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ION CHANNELS

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Section III

Second Messengers and Biochemical Approaches

[19] Protein Phosphorylation of Ligand-Gated Ion Channels

By ANDREW L. MAMMEN, SUNJEEV KAMBOJ, and RICHARD L. HUGANIR

Introduction

Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of chemical synapses in the nervous system. The major excitatory and inhibitory neurotransmitter receptors in the brain are ligand-gated ion channels. These receptors directly bind neurotransmitters, resulting in the opening of an intrinsic ion channel. The predominant ligand-gated ion channels in the nervous system are the nicotinic acetylcholine receptor, the glutamate receptors, the γ-aminobutyric acid ($GABA_A$) receptors and the glycine receptors. Biochemical and electrophysiologic studies of these receptors have demonstrated that they are multiply phosphorylated by a variety of protein kinases.[1] Phosphorylation of these receptors regulates many functional properties, including desensitization, open channel probability, open time, and subcellular targeting.[1] Because of the central role of ligand-gated ion channels in synaptic transmission, protein phosphorylation of these receptors is a major mechanism in the regulation of synaptic transmission and may underlie many forms of synaptic plasticity.[1-3] In this article we review a variety of techniques to examine the role of protein phosphorylation in the regulation of ligand-gated ion channel function. We review general strategies and methods for characterizing the phosphorylation state of ligand-gated ion channels, identifying phosphorylation sites on these channels, and analyzing the physiologic consequences of channel phosphorylation. To facilitate this discussion, we use the glutamate receptor subunit GluR1 as an example throughout this review.

Biochemical Characterization of Phosphorylation of Ligand-Gated Ion Channels

Ideally, the phosphorylation state of a ligand-gated ion channel should be investigated in the tissue(s) where it is endogenously expressed. For example, the phosphorylation state of glutamate receptors is often analyzed

[1] K. W. Roche, W. G. Tingley, and R. L. Huganir, *Curr. Opin. Neurosci.* **4**, 383 (1994).
[2] L. A. Raymond, C. D. Blackstone, and R. L. Huganir, *Trends Neurosci.* **16**, 147 (1993).
[3] R. A. Nicoll and R. C. Malenka, *Nature* **377**, 115 (1995).

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in brain slice preparations or in primary cultures of central neurons.[4] To identify and characterize specific phosphorylation sites at the biochemical and functional level, however, it is often necessary to study phosphorylation of wild-type and mutant recombinant channels. Because most primary cultures are difficult to transfect and, more importantly, have a background of wild-type channels, wild-type and mutant channels are typically expressed separately in heterologous systems such as the HEK293 or COS cell lines or in *Xenopus* oocytes. These heterologous expression systems simplify the study of channel phosphorylation by allowing biochemical and electrophysiologic comparisons between wild-type and mutant channels. However, to confirm the physiologic relevance of channel phosphorylation and its regulation in heterologous systems, it is always important to compare these findings with results obtained in cells where the channels are natively expressed.

The first step in characterizing the phosphorylation state of a given channel is to determine whether the protein of interest is indeed a substrate for protein kinases. To accomplish this, primary cultures or transfected cells expressing the channel are incubated with ortho[^{32}P]phosphate. The cells will incorporate the labeled phosphate into adenosine triphosphate (ATP) at the γ-phosphate position where it can be transferred to proteins by protein kinases. Care must be taken to allow enough time for the ortho[^{32}P]phosphate to reach equilibrium with the intracellular pools of ATP and the protein kinase substrates. For most cell types this takes around 4 hr and can be monitored by examining the ^{32}P incorporation into the substrate of interest. Following incubation of the cells with ortho[^{32}P]phosphate, the cells can be treated with activators of specific protein kinases for short periods of time or left untreated to examine the basal phosphorylation state of the substrate protein. After the cells are isolated, the ion channels are solubilized from the membrane with detergents and immunoprecipitated from the labeled cell extracts. The isolated channel is then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the dried gels exposed to film to detect ^{32}P incorporation into the channel.

