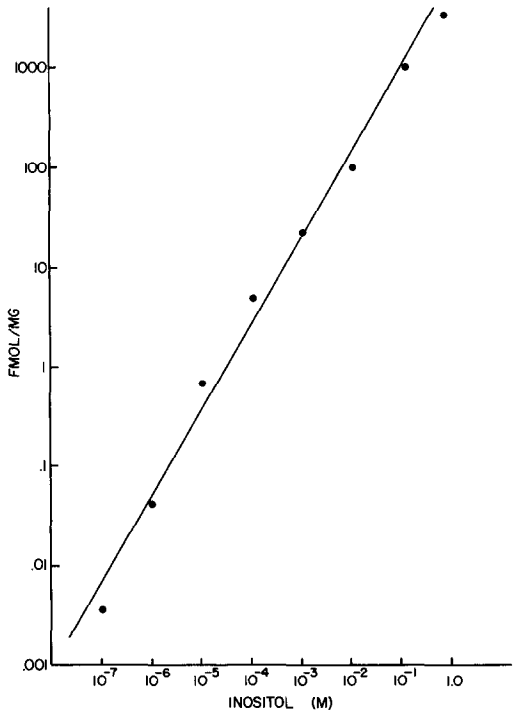

Fig. 2. Non-saturable uptake kinetics of MI uptake in chopped cerebral cortex. Values represent the accumulation of MI in the tissue as fmol/mg protein and are representative of a total of five experiments.

MI uptake. Choline was substituted for sodium to maintain osmolality. Both the removal of sodium and the addition of potassium resulted in an inhibition of MI uptake (sodium: $F = 26.0$, df 4/19, $P < 0.001$, potassium: $F = 21.0$, df 6/37, $P < 0.0001$). These data suggest that MI uptake into rat cortical slices is dependent upon the gradients for these monovalent cations. The following experiments were performed to determine if MI uptake could be modulated by pharmacologic agents known to affect intracellular sodium levels in neuronal slices.

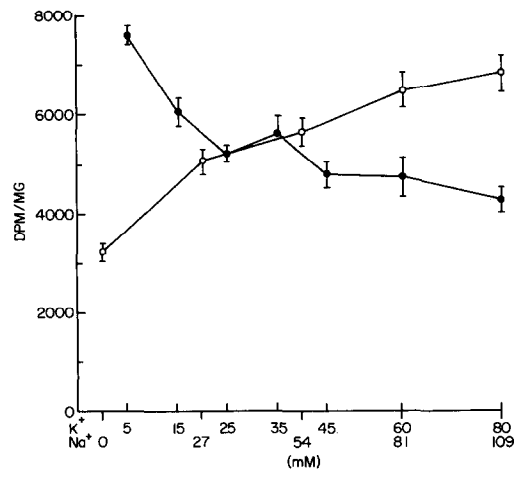

Fig. 3. Ionic requirements for MI uptake. Values represent accumulation of MI in the tissue expressed as dpm/mg protein. The final concentration of MI was 2.8×10^{-7} M (tracer only). Shown are dose–response relationships for Na^+ (○) and K^+ (●). The data are expressed as mean ± SEM of four experiments.

Effects of veratrine, ouabain and A23187 on MI uptake. Figure 4 demonstrates MI uptake in the presence of several concentrations of veratrine, ouabain or the calcium ionophore A23187. In panel A it can be seen that the uptake of MI was inhibited by veratrine between 10^{-5} and 10^{-3} M ($F = 21.8$, df 4/19, $P < 0.001$). Panel B depicts a more graded response to ouabain ($F = 7.9$, df 4/19, $P < 0.001$). The first ouabain concentration at which the inhibition was statistically significant was 10^{-4} M. Inhibition of MI uptake ($F = 8.3$, df 3/15, $P < 0.003$) was also observed in the presence of A23187. However, no significant inhibition was seen until a concentration of 10^{-3} M was reached.

Effects of metabolic inhibitors on MI uptake. Since several of the previous treatments would be expected to reduce ATP levels, it was of interest to determine whether inhibition of ATP formation would result in inhibition of MI uptake. In Fig. 5 it can be seen that 2,4-dinitrophenol (DNP) at concentrations from 10^{-5} to 10^{-3} M had no effect on MI uptake. The slight reduction of MI uptake at 10^{-3} M DNP may be due to inhibition of Na^+,K^+-ATPase resulting from reduced levels of ATP. Inhibition of glucose metabolism by 2-deoxyglucose would also be expected to lead to reduced levels of ATP. From the inset in Fig. 5, it can be seen that 2-deoxyglucose also did not alter the uptake of MI.

