Characterization of phosphotyrosine containing proteins at the cholinergic synapse

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Abstract Tyrosine phosphorylation has been associated with several aspects of the regulation of cholinergic synaptic function, including nicotinic acetylcholine receptor (AChR) desensitization as well as the synthesis and clustering of synaptic components. While some progress has been made in elucidating the molecular events initiating such signals, the downstream targets of these tyrosine kinase pathways have yet to be characterized. In this paper we have used molecular cloning techniques to identify proteins which are tyrosine phosphorylated at the cholinergic synapse. Phosphotyrosine containing proteins (PYCPs) were isolated from the electric organ of *Torpedo californica* by anti-phosphotyrosine immunoaffinity chromatography. Peptide sequencing and expression cloning then identified the isolated proteins. The proteins identified included heat shock protein 90, type III intermediate filament from *Torpedo* electric organ, α-fodrin, β-tubulin, actin and rapsyn. These tyrosine phosphorylated proteins may play a role in the regulation of synaptic function by tyrosine kinases.

Key words: Tyrosine phosphorylation; Cholinergic synapse; *Torpedo californica*

1. Introduction

Synapses are subject to complex regulation by multiple stimuli. One means of regulation may be tyrosine phosphorylation since some tyrosine kinases are expressed at their highest levels in the adult nervous system and are concentrated in presynaptic and postsynaptic membranes. Moreover, knockout mice deficient in the tyrosine kinase *fyn* have impaired long-term potentiation: the phenomenon by which synaptic connections are strengthened after a short burst of high frequency activity [1].

In order to elucidate the role of tyrosine phosphorylation in the central nervous system, some investigators have turned to the simpler models of the neuromuscular junction (NMJ) and the electric organ of *Torpedo californica*. The neurotransmitter receptor at these synapses is the pentameric ligand gated ion channel, the nicotinic acetylcholine receptor (AChR). Purified AChR from the *Torpedo* electric organ is indeed highly tyrosine phosphorylated with 1.5 mol of phosphotyrosine per mol of AChR [2].

Tyrosine phosphorylation has been implicated in several aspects of AChR regulation. First, tyrosine phosphorylation of the AChR itself is associated with a more rapid rate of desensitization of the channel [2]. Second, tyrosine phosphorylation also is involved in the regulation of the expression of the AChR subunit. During the development of the NMJ, the motor neuron releases a factor with AChR inducing activity (ARIA) which elicits a local increase in the expression of AChR [3]. This is particularly striking because it occurs at the same time as a marked decrease in AChR synthesis in extrasynaptic regions. Molecular cloning revealed ARIA to be a member of the neu ligand family [4]; it was subsequently shown that ARIA induces phosphorylation of erbB2 and erbB3, members of the epidermal growth factor receptor family of tyrosine kinases [5]. ARIA may therefore transduce its signal for the upregulation of AChR through a cascade of tyrosine phosphorylation.

Tyrosine phosphorylation is also associated with AChR clustering, another important aspect of synaptogenesis. AChR is initially distributed uniformly throughout the myotube surface. Upon innervation, the AChRs become clustered beneath the nerve terminal. The concentration of AChR at these sites is due in large part to the redistribution of AChR mediated by the extracellular matrix protein, agrin. Agrin is expressed in both motor neurons and muscle cells and is subject to alternative splicing. Interestingly, the neuronal isoform is much more active than the muscle isoform in clustering AChR. In the mature AChR clusters of the NMJ, the density of the AChRs in the crests of the postsynaptic membrane, which are precisely positioned underneath the presynaptic active zones is 10^4 molecules/μm^2; the crests contain more than 90% of the AChR molecules, even though they comprise less than 0.1% of the total muscle membrane [6].

AChR clusters at the rat NMJ have been shown to be associated with phosphotyrosine by double label immunofluorescence studies using fluorescein conjugated anti-phosphotyrosine antibodies and rhodamine conjugated α-bungarotoxin [7]. The role of tyrosine phosphorylation in initiating the redistribution of AChR was suggested by the finding that the treatment of *Xenopus* myoblasts with basic fibroblast growth factor (FGF) bound to latex beads results in AChR clustering at the point of contact with the bead; since FGF receptor contains a tyrosine kinase which is activated upon binding FGF, it is possible that local tyrosine phosphorylation of specific proteins can trigger clustering. Furthermore, such clustering is abolished by tyrosine kinase inhibitors [8].

The in vivo stimulus for AChR clustering, agrin, also appears to act via tyrosine phosphorylation. While it has been well established that agrin causes an increase in tyrosine phosphorylation associated with the receptor clusters [9], the mechanism of AChR clustering awaited the identification of the agrin receptor. Initial discoveries of agrin binding to α-dystroglycan caused great excitement [10]. α-Dystroglycan is an extracellular component of the dystrophin complex, which also includes several transmembrane and cytosolic peripheral proteins. It therefore seemed possible that agrin could cross-

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link cytoskeletal proteins and immobilize AChR. However, it was soon found that several forms of agrin, including muscle agrin, which is not active in clustering, could also bind α-dystroglycan with similar affinities, suggesting that α-dystroglycan binding alone does not induce AChR clustering [11]. Recent evidence suggests that agrin induced clustering is mediated by muscle specific kinase (MuSK). Neuronal agrin, but not muscle agrin, causes tyrosine phosphorylation of MuSK. Moreover, radiolabelled agrin can be crosslinked to MuSK [12]. The importance of agrin and MuSK is underscored by the fact that knock out mice deficient in either of these proteins are deficient in synaptogenesis [13,14].

In summary, tyrosine phosphorylation regulates the efficiency of synaptic transmission by modulating the gating properties, synthesis and distribution of the AChR. In order to better understand the role of tyrosine phosphorylation at the synapse, we have sought additional phosphotyrosine containing proteins (PYCPs) from postsynaptic membranes that may be involved in regulation of AChR and cholinergic synaptic function. By using both expression cloning and peptide sequencing, we found α-fodrin, type III intermediate filament from *Torpedo*, actin, β-tubulin and rapsyn to be tyrosine phosphorylated components of the synaptic membrane.

2. Materials and methods

2.1. Reagents

Agarose conjugated anti-phosphotyrosine antibody was obtained from UBI, anti-hsp90 from Stressgen, anti-tubulin from Chemicon, and anti-fodrin from ICN Biochemicals. A pan-intermediate filament antibody was used to detect type III intermediate filament from *Torpedo* [15]. All chemicals, unless otherwise indicated, were obtained from Sigma.

2.2. Subcellular fractionation of Torpedo electroplax

Postsynaptic membranes were prepared from *Torpedo californica* (Winkler Enterprises, San Pedro, CA) as previously described [7]. Briefly, the electric organ was dissected from *Torpedo californica* and homogenized in 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM EGTA, 20 mM benzamidine, 20 μg/ml each antipain, chymostatin, and pepstatin, 20 U/ml Traysolol, and 0.1 mM PMSF. The crude membrane fraction was collected by centrifugation in a GSA rotor, 6500 rpm/10′. Membranes were then fractionated on a discontinuous sucrose gradient: for analytical scale, 35% w/w, 37.5% w/w, and 41.5% w/w; and for preparative scale, 35% and 41.5%). While the 35% sucrose fraction is composed of non-synaptic proteins, the 41.5% is enriched in AChR and other synaptic proteins, and the 37.5% is mixed [16].

2.3. Phosphorylation and purification of PYCPs

The synaptic fraction was phosphorylated by incubating synaptic membranes at 0.5 mg/ml in phosphorylation buffer (20 mM Tris, pH 7.4, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM ouabain, 20 ng/ml NaN_3, 1 mM Na-orthovanadate, 0.1 mM dithiothreitol, 2 mM $MnCl_2$, 0.1 μM Walsh peptide, 20 μg/ml leupeptin and antipain, and 20 U/ml Traysolol) in the presence of 200 μM ATP. The membranes were then isolated by centrifugation in a Ti45 rotor, 40 K/20′, and resuspension at 2 mg/ml in wash buffer (20 mM Tris, 100 mM NaCl, 50 mM KCl, 1 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, and 2 ng/ml NaN_3) and solubilized by the addition of Triton X-100 to 1.5%. The insoluble fraction was removed by centrifugation in a Ti45.5 rotor at 20 K/20′/4°C (see below) and the soluble fraction was passed over an acetylcholine affinity column to deplete this mixture of the AChR. The flow through of this column was then passed over an anti-phosphotyrosine antibody column. After extensive washing with wash buffer+1% Triton X-100, the column was eluted using 100 mM phenyl phosphate in 200 mM ammonium bicarbonate, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM vanadate and 0.1% Triton X-100. The fractions of interest were pooled, concentrated and used for generating the polyclonal anti-PYCP antiserum (Hazelton).

2.4. Solubilization of the Triton insoluble fraction

The Triton insoluble pellet was resuspended in 2% SDS and then boiled. The SDS soluble fraction was diluted to a final concentration of 0.2% SDS/1% Triton with wash buffer, passed over an anti-phosphotyrosine antibody column, and eluted as above. SDS PAGE analysis showed two prominent bands at approximately 55 and 43 kDa which were selected for sequencing.

2.5. Expression cloning

Briefly, a *Torpedo* cDNA expression library in λgt11, generously provided by Dr. Kathryn Wagner, was plated at a density of 15 000 plaques per 100 mm plate and incubated 3.5 h at 42°C. Nitrocellulose filters soaked in 10 mM IPTG to induce the fusion protein were then laid over the plates, which were then incubated an additional 3.5 h; the filters were removed and soaked for 20′ in TBS+1% Tween-20. After blocking in 5% BSA/TBST for 60′, filters were incubated a 1:1000 dilution of anti-PYCP anti-serum with 1 mg/ml *Escherichia coli* extract (Promega). Filters were washed extensively with TBST, and incubated in alkaline phosphatase conjugated anti-rabbit secondary antibody (Promega) diluted 1:7500, and washed again with TBST. The filters were then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer's protocol (Promega).

To avoid cloning AChR, two additional nitrocellulose lifts were taken after the IPTG filter was removed. These were subject to hybridization with a mixture of full length probes encoding α, β, γ and δ AChR *Torpedo* cDNAs in 5×SSC/5×Denhardt's/0.5% SDS/0.1 mg/ml salmon sperm. These filters were then washed thrice in 0.5×SSC/0.2% SDS at 65°C, and subjected to autoradiography. Those clones which were recognized by the antibody but did not hybridize with the AChR probes were selected and plaque purified.

The insert of the purified phage was amplified by PCR using the flanking vector primers TTGACACCAGACCAACTGGTAATG and GTTGGCGACGACTCCTGGAGCCCG. The PCR products were band purified and subjected to automated sequencing using the primer GAGCCCGTCAGTATCGGC. Sequences were analyzed using the BLAST program.

2.6. Reverse Northern

Inserts from clones were amplified using PCR, as described above. The products were separated by electrophoresis and transferred to nitrocellulose. RNA was isolated form the electric organ and liver of *Torpedo* using the single step RNA isolation protocol using guanidinium thiocyanate described in *Current Protocols in Molecular Biology* (4.2.4–4.2.6) and used to make cDNA. The cDNA was radiolabelled as described above and hybridized at high stringency to the nitrocellulose filters.

2.7. Specificity of binding to the anti-phosphotyrosine antibody column

Triton soluble or Triton insoluble fractions (SDS solubilized as above) were passed over the anti-phosphotyrosine antibody columns in the presence or absence of 1 mM phosphotyrosine. The columns were washed extensively using wash buffer+1% Triton ± 1 mM phosphotyrosine and eluted with 100 mM phenyl phosphate as above. The elutions were concentrated, subjected to SDS-PAGE, transferred to PVDF, and blotted with the appropriate antibodies.

3. Results

3.1. Identification of PYCPs by expression cloning

In order to identify PYCPs we turned to a strategy of expression cloning. First, PYCPs were purified from the electric organ of *Torpedo californica*, a modified cholinergic synapse. While similar in composition to the neuromuscular junction, the electric organ is particularly well suited for biochemical studies as its innervated face poses a relatively large homogeneous area of synaptic membranes for protein purification and analysis.

Synaptic membrane fractions were isolated from electric organ homogenates and then incubated under phosphorylating conditions in vitro to increase the stoichiometry of tyrosine phosphorylation. As expected from previous studies,

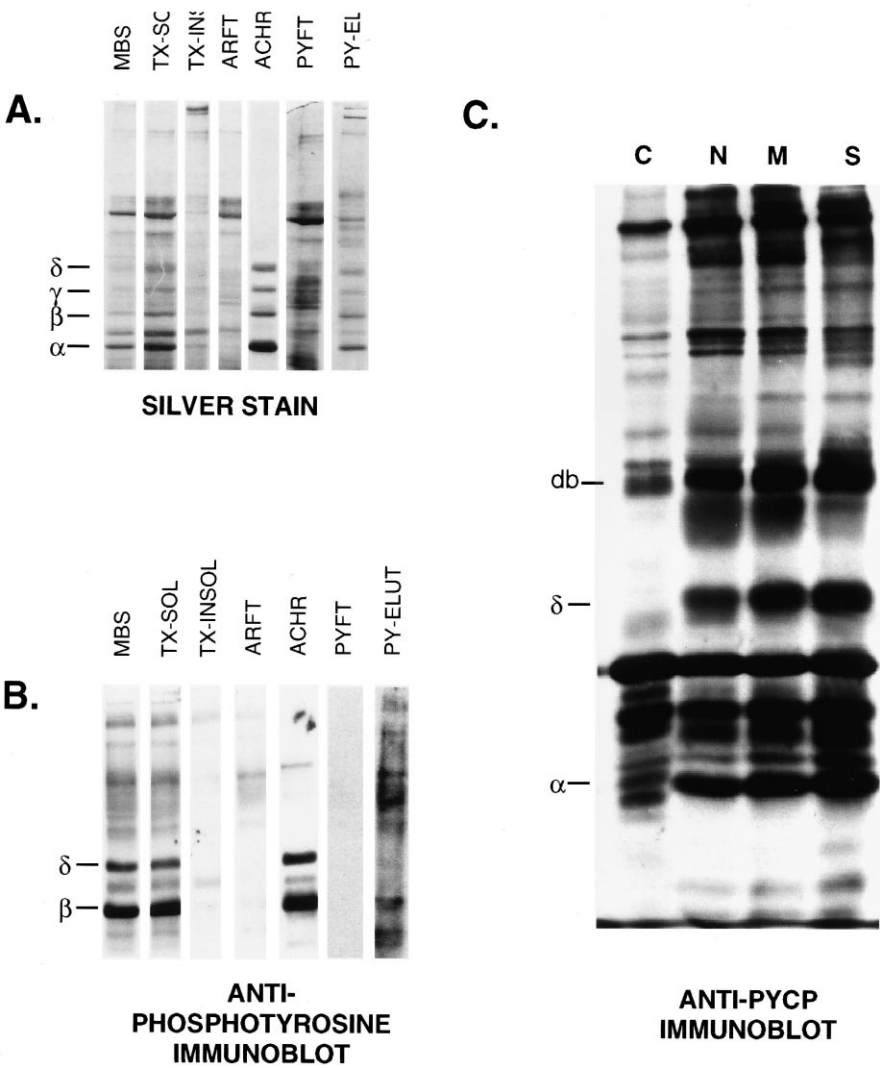

Fig. 1. PYCPs were purified from *Torpedo californica* electroplax; aliquots are shown by silver stain (A) and Western blot with anti-phosphotyrosine antibodies (B). The electric organ was dissected from *Torpedo californica* and homogenized. The membrane fraction was collected by centrifugation, fractionated on a discontinuous sucrose gradient and the AChR enriched fraction isolated. This fraction was phosphorylated by endogenous kinases in the presence of 200 μM ATP and tyrosine phosphatase inhibitors to increase the stoichiometry of tyrosine phosphorylation (MBS). The membranes were then solubilized by the addition of 1% Triton X-100. The insoluble fraction (TX-INSOL) was removed by centrifugation and the soluble fraction (TX-SOL) was passed over an acetylcholine affinity column to deplete this mixture of AChR. The flow through of this column (ARFT) was then passed over an anti-phosphotyrosine antibody column. The anti-phosphotyrosine antibody column was then eluted using 100 mM phenyl phosphate (PY-ELUT). Aliquots of the acetylcholine affinity column elution (ACHR) and the anti-phosphotyrosine antibody column flow through (PY-FT) are included in A and B. The elutions of the anti-phosphotyrosine antibody column were used to generate the anti-PYCP antiserum. *Torpedo* membranes were fractionated on a discontinuous sucrose gradient to yield cytosol (C) non-synaptic (N) mixed (M) and synaptic (S) membranes (C). The anti-PYCP, pre-incubated with purified AChR to attenuate the AChR signal, recognizes multiple proteins. The subunits of the AChR (α, β, γ and δ) and the 87 kDa tyrosine phosphorylated protein, dystrobrevin (db) [30] are indicated.

the major PYCPs of the electric organ were the β and δ subunits of the AChR (Fig. 1B, MBS). Since we were interested in identifying novel PYCPs, we removed the AChR by passing the preparation over an acetylcholine affinity resin. The affinity resin selectively bound the AChR (Fig. 1A,B, ACHR), leaving the affinity resin flow though (ARFT) relatively depleted of AChR (compare TX-SOL and ARFT in Fig. 1A,B).

The PYCPs were then purified from the ARFT by binding it to an anti-phosphotyrosine column. While the flow through of the anti-phosphotyrosine antibody column (PYFT) contained most of the proteins found in the ARFT (Fig. 1A), it was depleted of phosphotyrosine containing proteins (Fig. 1B). The elutions of the anti-phosphotyrosine antibody column (PY-ELUT) were composed of a mixture of PYCPs, shown in Fig. 1A,B. The PYCPs were concentrated and used to create a polyclonal anti-PYCP antiserum.

This antiserum is characterized in Fig. 1C. *Torpedo* electric organ homogenates were fractionated into cytosol (C), non-synaptic (N), mixed (M) and synaptic (S) membranes. The pre-immune serum only recognized a single band (data not shown). The anti-PYCP antibody, on the other hand, recognized many bands which were synaptically enriched (Fig. 1C), including the AChR itself, despite depletion of the AChR during the purification procedure. This was likely due to the abundance of AChR in synaptic membranes and its high stoi-

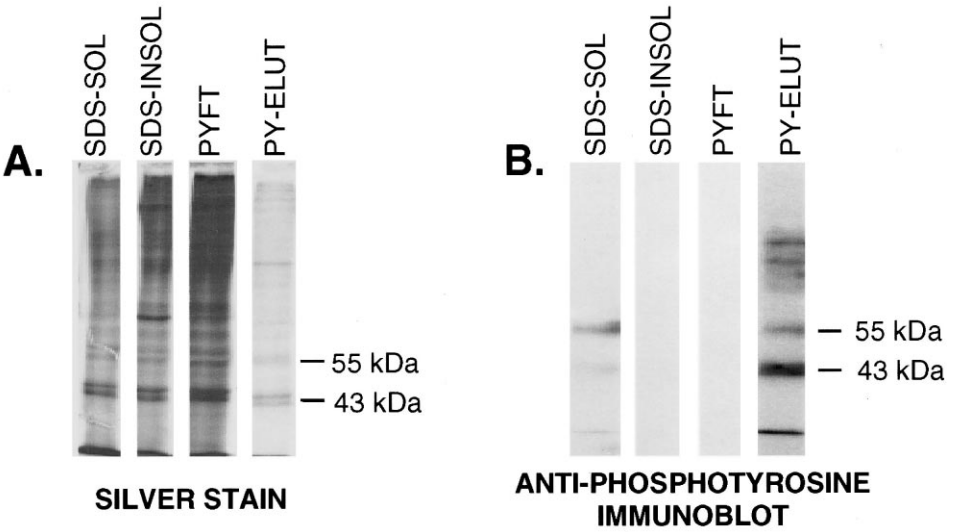

Fig. 2. Proteins from the Triton insoluble membrane fraction were collected as described in Fig. 1A, solubilized with SDS, and purified using an anti-phosphotyrosine antibody column. Aliquots of the SDS solubilized proteins (SDS-SOL) and column elutions (PY-ELUT), as well as the discarded SDS insoluble (SDS-INSOL) and column flow through (PY-FT) are shown by silver stain (A) and by immunoblotting with anti-phosphotyrosine antibodies (B). The prominent bands at ~55 and ~43 kDa were sequenced after tryptic digest; a peptide from the upper band was identified as β-tubulin, while peptides from the lower band were identified as rapsyn and actin.

chiometry of tyrosine phosphorylation. However, additional proteins were also detected, suggesting that this antiserum recognized several non-AChR, synaptically enriched tyrosine phosphorylated proteins. Preabsorption of the anti-PYCP antibodies with phosphotyrosine did not block the immunorecognition of the PYCPs, indicating that the antibodies were not recognizing phosphotyrosine itself.

The anti-PYCP anti-serum was used to screen a λgt11 cDNA expression library made from the electric organ of *Torpedo californica*. Of the 1.3 million phage screened, 70 non-AChR clones were isolated: 39 were novel, 12 encoded type III intermediate filament from *Torpedo*, 10 encoded heat shock protein 90 (hsp90), and nine encoded α-fodrin.

3.2. Identification of PYCPs by peptide sequencing

A complementary approach was simultaneously pursued. It was noted that the Triton X-100 pellet was enriched in two tyrosine phosphorylated proteins of 55 and 43 kDa (Fig. 1B, TX-INSOL). These proteins could, to a large extent, be solubilized by boiling in SDS (Fig. 2B, SDS-SOL). These SDS solubilized proteins were then purified using an anti-phosphotyrosine antibody column, as described above (Fig. 2A,B, PY-

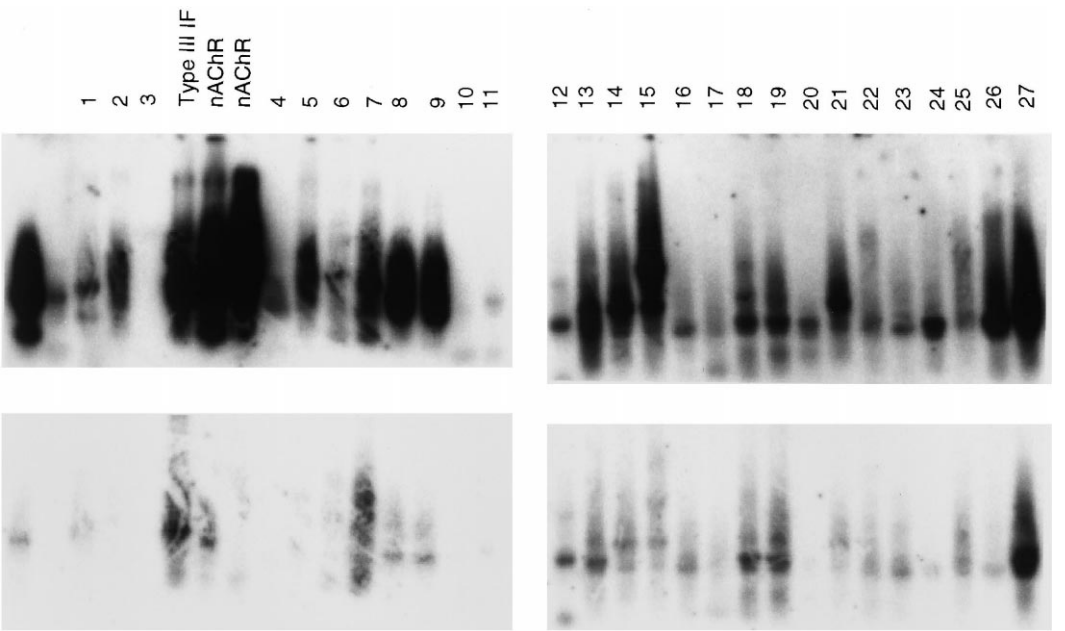

Fig. 3. Reverse Northern technique showing the differential expression of the various clones between electric organ and liver. Inserts from clones were amplified using PCR, separated by electrophoresis, and transferred to nitrocellulose. cDNA prepared from either electric organ or liver was radiolabeled and hybridized at high stringency to the nitrocellulose filters. Comparison of the signal intensities between the upper and lower panels suggests the relative levels of expression in the two tissues.

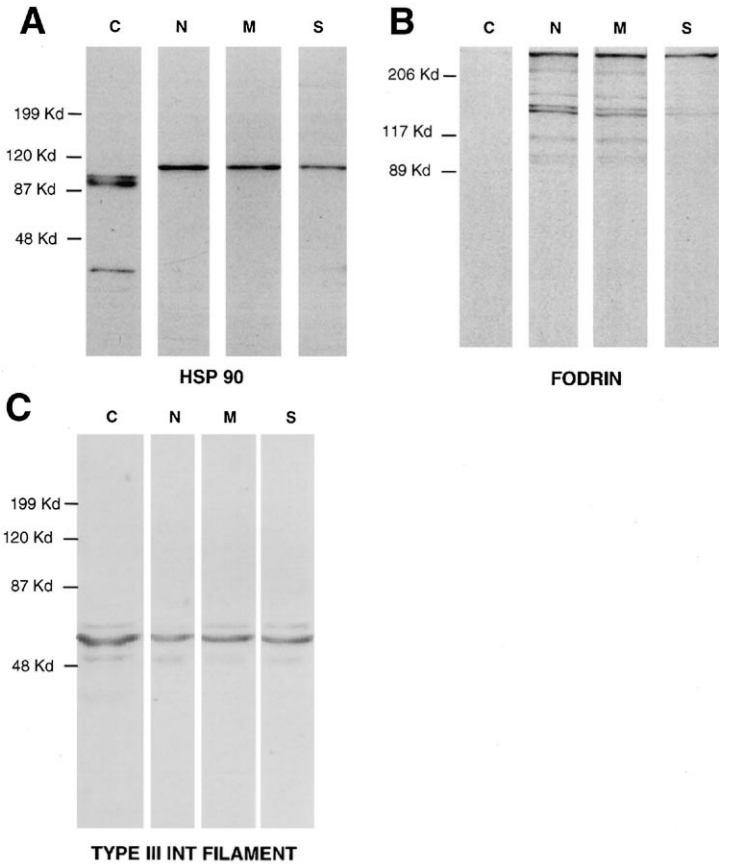

Fig. 4. *Torpedo* membranes were fractionated into cytosol (C), non-synaptic (N), mixed (M), and synaptic (S) on a discontinuous sucrose gradient, subjected to SDS-PAGE, transferred to PVDF, and probed with the appropriate antibodies against hsp90 (A), fodrin (B) and a pan-intermediate filament antibody (C).

ELUT). These bands were excised and subjected to automated sequencing. Analysis of the resulting peptide sequences revealed the 55 kDa protein to be β-tubulin, while sequences from the 43 kDa proteins were found to encode rapsyn and actin.

3.3. Tissue distribution of the identified clones

To further characterize the PYCPs identified, we examined their distribution. First, in the case of the novel clones, we wished to determine their relative distribution in electric organ versus liver. To do this, a reverse Northern blotting technique was used. The cloned fragments were subjected to electrophoresis, blotted to nitrocellulose, and probed with radiolabelled total cDNA from *Torpedo* electric organ or liver (Fig. 3). The signal intensities obtained after hybridization were then compared between the two tissues. Some of the clones appeared to be expressed at similar levels in electric organ and liver. Others, for example clone 5, seemed to be predominantly expressed in electric organ.

We also examined the distribution of fodrin, hsp90 and type III intermediate filament in the cytosol (C), non-synaptic (N), mixed (M), and synaptic (S) membrane fractions of electric organ, using antibodies selective for each protein. As can be seen in Fig. 4, all of the proteins were present in synaptic membranes. Interestingly, membrane associated hsp90 has a larger apparent molecular weight. It is possible that this is due to alternative splicing or post-translational modification such as phosphorylation.

3.4. Tyrosine phosphorylation of the PYCPs

Finally, to verify that the proteins cloned were tyrosine phosphorylated, *Torpedo* membranes were incubated under phosphorylating conditions, and solubilized with Triton X-100 (LOAD in Fig. 5A,B). The Triton insoluble fraction was then further solubilized with SDS (LOAD in Fig. 5C–E). Both were passed over the anti-phosphotyrosine antibody column. Where indicated phosphotyrosine (1 mM) was added prior to loading the column. The columns were eluted and subjected to immunoblotting with the antibodies indicated. As shown, fodrin, type III intermediate filament, actin, tubulin and rapsyn were all bound by the anti-phosphotyrosine antibody column (Fig. 5, unblocked), suggesting that they are indeed tyrosine phosphorylated. Hsp90 binding was not detectable under these conditions, perhaps due to the low stoichiometry of tyrosine phosphorylation and relatively low affinity for the antibody used. In all cases, 1 mM phosphotyrosine blocked binding to the column (Fig. 5, blocked), demonstrating the specificity of this interaction.

4. Discussion

Tyrosine phosphorylation has been implicated in several aspects of AChR regulation at the NMJ: namely, upregulation of AChR transcription, clustering of AChR beneath the nerve terminal, and AChR desensitization. The molecular events initiating these processes have gradually been characterized. ARIA, a member of the neu ligand family, which

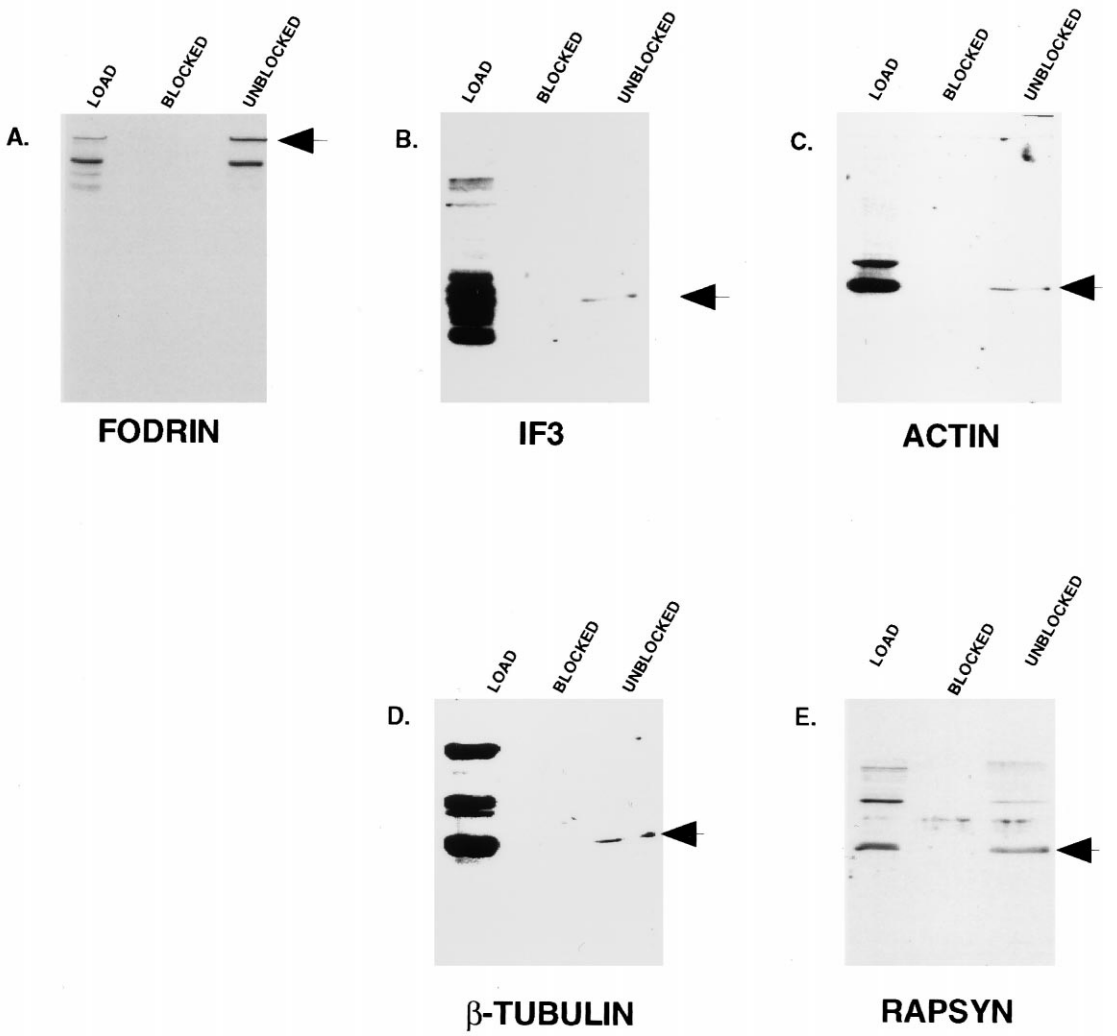

Fig. 5. Triton soluble or Triton insoluble fractions (SDS solubilized as above) were passed over the anti-phosphotyrosine antibody columns in the presence (blocked) or absence (unblocked) of 1 mM phosphotyrosine. The columns were eluted with 100 mM phenylphosphate, concentrated, subjected to SDS-PAGE, transferred to PVDF, and blotted with the appropriate antibodies. The ability of 1 mM phosphotyrosine to block binding showed that in each case, association with the anti-phosphotyrosine antibody column was specific.

increases the amount of α-subunit mRNA, causes phosphorylation of the receptor tyrosine kinases, erbB2 and erbB3 [3,5]. Agrin, the nerve-derived signal which causes aggregation of preexisting AChRs, activates the MuSK tyrosine kinase [12].

One important substrate of tyrosine kinases is the AChR itself. Tyrosine phosphorylation of the β-subunit in response to agrin treatment has been well established. However, two arguments suggest that tyrosine phosphorylation of proteins other than the AChR may be important in maintaining clusters. First, in cultured mouse muscle cells, tyrosine phosphorylation of the β-subunit in response to agrin appears to be only transient, returning to baseline within 24 h [17]. Second, co-expression of rapsyn and AChR subunits in heterologous cell systems results in patches of AChR which react with anti-phosphotyrosine antibodies; however, such reactivity is not abolished by mutation of the AChR tyrosine phosphorylation sites [18] suggesting the presence of other phosphotyrosine containing proteins.

In this paper synaptic tyrosine phosphorylated proteins were sought using the complementary approaches of expression cloning and peptide sequencing. The majority of identified proteins were novel, without significant homology to any known proteins. These are potential candidates for mediating the upregulation of AChR in response to ARIA and the redistribution of AChR in response to agrin; they may represent new substrates for the erbB2/3 and MuSK tyrosine kinases.

In addition, hsp90 and several cytoskeletal proteins were identified: namely, actin, β-tubulin, α-fodrin, and type III intermediate filament. While their exact functions at the synapse await further investigation, studies in other cell systems hint at their possible roles.

4.1. Fodrin

α-Fodrin, also known as non-erythroid α-spectrin, is a flexible rod-like molecule made up primarily of 22 closely related triple helical bundles, known as spectrin repeats, in addition to an SH3 domain, which may mediate interactions with other proteins. Adjacent to the SH3 domain is a calmodulin binding domain. α-Fodrin combines with β-fodrin to form dimers (reviewed in [19]). While fodrin has been shown by immunohistochemistry and immunoelectron microscopy to be local-

ized to the non-innervated face of the *Torpedo* electrocyte [20], we show here that fodrin can be detected in synaptic membrane fractions. This may indicate that there is a subpopulation of synaptic fodrin. In support of this is the fact that fodrin is highly expressed in brain [21]; it is detectable by immunocytochemistry at the neuromuscular junction [22]; and it is a major component of the post-synaptic density – the biochemical correlate of the synaptic cytoskeleton isolated from brain [23].

There is also evidence that the distribution of fodrin can be altered by various stimuli. For example, EGF can recruit fodrin to membrane ruffles in A-431 cells [24]. Interestingly, α-fodrin can be tyrosine phosphorylated in vitro by the protein tyrosine kinase, $pp60^{c-src}$ [25] and by a highly purified kinase from spleen [26]. These results suggest that fodrin may be regulated by tyrosine kinases. Since fodrin, like its erythrocyte homologue, may be involved in anchoring transmembrane proteins, restricting their motion through the plasma membrane, it is a potential candidate for mediating the effects of tyrosine kinases on membrane organization.

4.2. Rapsyn

Rapsyn is a synaptic, peripheral membrane protein originally identified in the electric organ of *Torpedo californica*. Several lines of evidence suggest that it is involved in the clustering of AChR at the synapse. First, it is present at a 1:1 stoichiometry with the AChR at the neuromuscular junction [27]. Second, it can induce the clustering of AChR when the two are coexpressed in heterologous cell systems [28]. Finally, knockout mice deficient in rapsyn fail to cluster AChR at the NMJ [29].

Previous studies have shown rapsyn not to be tyrosine phosphorylated [30]. This discrepancy may be due to the means of solubilizing rapsyn: here, rapsyn was solubilized by boiling in 2% SDS. These highly denaturing solubilization conditions suggest that a subpopulation of rapsyn, tightly associated with the cytoskeleton, may be subject to tyrosine phosphorylation.

4.3. Actin

Tyrosine phosphorylation of actin has best been studied in *Dictyostelium discoideum*. When these cells are returned from starvation to growth medium, there is rapid tyrosine phosphorylation of actin [31,32]. The strong increase in tyrosine phosphorylation is correlated with alterations in the actin cytoskeleton – loss of pseudopods and cell rounding. Similarly, readdition of oxygen to hypoxic endothelial cells also leads to tyrosine phosphorylation of actin and a concurrent reorganization of the actin cytoskeleton [33]. Thus, there is precedent for the reorganization of the actin cytoskeleton associated with increases in tyrosine phosphorylation.

4.4. β-Tubulin

Tyrosine phosphorylation of β-tubulin has been studied in several systems. For example, tubulin is highly tyrosine phosphorylated in neuronal cells in vivo [34]. When membrane fractions enriched in growth cones are activated by cell adhesion molecules such as L1 and NCAM (neural cell adhesion molecules), phosphorylation of tubulin is decreased [35], altering the ability of tubulin to polymerize [36]. Thus, tyrosine phosphorylation of tubulin may be important in the alterations of the cytoskeleton of growth cones in response to substrate recognition. Since agrin is also an extracellular matrix protein, it is conceivable that similar pathways, culminating in the tyrosine phosphorylation of tubulin, are invoked to restructure the synapse.

4.5. Type III intermediate filament

The third protein identified was type III intermediate filament from *Torpedo* [15]. Three intermediate filament components have been identified at the neuromuscular junction. First, desmin has been found to be concentrated at the neuromuscular junction [27]. Second, a 51 kDa protein isolated from *Torpedo* electric organ was found to be concentrated at the synapse [37]. Third, there is a lamin B immunoreactivity which is colocalized with the AChR [38]. Finally, there is an additional 41 kDa protein which is present in an innervation dependent manner at the neuromuscular junction [39]. Whether one or more of these described proteins is identical with the type III intermediate filament cloned here is not clear. Recently, the type III intermediate filament cloned here was isolated by a screen for proteins interacting with the rapsyn protein using the yeast two-hybrid system [40]. This offers the intriguing possibility that rapsyn may recruit intermediate filaments to the synapse to immobilize the signal transducing elements.

4.6. Hsp90

Hsp90 is a highly abundant cytosolic protein in virtually all cells, expressed at even higher levels when cells are exposed to heat. While the functions of other heat shock proteins are gradually being elucidated, the function of hsp90 remains unclear. It has been shown to prevent protein aggregation and misfolding, suggesting a role as a general chaperone; however, several features of hsp90 suggest that it may be an important player in phosphotyrosine mediated signaling.

First, hsp90 has been shown to be involved in the regulation of the glucocorticoid receptor: hsp90 is required for high affinity ligand binding and subsequent transcriptional activation (reviewed in [41]). Interestingly, the release of dioxin receptor from hsp90 is inhibited by genistein, a tyrosine kinase inhibitor [42]. Second, hsp90 has also been identified in a complex with $pp60^{v-src}$. The finding that hsp90 mutations in yeast alter the pattern of tyrosine phosphorylation in $pp60^{v-src}$ but not $pp160^{v-abl}$ transformed cells suggests that hsp90 interactions with tyrosine kinases may be highly specific [43]. Finally, the isolation of hsp83 (a member of the hsp90 family) as an enhancer of sevenless, a receptor tyrosine kinase involved in photoreceptor differentiation, also suggests that it may play a role in phosphotyrosine mediated signal transduction [44].

In summary, several known and novel proteins have been identified as potential tyrosine kinase substrates in the electric organ of *Torpedo californica*. Of the known proteins, several appear to be cytoskeletal proteins. These proteins may contribute to the intense anti-phosphotyrosine staining in the electric organ and at the neuromuscular junction. The time course and functional importance of tyrosine phosphorylation of these proteins awaits further study. However, it seems likely that specialization of the postsynaptic membrane involves tyrosine phosphorylation of various cytoskeletal elements in addition to the AChR.

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