Below is a protocol for the immunoprecipitation of ^{32}P-labeled glutamate receptors from transiently transfected HEK293 cells. The same protocol is used for labeling and immunoprecipitating glutamate receptors from primary cultures by eliminating the transfection step. This protocol may also be easily modified for the labeling and immunoprecipitation of other ligand-gated ion channels from a variety of cell types. Because of the relatively large amounts of radioactivity used in these experiments, special

[4] C. Blackstone, T. H. Murphy, S. J. Moss, J. M. Baraban, and R. L. Huganir, *J. Neurosci.* **14**, 7585 (1994).

care must be taken to avoid ^{32}P contamination and exposure. We recommend practicing the immunoprecipitations with unlabeled cultures prior to working with the labeled material.

Labeling and Cell Extract Preparation

Reagents

Immunoprecipitation buffer (IPB): 10 m*M* sodium phosphate (pH 7.0), 100 m*M* NaCl, 10 m*M* sodium pyrophosphate, 50 m*M* NaF, 1 m*M* sodium orthovanadate, 5 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* EGTA, 1 μM okadaic acid, 1 μM microcystin-LR, and appropriate protease inhibitors

DAY 1

1. Split HEK293 cells onto 100-mm tissue culture plates.

DAY 2

1. Transfect HEK293 cells with the appropriate cDNA. We typically use the calcium phosphate precipitation method to transfect HEK293 cells with 20 μg cDNA/plate.[5]

DAY 4

1. Remove the media from the plates and wash the cells twice with 5 ml phosphate-free MEM (Sigma St. Louis, MO).
2. Add 2.5 ml phosphate-free MEM to each plate.
3. Prelabel each plate with 2 mCi/ml of ortho[^{32}P]phosphate. We order ortho[^{32}P]phosphate as a 50 mCi/ml solution from NEN Dupont and add 100 μl to each plate. Return the plates to the incubator for 4 hr to allow the label to reach steady-state levels.
4. Activate endogenous protein kinases by treating cells with phorbol esters, forskolin, calcium ionophores, etc. Leave one plate untreated to examine basal phosphorylation.
5. Remove "hot" media and rinse each plate twice with 3–4 ml room temperature phosphate-buffered saline (PBS). Carefully dispose of the liquid radioactive waste from these washes.
6. Add 150 μl of room temperature IPB with 1% SDS to each plate and scrape with a cell scraper. Leave the extracts in the plates at this stage.
7. Dilute extracts by adding 750 μl of ice-cold IPB with 1% Triton X-100 to each plate and mix with a cell scraper.

[5] C. D. Blackstone, S. J. Moss, L. J. Martin, A. I. Levey, D. L. Price, and R. L. Huganir, *J. Neurochem.* **58**, 1118 (1992).

8. Transfer the labeled extract to a 15-ml conical tube on ice.
9. Carefully sonicate each sample with a probe sonicator at setting 6 for 20 sec, making sure not to contaminate area with ^{32}P, and return to ice. Sonication breaks up the DNA, making the solution less viscous and easier to work with. Samples can be frozen at this stage, if desired.