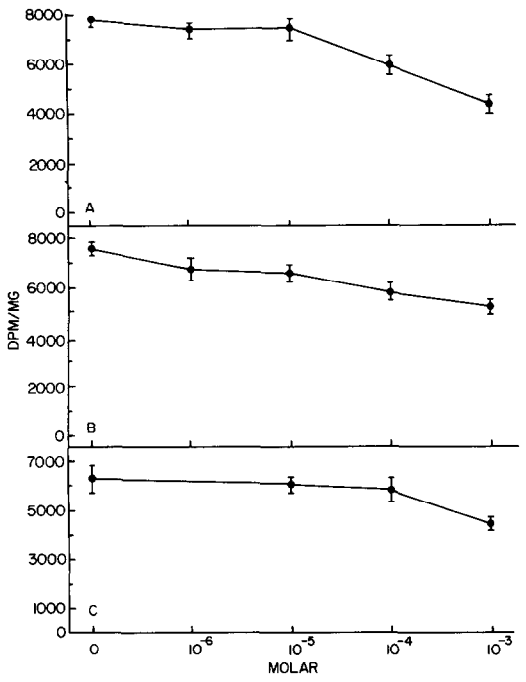

Fig. 4. Effects of agents which increase the intracellular content of Na^+ on MI uptake expressed as dpm/mg protein: (A) veratrine, (B) ouabain, and (C) A23187. The final concentration of MI was 2.8×10^{-7} M (tracer only). The data are expressed as mean ± SEM of four experiments.

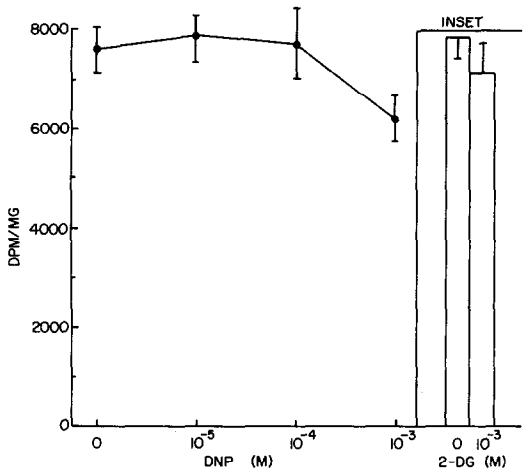

Fig. 5. Effect of 2,4-dinitrophenol (DNP) on MI uptake expressed as dpm/mg protein (●). Inset: Effect of 2-deoxyglucose (2-DG) on MI uptake.

Effect of ethanol on MI uptake. When the effects of ethanol alone and in combination with norepinephrine (10^{-4} M) were determined on the MI retained in an experiment in which the norepinephrine and MI were added simultaneously and incubated for 60 min, it was observed that ethanol both in the presence and absence of norepinephrine produced a decrease in MI which remained in the tissue (Fig. 6). This effect was statistically significant ($F = 18.8$, df 4/58, $P < 0.001$). In these experiments, the ice control blanks were not subtracted. When the initial uptake of MI was measured in the presence of ethanol, it was found that ethanol inhibited the nonspecific accumulation as indicated by the ice control blank ($F = 14.7$, df 3/15, $P < 0.001$) but had no effect on the net accumulation of MI (Table 1).

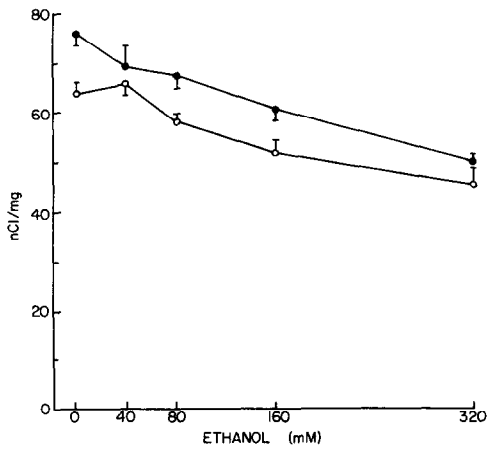

Fig. 6. Modification of extracellularly-bound MI with ethanol. Dose–response curve of ethanol on MI uptake expressed as nCi MI/mg protein in the presence (○) and absence (●) of 10^{-4} M norepinephrine. Samples were preincubated in ethanol for 10 min followed by simultaneous addition of norepinephrine or vehicle for 60 min. No ice control blank was used in this experiment. Values represent the mean ± SEM of four experiments.

