THE EFFECT OF $MnCl_2$ ON THE BASAL AND ACETYLCHOLINE-STIMULATED TURNOVER OF PHOSPHATIDYLINOSITOL IN SYNAPTOSOMES

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1. Introduction

Recently this laboratory has reported a defect in the acetylcholine (ACh)-induced turnover of phosphatidylinositol (PhI) in synaptosomes from galactose-toxic rats [1]. In order to assess the significance of this finding we have continued the study of synaptosomal PhI metabolism and have investigated the effects of a perturbation of this system by the addition of Mn^{2+}, a cofactor in the final step of PhI synthesis by CDP-diglyceride:inositol transferase.

This enzyme is very selective for Mn^{2+} as a cofactor, with Mg^{2+} able to substitute only at much higher concentrations [2]. The exchange of inositol into PhI [3] and the production of CDP-diglyceride from CMP and PhI [4] have also been shown to be dependent on Mn^{2+}. These reactions have been assumed to involve a reversal of the synthetic enzyme, but it was suggested [3] that a separate enzyme is involved. A reversal of the synthesis of PhI has been suggested as the pathway which is stimulated by the action of neurotransmitters and hormones in the 'phospholipid effect' [5].

We report here a striking effect of external Mn^{2+} on the turnover of the inositol portion of synaptosomal PhI, with a much lesser effect on its phosphate moiety. A comparison of the effects of Mn^{2+}, ACh and the two agents in combination suggests that at least in synaptosomes the reversal of the reaction of CDP-diglyceride:inositol transferase in not a rate limiting step which is accelerated by neurotransmitters.

2. Methods

Synaptosomes were prepared from cerebra of 4 young adult Sprague-Dawley rats (Charles River) as in [6] except that Ficoll was purified by deionization of a 40% solution with mixed-bed ion exchange resin (Amberlite MB-3). The basic incubation medium consisted of the following in the indicated mM concentrations: NaCl, 138; KCl, 5; $MgSO_4$, 2; $CaCl_2$, 1.3; $MnCl_2$, 0.001; sodium phosphate, 2; Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5, at pH 7.4. This buffer was equilibrated with O_2:CO_2 (95:5) at 0°C.

Synaptosomal suspension (3 ml) was placed in each of 8 flasks, providing duplicate incubations of 4 conditions for each experiment. Malate (1 mM), pyruvate (2 mM) and cytidine (1 mM) were added and the flasks were incubated at 37°C in a shaker bath. After a 10 min pre-incubation agents were added as indicated and the experiment was started by addition of 20–50 μCi carrier free $^{33}PO_4$ and 5 μCi [^{3}H]inositol (final con. 0.02 mM). The incubation was stopped by pipetting 2 ml suspension into 5 ml cold water. An additional 5 ml water was added and membranes collected by centrifugation at 40 000 × *g* for 20 min. Protein was determined on the remaining suspension [7].

The membrane pellet was resuspended in methanol: water 1:1 and extracted with methanol, chloroform and a 1:1 mixture of these using a glass/Teflon homogenizer (A. H. Thomas). Monogalactosyl diglyceride was added as an internal standard. The pellet after centrifugation was reextracted with acidified

solvents and the extract was washed with 50 mM HCl. Deacylation and recovery of the glycerophosphoryl esters was performed essentially according to [8], except that the Dowex columns were eluted with 3 ml water and the eluants were neutralized with pyridine prior to evaporation with nitrogen. Trimethylsilyl-derivatives were formed by stirring overnight with dimethylformamide:bis(trimethylsilyl)trifluoroacetamide: trimethylchlorosilane (1:1:0.2). Gas–liquid chromatography was performed on a Perkin-Elmer 3920 with glass-lined injection port and a 1:15 stream splitter post column. A 3 ft X 4 mm glass column packed with 3% OV-1 on 100/120 Gas Chrom Q was held at 160° C for 4 min, then raised to 300° C at 8° C/min. Peaks of interest were collected at the stream splitter and radioacitvity determined by liquid scintillation counting. Peak areas were determined by electronic integration (Minigrator). Each sample was run 2 times.

Substrates, acetylcholine, and eserine were from Sigma Chemical Co. Lipid standards from PL Biochemicals were purified by preparative thin-layer chromatography. Glass-distilled solvents from Burdick and Jackson were stored under nitrogen. Other chemicals were reagent grade.