Immunoprecipitation of Protein from Labeled Extracts

1. Transfer labeled extract to a 1.5-ml screwtop Eppendorf tube containing 200 μl of a 1 : 1 slurry of protein A-Sepharose beads (Sigma) suspended in IPB with 1% bovine serum albumin (BSA), 50 μl preimmune serum, 15 units DNase, and 150 μg RNase. The addition of DNase and RNase minimizes the contamination of the sample with ^{32}P-labeled DNA or RNA. Rotate tubes for 1 hr at 4°. This step preabsorbs any protein that may stick nonspecifically to the protein A-Sepharose beads.
2. Centrifuge at 2000 rpm for 1 min and add supernatant to a 1.5-ml screwtop Eppendorf tube containing 200 μl of the 1 : 1 slurry of protein A-Sepharose beads and the precipitating antibody. Rotate tubes for 2 hr at 4°.
3. Wash the beads with 1 ml each of IPB with 1% Triton X-100 (×2), IPB with 1% Triton X-100 and 500 m*M* additional NaCl (×3), and IPB (×2). Carefully dispose of these radioactive washes.
4. Elute immunoprecipitated material from beads with 150 μl SDS–PAGE sample buffer. Analyze samples by SDS–PAGE and stain the gel with Coomassie blue. Then, dry the gel between two sheets of cellophane (Bio-Rad, Richmond, CA) and expose to film.

In the experiment shown in Fig. 1, we transfected HEK293 cells with wild-type GluR1, a mutant form of GluR1 in which the serine at residue 845 has been converted to an alanine (GluR1 S845A), and used mock transfected cells as controls. These cells were labeled with ortho[^{32}P]phosphate, treated with phorbol ester (a PKC activator), forskolin (which indirectly stimulates PKA activity), and IBMX, and immunoprecipitate with anti-GluR1 antibodies as described above. Figure 1A shows that both the wild-type and mutant receptors are phosphorylated under these conditions. Although we have previously shown that serine-845 is a substrate for phosphorylation PKA[6] (and see below), in this experiment, the mutant receptor

[6] K. W. Roche, R. J. O'Brien, A. L. Mammen, J. Bernhardt, and R. L. Huganir, *Neuron* **16**, 1179 (1996).

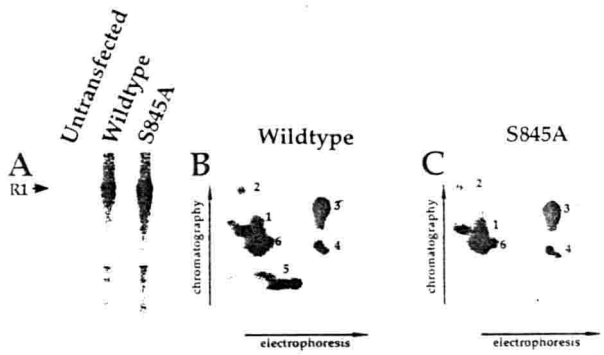

FIG. 1. Immunoprecipitation and mapping of [^{32}P]-labeled channels from transfected cells. QT6 cells were transfected with wild-type GluR1, mutant GluR1 (S845A), or mock transfected. These plates were incubated with ortho[^{32}P]phosphate for 4 hr, treated with 100 nM phorbol 12-myristate 13-acetate (PMA), 10 μM forskolin (FSK), and 100 μM IBMX (IBMX) for 15 min. GluR1 was immunoprecipitated from detergent extracts of each plate (A). Two-dimensional phosphopeptide maps were generated from immunoprecipitated GluR1 (B) and GluR1 S845A (C).

appears to be phosphorylated even more robustly than the wild type. This is because GluR1 contains phosphorylation sites for other protein kinases (including PKC[6]) and the mutant receptor was expressed at higher levels than the wild-type receptor in this experiment. This result exemplifies that careful controls to examine the expression level of your protein of interest need to be performed in order to quantitate phosphorylation. No labeled receptor was immunoprecipitated from mock transfected cells.

Phosphopeptide Mapping of Ligand-Gated Ion Channels

The next step in analyzing the phosphorylation of a ligand-gated ion channel involves subjecting the ^{32}P-labeled phosphoprotein to two-dimensional phosphopeptide map and phosphoamino acid analysis. In the first procedure, the labeled material is excised from the gel and digested with protease. The resulting peptides are spotted onto a cellulose thin-layer chromatography (TLC) plate and subjected to electrophoresis. This separates the proteolytic fragments according to charge. Next, the plate is subjected to ascending chromatography where the peptides are separated according to their solubility in the chromatography buffer. The TLC plates are then exposed to film in order to visualize the phosphopeptides. Each ligand-gated ion channel subunit will yield a unique map depending on the location of its proteolytic cleavage sites and phosphorylated residues.

Below is a protocol for the two-dimensional phosphopeptide map analysis of glutamate receptors. To achieve optimal separation of phosphopeptides generated from other ligand-gated ion channels, it may be necessary to employ different proteases, electrophoresis times, and ascending chromatography buffers. These issues—and phosphopeptide mapping in general—are discussed thoroughly elsewhere.[7]

Proteolytic Digestion

Reagents

Destain solution: 25% methanol/10% acetic acid in H_2O

1× Trypsin solution: 0.3 mg/ml trypsin TPCK (Sigma) dissolved in 50 m*M* NH_4HCO_3

10× Trypsin solution: 3 mg/ml trypsin TPCK dissolved in 50 m*M* NH_4HCO_3

Procedure

1. Cut the ^{32}P-labeled ligand-gated ion channel out of the dried acrylamide gel and place in a glass scintillation vial.
2. Wash each protein-containing gel fragment with 20 ml destain solution for 30 min (×3).
3. Wash with 20 ml 50% methanol for 30 min (×2).
4. Transfer the gel fragment to an Eppendorf tube and dry down in a Speed-Vac, approximately 2 hr.
5. To each tube, add 1 ml of 1× trypsin solution and incubate overnight at 37°. The next morning add 100 μl of a 10× trypsin solution and incubate an additional 3–4 hr at 37°.
6. Remove the supernatant from the tube and save. Add 1 ml H_2O to the gel fragment-containing tube and incubate 1 hr at 37°.
7. Combine the supernatants and dry down in a Speed-Vac. Resuspend the dried material in 0.5 ml H_2O and dry down again; repeat three times to completely sublimate the NH_4HCO_3.
8. Resuspend the dried material in 10 μl H_2O and microcentrifuge at 14,000 rpm for 10 min.
9. Remove the supernatant to a fresh Eppendorf tube and count in a scintillation counter (Cerenkov method).

[7] W. J. Boyle, P. V. D. Geer, and T. Hunter, *Methods Enzymol.* **201,** 110 (1991).

Two-Dimensional Thin Layer Chromatography

Reagents

Electrophoresis buffer (pH 3.4): acetic acid : pyridine : H_2O at 19 : 1 : 89
Chromatography buffer: pyridine : butanol : acetic acid : H_2O at 15 : 10 : 3 : 12
Basic fuchsin solution: 1 mg/ml solution
Phenol red solution: 1 mg/ml solution

Procedure

1. On a 20- × 20-cm Kodak (Rochester, NY) 13255 cellulose TLC sheet, make a single pencil mark at the sample origin, 10 cm from each side and 4 cm from the bottom of the sheet. Make two additional pencil marks 5 cm from each side and 4 cm from the bottom of the sheet.
2. Spot at least 250 cpm of the sample at the sample origin. However, do not spot more than about 2000 cpm of the sample per plate. Use only a fraction of the 10-μl sample if possible because overloading of the sample can cause streaking of the phosphopeptides. The sample should be spotted 1 μl at a time with drying in between (a hair dryer works much faster than air drying). Avoid gouging the TLC plate when spotting the sample.
3. Spot 1 μl each of basic fuchsin and phenol red solutions at the origin.
4. Prepare two sheets of 25- × 25-cm Whatman paper. Place a pencil mark 6.5 cm from the bottom and 12.5 cm from each side of one piece of Whatman paper; using the mark as its center, cut a 3-cm-diameter hole in this piece of Whatman paper. Place the spotted TLC plate on top of the intact piece of Whatman paper. Prewet the Whatman paper with the hole with pH 3.4 electrophoresis buffer. Lay the prewetted Whatman paper on the TLC plate so that the spotted sample appears in the middle of the hole. With a Pasteur pipette, dribble pH 3.4 electrophoresis buffer on the top piece of Whatman paper until the entire TLC plate underneath is wet. To avoid movement of the sample away from the origin, one should dribble electrophoresis buffer around the perimeter of the hole such that the diffusing buffer reaches the spotted sample from all sides simultaneously.
5. While still wet, place the TLC plate in an electrophoresis tank containing the electrophoresis buffer with the spotted sample in the middle between the two electrodes. Run at 500 V until the dyes reach the marks 5 cm from the edges of the plate (about 1.5 hr).

6. Remove the plate and allow to dry completely.
7. Place the plate in an ascending chromatography chamber with the bottom of the plate submerged in the chromatography buffer. When the buffer is 1 cm from the top of the plate (4–6 hr) remove it and allow to dry completely.
8. Wrap the TLC plate in Saran wrap and expose to film. For faster results place the wrapped plate in a PhosphoImager cassette.

The two-dimensional phosphopeptide map generated for each ligand-gated ion channel represents a highly reproducible "phosphopeptide fingerprint" of that channel. In the case of wild-type GluR1, phosphopeptide maps of the immunoprecipitated receptor from phorbol ester and forskolin treated HEK293 cells include seven distinct phosphopeptides (Figure 1B). However, this does not necessarily imply that there are seven distinct phosphorylation sites on GluR1; incomplete protease digestion is common and often generates multiple phosphopeptides, which include the same phosphorylation site. In fact, we have shown that GluR1 phosphopeptides 3, 4, and 6 all include the same PKC phosphorylation site.[6]

Phosphoamino Acid Analysis of Ligand-Gated Ion Channels

Serine, threonine, and tyrosine are each substrates for protein phosphorylation, and determining which of these amino acids are phosphorylated is an important step in characterizing the phosphorylation of a ligand-gated ion channel. To perform phosphoamino acid analysis, follow the protocol for proteolytic digestion of the sample described above and then use the isolated sample for acid hydrolysis to individual amino acids.

Phosphoamino Acid Analysis Protocol

Reagents

Electrophoresis buffer (pH 1.9): formic acid : acetic acid : H_2O at 1 : 10 : 89

Electrophoresis buffer (pH 3.4): acetic acid : pyridine : H_2O at 19 : 1 : 89

Procedure

1. Add at least 500 cpm of the sample to a 16- × 75-mm black-topped Kimax tube with a Teflon screw cap containing 0.5 ml 6 *N* HCl. Blow N_2 gently over the liquid before screwing the lid on tightly.
2. Place in a 105° oven for 1–2 hr and then transfer to a microcentrifuge tube and Speed-Vac until dry.
3. Resuspend the sample in 0.5 ml H_2O and redry.
4. Resuspend the sample in 10 μl H_2O and vortex 30 sec. Spin the

tube at 14,000 rpm for 10 min and transfer the supernatant to a second microcentrifuge tube.

5. Prepare a Kodak TLC 13255 cellulose sheet as follows: Mark the two side edges of the sheet with a pencil 4 cm from the bottom. Lay a ruler across the sheet between the two marks and, starting 2 cm from the side, make pencil marks every 4 cm. Up to five samples may be spotted at these five origins. Make additional marks 5 and 14 cm above where the samples will be spotted.
6. Spot the samples 1 μl at a time with drying in between.
7. Prepare fresh phosphoserine, phosphothreonine, and phosphotyrosine standards at 10 mg/ml in H_2O. Spot 1 μl of each on top of each spotted sample. Also spot 1 μl of phenol red on each sample.
8. Cut three pieces of Whatman paper: 25 × 25 cm, 25 × 17 cm, and 25 × 5 cm. Lay the TLC plate on the large piece of Whatman paper and prewet the others with the pH 1.9 electrophoresis buffer. Lay the 25- × 17-cm Whatman paper over the TLC plate such that its lower edge is 1.5 cm above the spotted samples. Lay the 25- × 5-cm Whatman paper such that its upper edge is 1.5 cm below the spotted samples. Drip pH 1.9 solution onto the prewetted Whatman papers until the TLC plate underneath is wet. The buffer should gradually diffuse from the upper and lower pieces of Whatman paper to wet the samples without moving them.
9. While wet, place the TLC plate in an electrophoresis tank containing pH 1.9 electrophoresis buffer. The samples should be nearest the cathode. Electrophorese the samples at 500 V until the phenol red dye reaches the first pencil mark, 5 cm above where the samples were spotted.
10. Without allowing it to dry, transfer the plate to an electrophoresis tank containing pH 3.5 electrophoresis buffer and electrophorese at 500 V until the phenol red dye reaches the next pencil mark, 14 cm above where the samples were spotted.
11. Dry the TLC plate, then dip in ninhydrin (1% in acetone) and allow to dry. The phosphoamino acid standards will turn purple in about 15 min. Phosphoserine migrates at the front, followed by phosphothreonine and, finally, phosphotyrosine.
12. Cover the plate with Saran wrap and expose to film.

Fusion Protein Phosphorylation Studies

Ligand-gated ion channels are composed of relatively large polypeptides which may contain many serines, threonines, and tyrosines. Thus, determining which residues are phosphorylated may seem like a daunting task.

However, if the protein kinase that phosphorylates the channel has been identified, one can begin by searching the protein for the appropriate protein kinase consensus sites.[8] Furthermore, if the topology of the channel in the membrane has been determined, candidate sites can be narrowed to those that are present on intracellular regions of the channel. Once regions likely to include phosphorylation sites have been identified, it is often useful to make fusion proteins corresponding to these regions and phosphorylate them *in vitro* with purified protein kinases. If the channel of interest has large continuous domains which might contain phosphorylation sites, it is helpful to generate several smaller fusion proteins (50–100 residues long) which span the length of the domain. These fusion proteins can then be analyzed by two-dimensional phosphopeptide mapping and the resulting maps compared to those generated from native channels or full-length recombinant channels expressed *in vivo*.

In Vitro Fusion Protein Phosphorylation

Reagents

5× PKA reaction buffer: 50 m*M* HEPES, pH 7.0, 100 m*M* $MgCl_2$
5× PKC and CaMKII reaction buffer: 50 m*M* HEPES, pH 7.0, 50 m*M* $MgCl_2$, 5 m*M* $CaCl_2$
ATP mixture: 250 μM ATP (2000 cpm/pMol)

Procedure

1. Add 10 μl (1 μg) of a 0.1 mg/ml solution of fusion protein substrate (dissolved in PBS) to a 1-ml microcentrifuge tube on ice.
2. Add 20 μl of the appropriate 5× reaction buffer to each tube.
3. For PKC reactions add 10 μl of a 500 μg/ml phosphatidylserine, 50 μg/ml diolein solution in H_2O. For CaMKII reactions add 10 μl of a 0.3 mg/ml calmodulin solution in H_2O.
4. Add H_2O to bring volume to 90 μl.
5. Add 9 μl ATP mixture.
6. Begin reaction by adding 1 μl of a 100 μg/ml solution of the desired protein kinase and incubate at 30° for 30 min. Terminate the reactions by adding 50 μl of 3× sample buffer and analyze by SDS–PAGE. Stain the gel with Coomassie blue, dry the gel between two sheets of cellophane, and expose to film. Cut out the appropriate band from the gel to perform phosphopeptide map and phosphoamino acid analysis as described above.

[8] R. B. Pearson and B. E. Kemp, *Methods Enzymol.* **200,** 62 (1991).

When comparing fusion protein maps with maps from full-length channels, one should look for phosphopeptides that are of similar shape and comigrate on TLC plates. