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GUANINE NUCLEOTIDES STIMULATE HYDROLYSIS OF PHOSPHATIDYL INOSITOL BIS PHOSPHATE IN HUMAN MYELIN MEMBRANES

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SUMMARY: Phosphodiesterase activity was stimulated in myelin membranes in the presence of guanine nucleotide analogues. This activity was reduced in myelin membranes which had been adenosine diphosphate ribosylated in the presence of cholera toxin which ADP-ribosylated three proteins of Mr 46,000, 43,000 and 18,500. Aluminum fluoride treatment of myelin had the same stimulatory effects on phosphodiesterase activity as did the guanine nucleotides.

INTRODUCTION: Myelin is a specialized multilammelar membrane system which facilitates saltatory conduction of impulses along nerve axons. Although myelin has been considered an electrical insulator, it is now viewed in a different way owing to the discovery of enzymes involved in posttranslational modifications, reutilization of myelin components and signal transduction (for review see ref. 1). The recent discovery of the stimulation of phosphoinositide hydrolysis by muscarinic agonist further indicates that myelin is a metabolically dynamic membrane (2,3). In brain tissues, phosphoinositides appear to be located predominantly in myelin (4) and a phosphodiesterase activity has been identified in this specialized membrane (2,5).

The catabolism of the phosphoinositides phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP), is catalyzed by phosphatidylinositol (PI) phosphodiesterase(s) (PLC) (EC 3.1.4.10) and phosphomonoesterases probably found in all animal tissues (6). Phosphodiesterase action on PIP_2 results in the formation of diacylglycerol (DG) and water soluble inositol 1,4,5-trisphosphate (IP_3), and DG and inositol 1,4-bisphosphate (IP_2) if formed from PIP. The IP_3 may then act as a messenger to release Ca^{2+}, and the DG may stimulate protein kinase C (7). Recent evidence in a variety of cells has suggested that receptors are coupled to PLC via a guanine nucleotide binding regulatory protein (G-protein) (8,9,10). Modification of G-proteins with adenosine diphosphate ribose, by cholera toxin reduces their stimulatory activity on their effectors (11).

Myelin has been shown to contain large amounts of the substrates for enzymes which are known to be involved in signal transduction via the inositol lipid pathway. Furthermore it has been suggested that phosphorylation, via protein kinase C, and dephosphorylation of the cytoplasmic myelin basic protein occurs in response to an external electrical impulse (12). In this paper, we demonstrate that aluminum fluoride, and analogues of guanine nucleotides e.g. GTPγS

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stimulate the PLC activity of myelin, while ADP-ribosylation interferes with the GTPγS effect although it is not completely abolished.

MATERIALS AND METHODS

Preparation of myelin

Myelin was prepared from white matter of normal human brain according to the procedure of Lowden *et al* (13). Briefly, 35g of white matter was processed through two sucrose gradient ultracentrifugation steps. The myelin banded at the boundary phase between the 0.25 M and 0.88 M sucrose was subjected to water shock and centrifugation to remove the axolemma. After a third sucrose gradient centrifugation step, the purified myelin was washed several times with water and stored at -20°C.

PI phosphodiesterase assay

PLC activity was assayed in duplicate at 37°C, in a final volume of 0.5 mL, containing (unless otherwise noted) 50mM Tris-Cl, pH 6.8, 1μM $MgCl_2$, 10μM $CaCl_2$, 0.1% deoxycholate and ~5000 cpm [^{3}H]PIP_2. Assays were initiated by the addition of 300-500 μg of myelin protein to tubes which contained the above reagents in the presence or absence of guanine nucleotides or aluminum fluoride. Incubations were terminated by the addition of 0.5 mL ice cold chloroform/methanol (1:2 vol/vol). After 10 min. on ice the [^{3}H]inositol phosphates were separated from the [^{3}H]phospholipids by the addition of 0.5 mL chloroform containing 0.5% 1N HCl. Tubes were vortexed and centrifuged for 5 min. at 9000 x g to separate the phases and aliquots from each phase were measured for radioactivity. Blank assays were performed by the addition of myelin proteins after chloroform/methanol addition. Identification of products in the aqueous phase was by paper chromatography using Whatman 3MM paper. The solvent system employed was methanol, 88% formic acid, water (16:3:1, v:v:v). Product determination was by the silver stain method of Benson *et al* (14).

Cholera toxin treatment

Cholera toxin (List Biological) was preactivated by incubation with 20 mM dithiothreitol (DTT) and 2.5 mM potassium phosphate, dibasic, pH 7.5, as described by Bokoch *et al* (15) for 30 minutes at 30°C. Incubation of 600 μl of myelin membranes (3 mg protein) with cholera toxin contained 10 mM thymidine, 1mM ATP, 100 μM GTP, 2.5 mM $MgCl_2$, 1mM EDTA, 1mM DTT, 400 μM ADP-ribose and 10 μM [^{32}P]NAD^+ (0.5 μCi/assay), 100 μg cholera toxin, 100mM potassium phosphate buffer, pH 7.5. In all experiments controls contained an equivalent volume of the preincubation buffer without the toxin. After a 30 minute incubation, a 200 μL aliquot was used to carry out the PI phosphodiesterase assay as described above. To identify the proteins which were ADP-ribosylated, the incubation was carried out for 90 minutes at 30°C. The reaction was terminated by the addition of 1 mL 10% polyethylene glycol 8000 (PEG 8000). The membranes were then washed five times, 1 mL each time, with H_2O and solubilized in gel sample buffer containing 2% (wt/vol) sodium dodecylsulfate and 2% (vol/vol) 2-mercaptoethanol. Solubilized membranes were heated in boiling water for 5 minutes and subjected to electrophoresis on a 12.5% (wt/vol) polyacrylamide slab gel. The dried gel was exposed to Kodak X-Omat R film for 24 hours.

RESULTS

The effect of different nucleotides on [^{3}H]PIP_2 hydrolysis

The effect of ATP, GDP and GTPγS on the hydrolysis of [^{3}H]PIP_2 is shown in Figure 1. When 10 μM GTPγS was added either at zero time (curve 1) or 5 min after beginning the incubation (curve 2), a large stimulation of [^{3}H]PIP_2 hydrolysis was observed, from 6% of the initial radioactivity to a maximum of 40% after 15 min of incubation. In the absence of added nucleotides a much smaller increase in [^{3}H]PIP_2 hydrolysis was observed (curve 5), representing the endogenous level of activity of the phosphodiesterase in myelin. Neither 10 μM GDP nor 10 μM ATP increased the [^{3}H]PIP_2 hydrolysis over the endogenous level (curves 3 and 4). The hydrolysis product, IP_3, was identified by paper chromatography as described in Materials and Methods. Hydrolysis of [^{3}H]PIP was also observed upon addition of 10 μM GTPγS to myelin

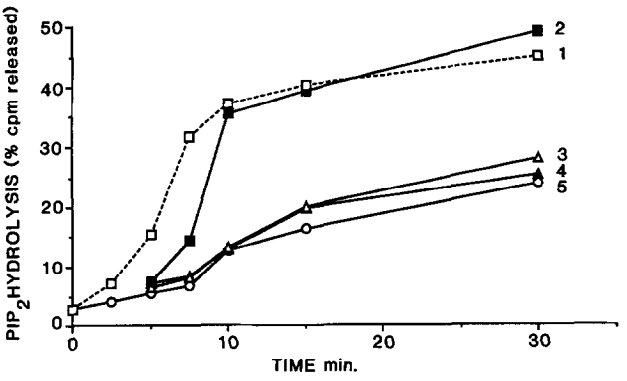

Fig. 1 Time course of phosphodiesterase activity in myelin membranes. PLC activity was assayed as described in the Methods section with [^{3}H]PIP_2 as substrate. Water (○), 10 μM-ATP (▲), 10 μM-GDP (△) or 10 μM-GTPγS (—□—) was added at 0 time or 5 min (—■—). The radioactivity in the aqueous phase was expressed as a percentage of the total cpm; % cpm in aq. phase = cpm in aq.phase/(cpm in aq. phase + cpm in organic phase).

membranes. However, this stimulation was about 25% of that observed for [^{3}H]PIP_2 in the presence of the same guanine nucleotide analogue (data not shown).

The effect of AlF_4 on [^{3}H]PIP_2 hydrolysis

Activation of PI phosphodiesterases in myelin by GTPγS suggested the involvement of a G protein. A second characteristic of G proteins is activation by F^- in the presence of Al^{3+}. The addition of increasing amounts of $Al(NH_4)SO_4$ to 5mM KF resulted in an increase in [^{3}H]PIP_2 hydrolysis by the myelin suspension (Fig. 2). KF (5mM) alone did not have a significant effect on the hydrolysis of [^{3}H]PIP_2. In the presence of either increasing amounts of $Al(NH_4)SO_4$ or $Al(NH_4)SO_4$ with 5 mM KCl, a significant decrease of activity was observed. These data (Fig. 1 and 2) suggest that a G protein is involved in the effects observed.