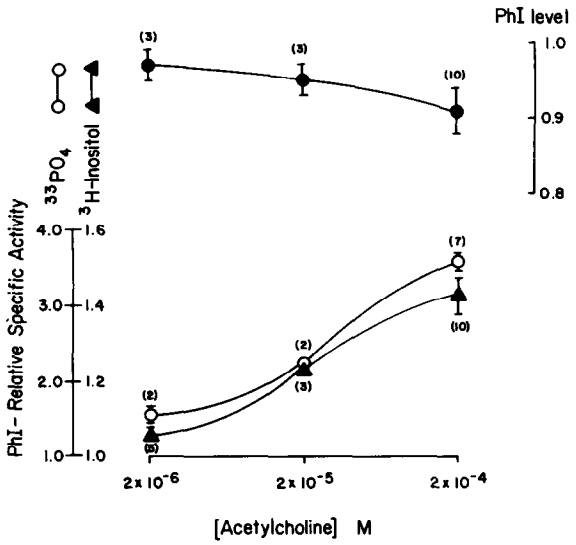

Fig.1. The effect of acetylcholine on synaptosomal PhI. Synaptosomes were incubated for 1 h with the indicated concentrations of ACh and 0.2 mM eserine sulfate. PhI levels were calculated as nmol/mg protein and specific radioactivities as dpm/peak area in arbitrary units. Values for the control samples were defined as 1, and the values of other samples are expressed relative to these. Numbers in parentheses indicate the number of experiments performed. Bars indicate the standard error of the mean except where $n = 2$, in which case the range is given.

3. Results

Our analysis of glycerophosphate esters derived from phospholipids allows the simultaneous determination of PhI levels and specific radioactivity (dpm/peak area) of PhI, diacyl-glycerophosphorylethanolamine (PhE) and phosphatidyl serine (PhS). The effect of ACh on synaptosomal PhI is shown in fig.1. Labelling by both $^{33}PO_4$ and [^{3}H]inositol was stimulated by ACh with the effect on both isotopes rising roughly in parallel. Note however, the difference in the magnitude of the effects. While inositol incorporation is stimulated maximally by 40–50%, phosphate radioactivity was increased 3–4-fold by 0.2 mM ACh. The dose relationship of this response was similar to that in [9,10] for synaptosomes, however, the magnitude of the stimulation of phosphate incorporation was somewhat greater in our hands. This and the decrease in PhI levels at high ACh concentrations [1] may be attributable to the greater purity and metabolic integrity of synaptosomes obtained from a 5-step Ficoll gradient.

Control levels of PhI for 10 expt were 11.52 ± 0.32 nmol/mg protein. In order to assess the specificity of the ACh effect on PhI metabolism, the phosphate incorporation into PhE and PhS was also determined. Peaks for these compounds were collected together. Specific radioactivity of phosphate did not differ from control values at any dose of ACh. At 0.2 mM ACh the value was 1.03 ± 0.07 times control.

Addition of $MnCl_2$ to the incubation medium had a striking concentration-dependent effect on labelling of PhI by inositol (fig.2). The specific radioactivity was doubled at 50 μM (not shown) and was increased nearly 20-fold at 1 mM $MnCl_2$. PhI levels were decreased slightly at higher Mn^{2+} concentrations. In contrast, phosphate labelling of PhI was only slightly affected (15%) at all Mn^{2+} concentrations tested. Phosphate labelling of PhE and PhS was even less affected (1.03 ± 0.04 at 1 mM $MnCl_2$), indicating a specific effect on PhI. Inositol transport into synapto-

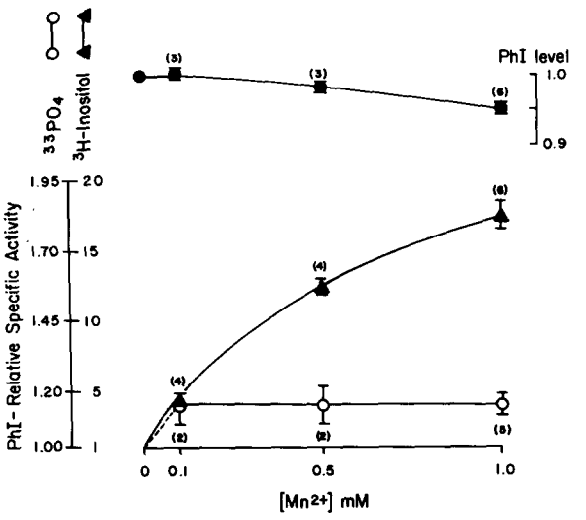

Fig.2. The effect of Mn^{2+} on synaptosomal PhI. Conditions as in fig.1.

somes [11] was not affected by 0.5 mM $MnCl_2$ (S.-M. Hwang, personal communication).

A comparison of the effects of both ACh and Mn^{2+} is presented in fig.3. The stimulation by Mn^{2+} of inositol incorporation into PhI was far greater than that

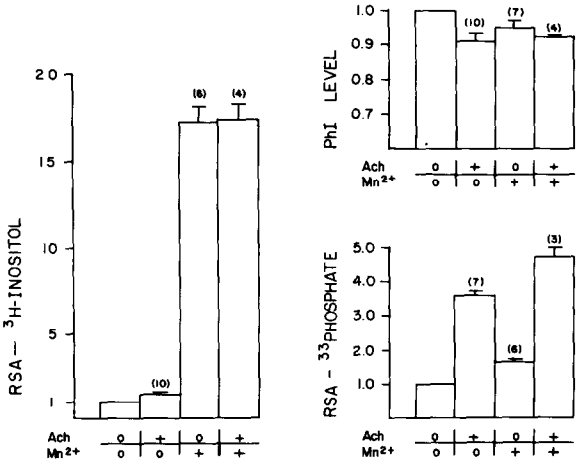

Fig.3. Comparison of the effects of ACh and Mn^{2+} on synaptosomal PhI. Parameters were calculated as in fig.1. Acetylcholine when present was at 0.2 mM; Mn^{2+} was $MnCl_2$ at 1 mM. Bars indicate the standard error of the mean for the number of experiments in parentheses. RSA = relative specific activity relative to control samples with neither agent.

produced by ACh. At lower Mn^{2+} concentrations (not shown) the ACh-stimulated inositol labelling parallels that found in the absence of ACh as shown in fig.2. Thus, the percentage of stimulation by ACh steadily decreases as the Mn^{2+} concentration is raised.

The major effect of ACh on PhI metabolism is on its labelling by phosphate. There is a less striking effect of Mn^{2+} on this parameter as well. It is of interest to the mechanism of ACh-induced turnover of PhI that addition of ACh in the presence of 1 mM $MnCl_2$ produced a 4.1-fold stimulation of phosphate incorporation compared to a 3.6-fold stimulation in control (1 μM Mn^{2+}) samples. This effect was also seen at 0.5 mM $MnCl_2$, but was not apparent at 0.1 mM. Again the effect appears to be specific to PhI, since the specific radioactivity of the peaks for PhE and PhS was 1.16 ± 0.10-times control in the presence of both agents. The decrease in PhI levels seems to be a result primarily of the action of ACh.

4. Discussion

We interpret the effects of $MnCl_2$ on the inositol labelling of PhI as the specific stimulation of an enzyme or enzyme system which accomplishes the exchange of free inositol into the phospholipid. That this does not reflect an overall stimulation of phospholipid metabolism is shown by the lack of effect on labelling of PhE and PhS. The turnover of PhI induced by Mn^{2+} does not involve a diglyceride intermediate, since phosphate labelling of PhI is only slightly increased. This also argues against Mn^{2+} acting to release a neurotransmitter which is 20-times as active as ACh, since this should produce an equally large stimulation of phosphate labelling. That synaptosomes exposed to these concentrations of $MnCl_2$ remain intact and metabolically active is shown by the lack of effect on inositol transport, and by the ability of these synaptosomes to respond to ACh with phosphate labelling.

The most likely site of action for Mn^{2+} is at the enzyme CDP-diglyceride:inositol transferase which may act to reverse the synthetic reaction [2,5] or at a separate Mn^{2+}-requiring exchange enzyme [3]. Our data then indicate that this enzyme normally operates at only a fraction of its maximal capacity. This enzyme might then be a candidate for the rate-limit-

ing step in the neurotransmitter-stimulated turnover of PhI [5], with the decreasing stimulation of inositol incorporation by ACh at increasing $MnCl_2$ concentrations reflecting a common site of action for these two agents. However, the ACh-induced stimulation of phosphate incorporation into PhI is not impaired at high Mn^{2+} concentrations, suggesting that the rate limiting step which is stimulated by transmitter involves production of a diglyceride intermediate rather than of CDP-diglyceride. This intermediate might arise by hydrolysis of PhI or of CDP-diglyceride, or from phosphatidic acid (PhA) as has been suggested [9,12]. Stimulation of phosphate incorporation into PhI by Mn^{2+} may indicate a preference for this metal cofactor by cytidyl transferase [13].

While it is commonly accepted that in most tissues increased labelling of PhI occurs as a consequence of PhI breakdown, it has been proposed [9,12] that a different mechanism, involving a primary effect on PhA hydrolysis, produces the 'phospholipid effect' in synaptosomes exposed to neurotransmitters. This is based in part on the greater effect on phosphate labelling of PhI relative to inositol in synaptosomes exposed to neurotransmitter [9]. This result, which we confirm, is difficult to reconcile with a primary effect of neurotransmitter on PhI breakdown. It is known that neither ATP specific radioactivity [9] nor inositol transport [11] are affected by neurotransmitters, therefore resynthesis of PhI from diglyceride should show the same percentage of stimulation for both isotopes. However, our studies suggest that inositol exchange into PhI may result in a basal incorporation of inositol into PhI which is much greater than its cyclical breakdown and resynthesis through a diglyceride intermediate. An effect of neurotransmitter on diglyceride production in synaptosomes would then be expected to produce a much greater increment of phosphate labelling of PhI relative to inositol incorporation through a cyclical pathway of resynthesis.

Acknowledgement

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