Vol. 147, No. 3, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1987 Pages 1251-1258

PHOSPHORYLATION OF PHOSPHATIDYLINOSITOL ASSOCIATED WITH THE NICOTINIC ACETYLCHOLINE RECEPTOR OF TORPEDO CALIFORNICA

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Received August 21, 1987

When isolated, detergent solubilized and affinity chromatographically purified nicotinic acetylcholine receptor of Torpedo californica electric organ is incubated with $[\gamma\text{-}^{32}P]ATP/Mg^{2+}$, phosphatidylinositol 4-phosphate (PIP) is formed from receptor associated phosphatidylinositol (PI). This receptor associated endogenous kinase activity is enhanced by orthovanadate and, remarkably, also by acetylcholine. Exogenously added PI-kinase only increases the phosphorylation rate if vanadate is present. PIP as the main phosphorylation product (up to 95%) remains bound to the ß-, γ - and δ-subunits of the receptor and to the receptor associated ν - protein. The α-subunits do not carry ^{32}P phosphate; no phosphatidylinositol 4,5-bisphosphate formation has been observed. Concomitant to lipid phosphorylation tyrosine and serine residues are phosphorylated (5% of total incorporated ^{32}P phosphate).

Transmembrane signalling is frequently mediated by transmitter/hormone membrane receptors mobilizing Ca^{2+} fluxes. The functionally important changes in the intracellular Ca^{2+} level appear to be initiated in the plasma membrane by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) yielding inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG). IP_3 has been shown to be involved in Ca^{2+} release from intracellular stores and DG activates the phospholipid sensitive protein kinase C (for review see ref.1).

The oligophosphoinositides and phosphatidylinositol (PI) strongly interact with membrane proteins and may thus be considered as lipophilic membrane localized effectors rather than just metabolites [2]. Structurally, PI is tightly bound to, and is particularly effective in preserving the stability, of the nicotinic

0006-291X/87 $1.50

acetylcholine receptor (nAcChR) [3], a neurotransmitter dependent, rapid ion channel protein [4].

When plasma membrane fragments of Torpedo japonica electrocytes, which are rich in the nAcChR were incubated with [γ-^{32}P]ATP, the formation of phosphatidyl-inositol 4-phosphate (PIP) and of PIP_2 has been observed indicating the presence of lipid kinase activities [5,6].
In contrast to the muscarinic AcChR known to mobilize Ca^{2+} fluxes by PIP_2 hydrolysis [7], a possible relationship between oligophosphoinositide formation and nAcChR function has not been clarified.

The receptor rich membranes of fish electric organs have been shown to contain endogenous cAMP dependent protein kinase [8,9], Ca^{2+}/calmodulin dependent protein kinase [10], Ca^{2+}/phospholipid dependent protein kinase [11] and tyrosine specific protein kinase activities [12]. The nAcChR protein is directly phosphorylated by these kinases on seven different phosphorylation sites [12].

In this study we demonstrate that the isolated, detergent solubilized and affinity chromatographically purified nAcChR of Torpedo californica electric organ carries endogenous, acetylcholine sensitive, kinase activity leading to phosphorylation of receptor associated PI to PIP. The PIP formed remains bound to the nAcChR subunits and the receptor associated ν-protein. Since the ν-protein is suggested to connect the nAcChR with the cytoskeleton [4], phosphorylation may be an important regulatory factor in the coupling between the membrane bound receptor and cytoskeletal elements.

Materials and Methods

[γ-^{32}P]ATP was synthetized from ortho-[^{32}P]phosphate (carrier free, NEN) and Boehringer-Mannheim reagents [13]. PI, PIP and PIP_2 (Sigma) were used as standards. [^{125}I]α-Btx (α-Btx) from NEN was applied to determine the nAcChR concentration as described in [14]. Protein concentration was determined according to Lowry et al. [15]. Thin layer chromatography (TLC) was carried out on oxalate treated Silicagel plates (Merck) according to [16]. Two dimensional thin layer (2D-TL) electrophoresis was performed with the FBE 3000 Pharmacia flat bed apparatus. SDS polyacrylamide gel electrophoresis was carried out as described in [17].

Phosphorylase kinase was prepared according to [18] as modified in [19] . This Ca^{2+}/calmodulin dependent protein kinase exhibits, as side activity, PI kinase activity ; the specific lipid kinase activity associated with the phosphorylase kinase preparations is about 1 nmol min^{-1} mg^{-1} [20,21]. In the experiments presented in this paper phosphorylase kinase has been used as exogenously added PI kinase.

2.1. Membrane fragments of Torpedo californica electric organ, rich in nAcChR, were prepared according to [14] . The specific activity was 0.8 - 1.1 nmol Btx/mg protein; the protein concentration varied between 1-2 mg/ml; residual acetylcholinesterase activity [22] was 3-10 umol min^{-1} mg^{-1}, assayed in 0.05 % Triton X 100 [14] .

2.2. Isolated nAcChR protein was prepared according to [14] ; the specific activity in the presence of detergent was 6 nmol α-Btx/mg, corresponding to about 2 moles α-Btx bound to one mole of receptor protein ($M_r \approx 300,000$); the protein concentration was 1 mg/ml. For the phosphorylation studies nAcChR was concentrated to ca. 3 mg protein/ml in 70 % glycerol, 10 mM Pipes buffer (pH 6.8), 0.02 % Lubrol WX.

The standard phosphorylation mixture, 200 ul, finally contained 1 mM DTE, 100 mM KCl, 100 mM Tris-HCl, 10 mM EDTA, 1 mM EGTA, 10 mM [γ-^{32}P]ATP, pH 7.5, 0.22 - 0.31 mg protein; 300 uM orthovanadate, if indicated. (Endogenous ATPases of the membrane fragments hydrolyses ATP; to keep the ATP/Mg^{2+} concentration optimal for the lipid kinase during the whole incubation time of 3 hours a relatively high ATP/Mg^{2+} concentration has been chosen). The mixture was preincubated at 30° C for 2 min; after 1 min aliquots of 15 ul were taken to determine the radioactivity bound unspecifically. At the second minute the phosphorylation was started by addition of 20 mM $MgCl_2$ yielding 1.2 mM free Mg^{2+} and 1.6 nM free Ca^{2+}. The free metal ion concentrations were calculated using the stability constants from [23] . Bound radioactivity was assayed according to [24] . PIP was extracted from the phosphorylated protein as described in [24] with the following slight modifications: the protein after phosphorylation was precipitated by perchloric acid at a final concentration of 7% in the presence of 6-7 mg ml^{-1} bovine serum albumine and centrifuged for 5 min at 5,000 rpm. The precipitate was washed three times with 10% perchloric acid and finally with water. The precipitate was dried in vacuo and preextracted with chloroform/methanol (2:1, v/v). Then the precipitate was extracted with chloroform/methanol/concentrated HCl (40/20/1, v/v). The extract was dried under nitrogen stream and resolubilized in chloroform/methanol/water, (75/25/2, v/v) for TLC. 2D-TL electrophoresis of acid hydrolysate of receptor has been performed as described in [24] except that the protein was hydrolyzed in 6 N HCl for 6 hours and the sample was not filtered.

Results

In Fig. 1 it is shown that the isolated detergent solubilized and affinity chromatographically purified nAcChR (protein/lipid particles, molar ratio protein/lipid 1:40 [3]) incorporate ^{32}P phosphate from [γ-^{32}P]ATP either by endogenous kinase activity or catalyzed by added exogenous kinase. The initial rate of the endogenously catalyzed ^{32}P incorporation into the receptor/lipid particles in the absence of orthovanadate (VO_4^{3-}) is 1.7 ± 0.2 pmol min^{-1} mg^{-1}. If 0.3 mM of VO_4^{3-} is present, the initial phosphorylation rate is increased by

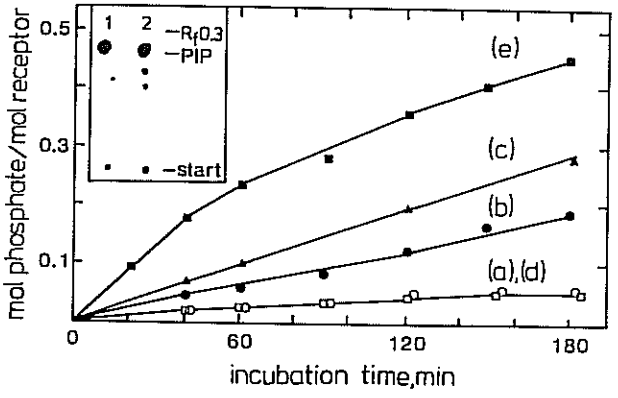

Figure 1.
Phosphorylation of the isolated, detergent solubilized, nAcChR of Torpedo californica.(a) ○, endogenous kinase activity in absence and presence of 0.5 mM Mn^{2+}; (b) ●, enhancement by 0.3 mM VO_4^{3-}; (c) ▲, further enhancement by 20 µM AcCh in presence of 0.5 mM Mn^{2+}; (d) □, as in (a) with exogenously added PI kinase; (e) ■, as in (d) in the presence of 0.3 mM VO_4^{3-}. Phosphate incorporation contributed by autophosphorylation of the exogenously added kinase was substracted to give the values shown. Inset: Identification of the labelled phospholipid formed after phosphorylation by (1) endogenously present and by (2) exogenously added PI kinase both in the presence of VO_4^{3-}. For experimental details see Materials and Methods.

a factor of two. When exogenous kinase is added in the absence of VO_4^{-3} no additional ^{32}P incorporation occurs. However, in the presence of VO_4^{3-} the exogenous kinase leads to an about 10-fold increase of the ^{32}P incorporation rate.

It is remarkable that the presence of the natural neurotransmitter acetylcholine (AcCh, 20 µM) increases the initial endogenous phosphorylation rate about two-fold compared to the rate in the presence of VO_4^{3-}.

Fig.2 a shows the result of the SDS polyacrylamide gel electrophoresis of nAcChR after endogenous phosphorylation in the presence of VO_4^{3-}. The receptor subunits ß, γ, δ and the receptor associated ν-protein, but not the α-subunits, are labelled with ^{32}P phosphate under our experimental conditions.

After phosphorylation in the presence of VO_4^{3-} approximately 95 % of the receptor bound radioactivity can be extracted by chloroform/methanol/conc.HCl

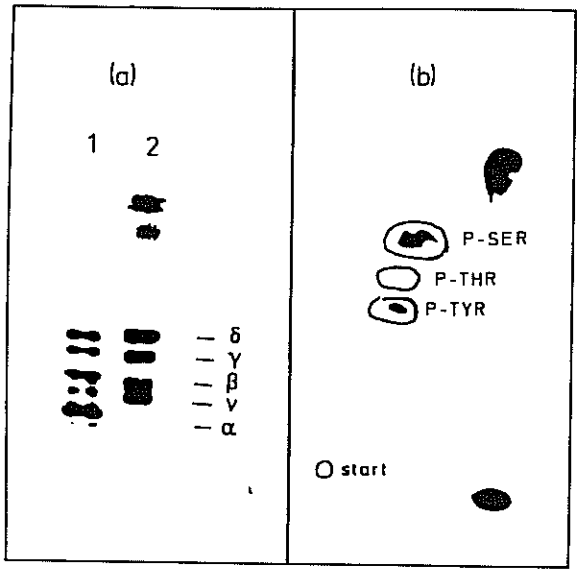

Figure 2.
(a) SDS polyacrylamide gel electrophoresis of the isolated nAcChR after phosphorylation by endogenous kinase. (1) Coomassie blue staining of the α_{32}, β-, γ- and δ-subunits and of the receptor associated ν_{32}-protein; (2) ^{32}P autoradiography of the separated subunits. (b) 2D-TL ^{32}P autoradiogram of the autophosphorylated (in the presence of VO_4^{3-}) receptor amino acids after lipid extraction; there is no ^{32}P phosphothreonine.

(100:100:0.6,v/v), suggesting that the main phosphorylation product is of lipidic character. Actually, TLC reveals that the main radioactively labelled product is PIP; no PIP_2 has been found (Fig.1 inset). The other radioactive compounds are lysoforms, artificial products of the acidic extraction procedure. The phosphorylated protein after lipid extraction ($\simeq$ 5% of the total bound radioactivity) has been subjected to partial acid hydrolysis in the presence of 6N HCl. Autoradiography following separation of the liberated amino acids shows phosphoserine and phosphotyrosine as the only labelled amino acids (Fig.2b). No indications have been found for enhanced serine and/or tyrosine phosphorylations in presence of VO_4^{3-}.

As is the case of the isolated nAcChR receptor rich membrane fragments (microsacs) also show ^{32}P incorporation, sensitive to VO_4^{3-} and AcCh. Here also 95% of the membrane bound radioactivity is PIP (data not shown).

Discussion

It has been previously shown that nAcChR rich membrane fragments of Torpedo japonica electric organ exhibit endogenous PI and PIP kinase activities [5,6]. These lipid kinase activities are either associated with the nAcChR or, more likely, come from a minor contaminant of the membrane fragments.

The main result of our study is that in Torpedo californica it is the isolated, detergent solubilized and affinity chromatographically purified nAcChR, inclusively the receptor associated ν-protein, that carries the kinase activity. This intrinsic kinase activity is enhanced by VO_4^{3-} and reaches values of 2.5-3 pmol min^{-1} mg^{-1}. Most interestingly, the endogenous kinase activity is affected by the neurotransmitter AcCh, similar to the insulin sensitivity of the PI kinase activity associated with the insulin receptor [25]. Exogenous PI kinase in the presence of VO_4^{3-} enhances the ^{32}P incorporation into the AcChR to 12-15 pmol min^{-1} mg^{-1}. VO_4^{3-} presumably exposes PI to the AcChR, perhaps through a conformational change of the protein, comparable to the observation on the sarcoplasmic reticular Ca^{2+} transport ATPase [21]. The lower PI kinase activity (3 pmol min^{-1} mg^{-1}) of the isolated, detergent solubilized nAcChR compared to the membrane fragments (10 pmol min^{-1} mg^{-1}) may be due to protein modification by the unavoidable detergent treatment during isolation and purification. However, the measured PI kinase activity is also a function of the substrate concentration. Therefore, the higher PI kinase activity of the receptor rich membrane fragments in comparison to that of the isolated receptor presumably results from an enhanced PI availability rather than an increased amount of kinase.

Unlike to Torpedo japonica membrane fragments we have not detected any PIP_2 formation by Torpedo californica receptor associated kinase under our phosphorylation conditions. Either PIP kinase is missing or the amount of the produced PIP is far below the Km value of the PIP kinase. Concomitant to the PI phosphorylation tyrosine and serine kinase activities have been found in isolated nAcChR inclusively the receptor associated ν-protein. Recent observations of

Fukami et al. indicate that the tyrosine specific protein kinase in the nAcChR rich membranes of Torpedo japonica and the intrinsic PI kinase activity are different enzyme entities [26].

It is surprising that the receptor subunits heavily labelled with ^{32}P lose about 95% of their radioactivity after extraction with organic solvent. This observation indicates that oligophosphoinositides remain associated with proteins even after electrophoretic separation in presence of SDS. Therefore, care has to be taken in analyzing phosphorylation patterns of membrane proteins in gels.

At present it cannot be definitely decided whether the 5% remaining ^{32}P radioactivity after lipid extraction and the ^{32}P carrying hydrolysis products phosphoserine and phosphotyrosine result from autophosphorylated nAcChR and ν -protein. The ν -protein has been suggested to crosslink the $\alpha_2\beta\gamma\delta$ channel complex with cytoskeletal elements[4]. Therefore the phosphorylation of the ν -protein associated acidic phospholipid might be an important regulatory coupling factor between the membrane bound nAcChR and the cytoskeleton.

ACKNOWLEDGMENTS

We thank Professor Heilmeyer,L.M.G.jr. and Professor H.W.Chang for instructive discussions, P.Berth, M.Cochu, M.Preiß, U.Siemen, A. Tiemann for excellent technical assistance. We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (SFB 223/D3 to E.N.).

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