Table 1. Effect of ethanol on the initial uptake and accumulation of MI at 37° and 4°

Ethanol concentration (mM)	MI (dpm/mg protein)	
	37°	4°
0	12,000 ± 411	1,970 ± 109
80	11,800 ± 327	1,520 ± 108
160	11,000 ± 787	1,340 ± 118
320	13,100 ± 929	1,120 ± 41

Values are the mean ± SEM; N = 3–4.

DISCUSSION

The uptake of MI into chopped cerebral cortex was linear with protein, not saturated at 0.78 M MI, and was not inhibited by the metabolic inhibitor DNP. This suggests that MI is not taken up by an active transport system. These results are consistent with those obtained in rat brain synaptosomes [7]. However, there appear to be differences across species with regard to the uptake of MI into isolated brain tissue since in rabbit brain slices [8] the uptake of MI was found to be saturable and inhibited by metabolic inhibitors. When the uptake of MI was measured in rat sciatic nerves, it was found that the endoneurial preparations accumulate MI by an energy-dependent saturable system [11]. In most peripheral tissues, however, the uptake of MI is not inhibited by analogs of glucose and occurs by an energy-independent nonsaturable system. This includes skeletal muscle [12] and isolated liver parenchymal cells [13]. The major exception appears to be the kidney in which transport of MI in cortex slices [14] and brush border membranes vesicles [15] has been shown to be saturable, sodium dependent and inhibited by glucose analogs and metabolic inhibitors.

It appears as if a sodium gradient in chopped brain tissue is required for maximal uptake and accumulation of MI. If this gradient was decreased by lowering the concentration of sodium in the incubation medium, the initial uptake and accumulation of MI were inhibited. Depolarization of the neurons or their nerve endings in brain cerebral cortex with elevated potassium ions in the incubation medium also led to inhibition of MI uptake. It is known that depolarization leads to an increase in the intracellular content of sodium ions [16]. This could explain the decreased MI uptake in the presence of elevated extracellular potassium. Most of the drugs which decreased the uptake of MI are also known to disrupt the sodium gradient. Veratrine acts by opening sodium channels [17, 18], while ouabain produces its effect by inhibition of Na^+,K^+-ATPase and thereby increasing the intracellular concentration of sodium [19, 20]. The high concentration of A23187 would also lead to increased levels of intracellular sodium [20]. All of these drug manipulations would be expected to decrease the sodium gradient.

The question of active transport of MI was approached in two ways. First, DNP did not reduce MI uptake at any concentration tested. The uptake of MI thus appears to be energy-independent as

measured by the availability of intracellular ATP levels. An inhibitor of glucose uptake and glycolysis, 2-deoxyglucose, did not reduce MI uptake which suggests that interference with ATP formation from glucose has no effect on MI uptake. The slight reduction in MI uptake seen at 10^{-3} M DNP is most easily explained by a reduction in Na^+,K^+-ATPase activity, resulting in slightly elevated intracellular sodium levels [21]. Thus, these data suggest that the effects of potassium, veratrine or A23187 are not mediated by a reduction in ATP levels directly but indirectly through an elevation in intracellular sodium.

The problem of determining a proper control for non-specific binding has been dealt with by several different approaches. Estimation of extracellular space [7, 12, 13], high concentrations of unlabeled MI [8, 22], and sodium-free [23] samples have all been used in various studies as possible control procedures. We chose the ice control blank since it limits radioactivity to the outside of the cell and thus transport of MI across the plasma membrane (regardless of mechanism) can be differentiated from extracellular binding. Fifty percent of the uptake seen in chopped tissue was also present in a water-lysed homogenate. This indicates that MI uptake, mediated by passive diffusion, is responsible for a large portion of MI accumulation in brain slices. A passive diffusion mechanism of MI uptake has been suggested previously [15].

Hypotonic homogenization of these slices followed by resuspension in normal buffer provided some information concerning intracellular MI binding. A water-lysed homogenate would be expected to expose all intracellular and extracellular binding sites without forming vesicles. It is clear that the majority of MI uptake mediated by passive diffusion was sequestered by intracellular binding sites. A role for tubulin in providing MI intracellular binding sites has been discussed [12].

Since ethanol is well known to perturb neuronal membranes by increasing their fluidity [24], it is not surprising that it should have a selective effect on the ice control binding blank. These data support the contention that this source of MI binding/uptake represents association with the extracellular membrane.

In conclusion, the present study supports and extends earlier studies on the characteristics of MI uptake. It is clear that uptake of MI occurs by both sodium-dependent and sodium-independent systems. The uptake of MI can be altered by drugs that change the sodium gradient across the membrane. MI uptake in rat brain slices is not saturable, does not require metabolic energy and does not occur via a glucose transport system.

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