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# PROTEIN PHOSPHATASE ACTIVITY IN ACETYLCHOLINE RECEPTOR-ENRICHED MEMBRANES

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SUMMARY

Phosphorylation of the acetylcholine receptor (AChR)* has been demonstrated in AChR-enriched membranes prepared from the electric organ of Torpedo californica. In this report we show that the same AChR-enriched membrane fraction also contains phosphoprotein phosphatase activity which dephosphorylates both the endogenous AChR and exogenous phosphorylated casein. Release of [$^{32}$P] $PO_4$ from phosphorylated casein was shown to be inhibited by $F^-$ as well as GTP. cAMP and cGMP were without effect. Dephosphorylation of the membrane-bound AChR was also inhibited by $F^-$. This phosphorylation-dephosphorylation mechanism may play a role in mediating the function of the AChR at the synapse.

## INTRODUCTION

Neurotransmitters, growth factors, polypeptide hormones and mitogens may act through regulating the level of phosphorylation of one or more membrane proteins (1-3). Acetylcholine-receptor enriched membranes provide one of the best model systems to study reversible phosphorylation of membrane receptor proteins and the role of phosphorylation in regulating the response of membrane receptors to external signals. In this preparation the AChR constitutes over 80% of the protein in these receptor-enriched membranes (4). There are only three other major polypeptides present which appear to correspond to protein kinase, protein phosphatase and NaK-ATPase activities (Gordon et al., manuscript submitted for publication).

We have recently shown that the membrane-bound acetylcholine receptor (AChR) in receptor-enriched membranes from Torpedo californica is phosphorylated in situ by a membrane protein kinase (5). Moreover, phosphorylation

* Abbreviation: AChR, acetylcholine receptor; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-N, $N^1$-tetraacetic acid; DTT, dithiothreitol

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is specifically stimulated by 100 mM $K^+$ and inhibited by cholinergic ligands (2). Covalent modification of the receptor protein in situ may be related to the mechanism by which acetylcholine produces changes in the properties of the post-synaptic membrane. If phosphorylation of the AChR is an important regulatory event at the synapse, then dephosphorylation of the AChR must also occur in situ. In this report we demonstrate that AChR-enriched membranes contain significant protein phosphatase activity which can dephosphorylate both the endogenous phosphorylated AChR and exogenously added phosphorylated casein.

## METHODS

AChR-enriched membranes (d=1.14-1.17) were prepared from the electric organ of Torpedo californica as previously described (2).

Preparation of [$^{32}$P] $PO_4$-Casein. AChR-enriched membranes containing protein kinase activity were used to phosphorylate casein for use as a substrate for the phosphoprotein phosphatase. These membranes had been stored at -20° for 30 days. Phosphorylation of casein was carried out for 1 hr at 37° in a reaction mix of 2 ml containing 10 mg casein, 20 μCi [$\gamma$-$^{32}$P] ATP, 1 mg AChR-enriched membranes, 1 mM ouabain, 0.25 mM ethylgeneglycol-bis (β-aminoethyl ether)-N, $N^1$-tetraacetic acid (EGTA), .005% Triton X-100, 10 mM $MgCl_2$ and 15 mM Tris-HCl, pH 7.4. The reaction was stopped by centrifugation at 48,000 X g for 60 min to remove the membranes. In order to separate [$^{32}$P] $PO_4$-casein from radioactive ATP, the supernatant was applied to a 1.5 X 30 cm Sephadex G-25 column previously equilibrated with 15 mM Tris-HCl, pH 6.8. The eluate was monitored by Cerenkov counting of 50 μl aliquots in 3 ml of water in a Beckman LS 233 scintillation counter and the first peak used for the phosphatase assay.

Exogenous phosphoprotein phosphatase activity was determined by the method of Graham et al (6). AChR-enriched membranes containing 0.1 mg protein were incubated in duplicate with 50 μg of [$^{32}$P] $PO_4$ casein at 37° for 20 min in a total volume of 100 μl containing 4 mM dithiothreitol (DTT), 0.1% Triton and 15 mM Tris-HCl, pH 6.8. The reaction was stopped with 3 ml of an ice cold solution containing Norit A (40 mg/ml), 0.1 M HCl, 0.2 mg/ml bovine serum albumin, 1 mM $NaP_i$, and 1 mM $NaPP_i$. After holding on ice for 10 min the suspension was filtered on Millipore filters (0.45 μm) and the residue washed twice with 3 ml of 0.01 N HCl-1 mM $NaP_i$. Releasable $^{32}$P not absorbed to the charcoal was determined by measurement of Cerenkov radiation in the filtrate.