The effect of ADP-ribosylation on the activity of PI phosphodiesterase

Freshly isolated human myelin was incubated with cholera toxin and NAD^+ under ribosylating conditions. The effect of GTPγS on the hydrolysis of [^{3}H]PIP_2 in ADP-ribosylated membranes is shown in Figure 4B. There is a two fold increase in the hydrolysis of [^{3}H]PIP_2 over that of ADP-ribosylated membranes incubated under identical conditions but in the absence of GTPγS (Fig. 4A). The effect of GTPγS on the hydrolysis of [^{3}H]PIP_2 in non-ADP-ribosylated membranes is shown in Figure 4C. This effect was twice that observed in Figure 4B and four times that of Figure 4A. Extent of hydrolysis in the absence of toxin is the same as that shown in Figure 4A. Therefore, ADP-ribosylation of myelin membranes decreases the stimulatory effect of GTPγS.

Identification of ADP-ribosylated myelin proteins

As G proteins are known to be ADP-ribosylated by either cholera or pertussis toxins, or both (11), and since it has been reported that cholera toxin ADP-ribosylates isolated myelin basic protein (16) and several rat myelin proteins *in situ* (17), it was attempted to detect proteins in human myelin which were ADP-ribosylated by the cholera toxin. Freshly isolated myelin was ADP-ribosylated with cholera toxin as described in Methods. The Coomassie blue-stained SDS polyacrylamide gel

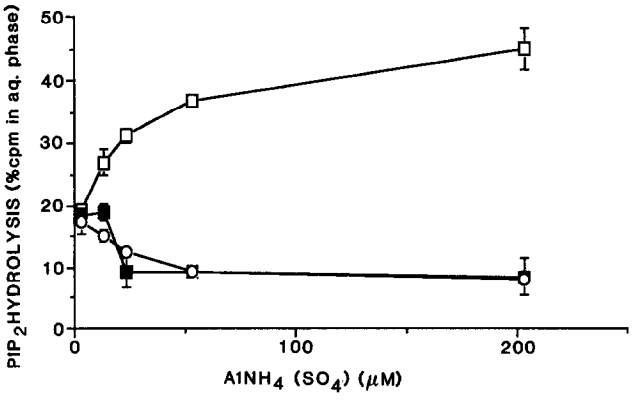

Fig. 2 The effect of fluoride on [^{3}H]PIP_2 hydrolysis in myelin membranes. Assays were performed as described in Methods. 5 mM-KF (□), 5 mM-KCl (■) or water (○) were added to myelin membranes which contained increasing concentrations of $AlNH_4(SO_4)$. PLC activity was assayed as described in Materials and Methods.

and corresponding radioautograph is shown in Figure 3. Proteins of M_r 46,000, 43,000 and 18,500 were extensively ADP-ribosylated by cholera toxin and [^{32}P]NAD^+ (Fig. 3A). The corresponding Coomassie blue stained gel is shown in Figure 3C. The Wolfgram doublet is

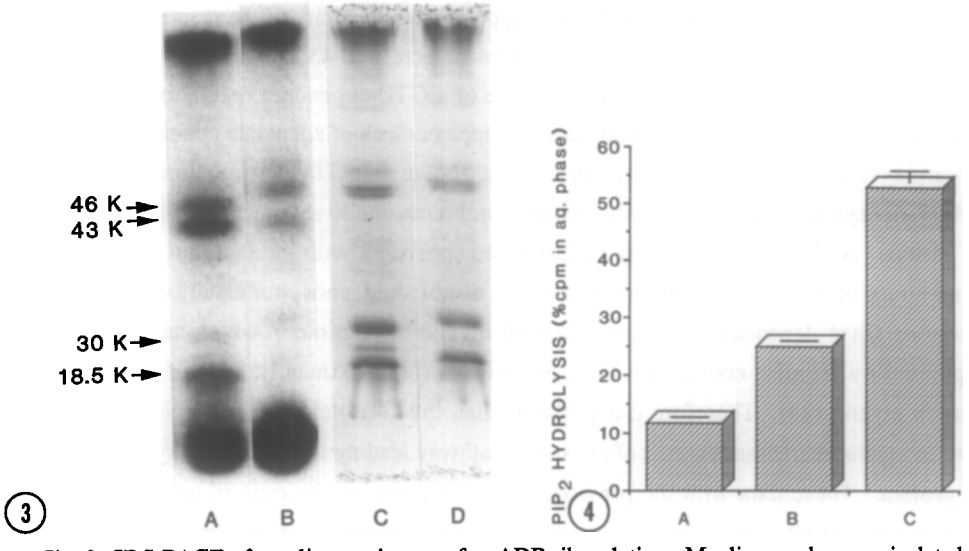

Fig. 3 SDS-PAGE of myelin membranes after ADP-ribosylation. Myelin membranes , isolated from human brain, were incubated with cholera toxin and [^{32}P]NAD^+ and then analyzed by SDS-PAGE on 12.5% acrylamide gels. The figure shows the autoradiograms of ADP-ribosylated (A) and non-ADP-ribosylated (no cholera toxin added) (B) myelin membranes, and their respective Coomassie blue stained SDS gel patterns (C & D).

Fig. 4 The effect of GTPγS on [^{3}H]PIP_2 hydrolysis by PLC in myelin membranes. 10 μM-GTPγS was added to ADP-ribosylated (B) and non-ADP-ribosylated (C) myelin membranes. [^{3}H]PIP_2 hydrolysis was assayed as described in Methods. Results were compared with PLC activity assayed in ADP-ribosylated myelin membranes in the absence of GTPγS (A).

clearly observed at 46 kDa as two sharp bands (Fig. 3C), which were ADP-ribosylated as seen in the autoradiograph (Fig. 3A). The radioactive band at 43 kDa does not correspond to a Coomassie blue-stained component. The proteolipid protein (PLP) is seen as a 30 kDa band on the Coomassie blue stained gel (Fig. 3C) but a radioactive band was not found on the autoradiograph (Fig. 3A). Therefore PLP is not a substrate for ADP-ribosylation by cholera toxin. Noteworthy, MBP was ADP-ribosylated (Fig. 3A & C). The sharp band on the Coomassie blue stained gel between 30 kDa and 18.5 kDa (Fig. 3C), is not present in Figure 3D which represents myelin incubated with [^{32}P]NAD$^+$ in the absence of toxin. Therefore this band was ascribed to the toxin. The radioautograph of this gel showed a small amount of non specific labelling in the 43-50 kDa range.

DISCUSSION: There presently exists a significant amount of evidence to support the theory that a G-protein is involved in receptor mediated activation of PLC, even though the identity of this signal transducer remains unkown (rev. 18 & 19). Our results show that in normal human myelin, both GTPγS and AlF_4 stimulated the phospholipase C activity as determined by the hydrolysis of [^{3}H]PIP_2 , suggesting that a G-protein was involved in this process. Studies of several other cell types have also demonstrated increased polyphosphoinositide breakdown and IP_3 release from endogenously labelled membranes incubated with guanine nucleotides (8-10, 20, 21). ADP-ribosylation of myelin membranes has identified several substrates for the cholera toxin mediated reaction of M_r 46,000, 43,000 and 18,500. No ADP-ribosylation of the proteolipid proteins, lipophilin and DM-20 was observed (Fig. 3), since a radioactive band was not detected at a M_r 30,000 and 24,000 respectively. Since the proteolipids account for about 50% of the total myelin protein, this lack of ADP-ribosylation, supports the specificity of the reaction of the myelin basic protein, Wolfgram doublet and the yet to be uncharacterized 43 kDa protein.

Tamir and Gill (22), have shown the presence of a GTP-dependent regulatory component of adenylate cyclase in myelin from rat brain despite apparent lack of adenylate cyclase activity in this membrane system. In this communication we have shown that GTPγS stimulates PLC activity in both ADP-ribosylated and non ADP-ribosylated membranes and to a greater extent in the latter than in the former. A G-protein regulating PLC is more consistent with the fact that myelin has very high amounts of inositol phospholipids and an established phosphodiesterase activity (2,5). Futhermore, Ca^{++}-dependent phosphorylation of myelin basic protein, which has been shown to be regulated by impulse conduction (23), accounts for approximately 80% of the total kinase activity in myelin (24). Therefore it is probable that the G-protein regulated phosphodiesterase activity in myelin membranes is part of the signal pathway leading to the phosphorylation of myelin basic protein. These data with the previously reported specific binding of azido-GTP to MBP (16), implicates MBP in some manner as part of a signal transduction system in myelin. Further studies are required to elucidate its specific role and to account for the ADP-ribosylation of proteins in the 43-46 kDa range on the SDS gels.

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