In order to prove that the radioactive product released from casein was [$^{32}$P] $PO_4$, we extracted the labeled material into isobutanol in the presence of ammonium molybdate (6). The dephosphorylation reaction was stopped by addition of 0.60 ml cold 25% trichloroacetic acid. 400 μg bovine serum albumin in 0.10 ml was added as carrier and the mixture kept at 0° for 10 min. After centrifugation at 2500 rpm in a Sorvall GLC centrifuge for 10 min, 0.6 ml of the supernatant was mixed with 0.4 ml of 4% ammonium molybdate in 2M $H_2SO_4$ and extracted with 2.0 ml of isobutanol. Following centrifugation at 2500 rpm for 10 min to promote phase separation, 1.0 ml of the isobutanol phase was withdrawn for scintillation counting in 10 ml of Instagel (Packard).

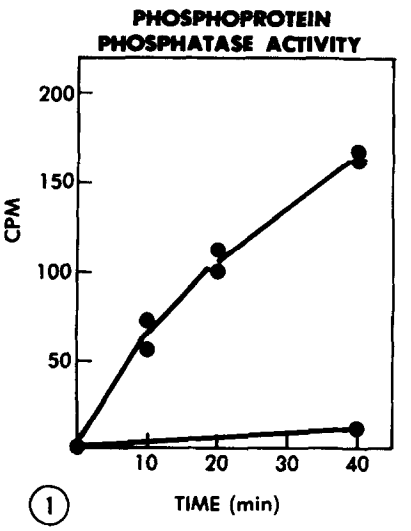
Endogenous Dephosphorylation of the AChR. Membranes were initially phosphorylated as described (2). The reaction mix containing AChR-enriched membranes (2.1 mg protein), 0.25 mM EGTA, 20 mM $MgCl_2$, 100 mM KCl, .005% Triton X-100, 1 mM ouabain and 62.5 mM Tris-HCl, pH 6.8 in a final volume of 2 ml. The reaction was initiated by addition of ($\gamma$-$^{32}$P) ATP (40 µCi) to a final concentration of 6 µM and allowed to proceed for 10 min on ice. The reaction was stopped by the addition of a solution containing EDTA (neutralized with Tris base) and NaF to final concentrations of 40 mM and 0.1 M respectively. NaF was present in the stop solution to prevent dephosphorylation. The mixture was then centrifuged at 48,000 X g for 10 min to separate the phosphorylated membranes from the excess ($\gamma$-$^{32}$P) ATP, and the pellets rinsed twice in the cold by floating the residual buffer on 2.5 ml of 60% sucrose. The pellet was then quickly resuspended in 62.5 mM Tris-HCl, pH 6.8 containing 2 mM ouabain and 8 mM DTT. 50 µl aliquots of the membrane suspension were pipetted into 50 µl of 200 mM NaCl or NaF. The tubes were then placed at 37° and the dephosphorylation reaction allowed to proceed for 10 min. For measurement of released ($^{32}$P) $PO_4$, the reaction was stopped by the addition of 0.6 ml of cold 25% trichloracetic acid followed by 0.1 ml of bovine serum albumin (4 mg/ml). Isobutanol extraction was carried out as described above. For experiments in which the level of phosphorylation of the AChR was to be quantitated directly the reaction was stopped with EDTA at a final concentration of 40 mM. After 0.5 min at room temperature, SDS and 2-mercaptoethanol were added to a final concentration of 3.7% and 4% respectively. After another 0.5 min, the solution was boiled for 1 min. The samples were then subjected to SDS gel electrophoresis and autoradiography as previously described (2).

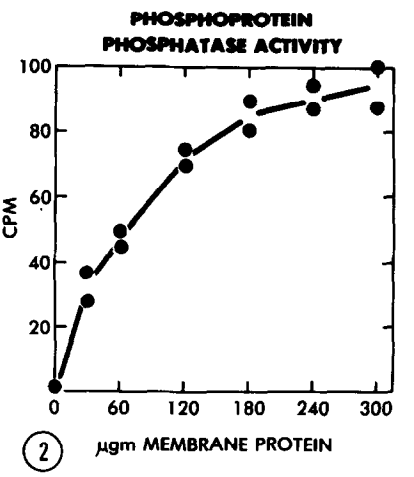
α-Casein (C-3883) and ATP were purchased from Sigma Chemical Company (St. Louis, Mo.). [$\gamma$-$^{32}$P] ATP (30 Ci/mmole) was purchased from New England Nuclear.

## RESULTS AND DISCUSSION

The results in this study indicate that phosphoprotein phosphatase activity is demonstrable in AChR-enriched membranes with either exogenous or endogenous phosphorylated substrates. Fig. 1 shows the time course for release of $^{32}$P from phosphorylated casein after incubation with AChR-enriched membranes. Boiled membrane controls or phosphorylated casein without membranes did not show release of radioactivity over this same time interval. The non-linearity of this time course may be due either to the heterogeneity of the phosphoester bonds of the casein or to the presence of more than one protein phosphatase in the membranes. Release of $^{32}$P from casein was proportional to the concentration of membrane protein (Fig. 2). The pH optimum for the reaction was 5-6.5 (Fig. 3) indicating this enzyme is not an alkaline phosphatase. Decreased activity at pH 4.5 may be due to enzyme denaturation.

Radioactive $^{32}$P released from casein after incubation with membranes could be due to proteolytic breakdown of casein into labeled amino acids. To



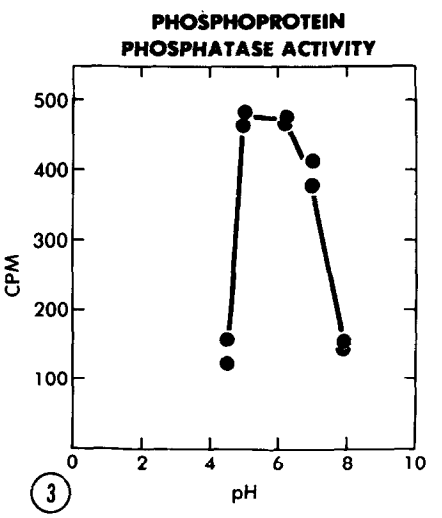




Fig. 1 Time course of phosphoprotein phosphatase activity with [$^{32}$P] $PO_4$-casein as substrate. Assay as described in Methods. Lower curve is a control in the absence of membranes.

Fig. 2 Phosphoprotein phosphatase activity as a function of membrane protein concentration. Assay as described in Methods for the indicated amount of membrane protein. Blank values with membranes omitted have been subtracted.

Fig. 3 Phosphoprotein phosphatase activity in AChR-enriched membranes as a function of pH.

investigate this possibility, we found that casein migrated as one band on SDS acrylamide gel electrophoresis and that the pattern was unchanged after

incubation with membranes (data not shown). In addition all of the $^{32}P$ released from casein was extractable into isobutanol in the presence of ammonium molybdate and sulfuric acid. This confirms that the label was inorganic phosphate (6).

Radioactive phosphate released during the incubation could also be derived from the hydrolysis of any free [$\gamma$-$^{32}P$] ATP which might not have been separated from the ($^{32}P$) $PO_4$-casein. $Na^+$-$K^+$ ATPase is the only ATPase present in the acetylcholine receptor-enriched membranes and is virtually completely inhibited by 0.1 mM ouabain (unpublished observations). Therefore, we examined the effect of 0.1 mM ouabain and found there was no change in the release of isobutanol extractable ($^{32}P$) $PO_4$ from casein. Moreover, if release of ($^{32}P$) $PO_4$ were due to $Na^+$-$K^+$ ATPase activity, the reaction should be stimulated by $K^+$ (7). However, no changes in phosphorylation were observed when KCl was substituted for NaCl during the dephosphorylation reaction. These experiments indicate that $Na^+$-$K^+$ ATPase activity could not account for the release of ($^{32}P$) $PO_4$ in our experiments.

We also tested the effects of several agents known to alter protein phosphatase activity in other preparations. Table I shows that 100 mM fluoride, a specific inhibitor of protein phosphatase, caused an 84% reduction in phosphatase activity while chloride produced only 23% inhibition. This result is consistent with the findings of Teichberg and Changeux (8). They incubated Triton-solubilized AChR-enriched membranes prepared from Electrophorus electricus with [$\gamma$-$^{32}P$] ATP and then measured $^{32}P$ incorporation into trichloracetic acid insoluble material. In this indirect assay they found more incorporation of $^{32}P$ in the presence of NaF than in the presence of NaCl. In our study, DTT, which is known to stimulate phosphatase activity (6), increased enzyme activity by more than 3-fold. cAMP and cGMP were without effect but ATP, GTP and inorganic phosphate inhibited dephosphorylation of casein.

Fluoride also had a striking effect on endogenous dephosphorylation of the AChR. This conclusion is based on two different assays for endogenous

TABLE I

Casein Phosphatase Activity

| Additions | mM | Relative Activity (%) |
|---|---|---|
| None | -- | 100 |
| NaF | 10 | 50 |
| NaF | 100 | 16 |
| NaCl | 100 | 77 |
| DTT | 4 | 340 * |
| $Mg^{++}$ | 10 | 77 |
| $Mn^{++}$ | 10 | 58 |
| cAMP | $10^{-3}$- $10^{-6}$ | 100 |
| cGMP | $10^{-3}$- $10^{-6}$ | 100 |
| ATP | 1 | 11 |
| GTP | 1 | 15 |
| $P_i$ | 10 | 46 |

Membranes were incubated in duplicate with ($^{32}$P) $PO_4$ casein as described in Methods. Replication was always within 5% of the mean.

* Relative to a control without DTT. All other samples contained 4 mM DTT.

phosphatase activity. In the first, we measured the time-dependent release of ($^{32}$P) $PO_4$ from AChR-enriched membranes that had been phosphorylated in the presence of [$\gamma$-$^{32}$P] ATP. Table IIA shows that ($^{32}$P) labeled inorganic phosphate was released from the phosphorylated membranes indicating endogenous phosphoprotein phosphatase activity with endogenous phosphorylated membrane proteins as substrate. This reaction is also inhibited by fluoride ion.

In the second assay, we measured the level of phosphorylation of the AChR directly after incubation of the phosphorylated membranes at 37$^{O}$ for 10 min. Table IIB shows that the 65,000 M.W. subunit of the AChR has less ($^{32}$P) $PO_4$ incorporated after incubation at 37$^{O}$ for 10 min. The dephosphorylation of the AChR is inhibited by 0.1 M NaF.

TABLE II

Dephosphorylation of the Membrane Bound AChR

| Condition | A<br>pmoles ($^{32}$P) $PO_4$ released | B<br>($^{32}$P) $PO_4$ in the 65,000 M.W. subunit of the AChR<br>$A_{700}$ |
|---|---|---|
| zero time control | 0.57 ± .03 | 4.5 |
| NaCl (0.1 M) | 0.74 ± .02 | 2.8 |
| NaF (0.1 M) | 0.50 ± .03 | 4.2 |

Membranes were incubated with [$\gamma$-$^{32}$P] ATP as described in Methods for endogenous dephosphorylation. A. ($^{32}$P) $PO_4$ release is measured in triplicate by isobutanol extraction as described. B. ($^{32}$P) $PO_4$ incorporated in the 65,000 M.W. component of the AChR is measured by SDS electrophoresis as described. After autoradiography of the dried gels, the film was scanned in a Gilford spectrophotometer with a linear transport attachment. Reported values are arbitrary units for the Absorbance at 700 nm of the 65,000 M.W. subunit of the AChR.

This study demonstrates that AChR-enriched membranes contain significant phosphoprotein phosphatase activity which can dephosphorylate both casein and the endogenous membrane-bound AChR. We have previously shown that these membranes also contain protein kinase activity which phosphorylates the AChR in situ (5). Therefore, the enzymes which phosphorylate and dephosphorylate the AChR are found in the same AChR-enriched membrane preparation. This suggests that changes in the level of phosphorylation of AChR could play a role in the function of the AChR at the synapse. In contrast to other studies on phosphorylation and dephosphorylation of membrane proteins, the substrate in our system, the AChR, is a protein of known function. In these experiments the membrane-bound AChR is virtually the only substrate for the phosphorylation-dephosphorylation reaction. This model system has great potential for elucidating the role of reversible phosphorylation of receptor proteins in the regulation of membrane function.

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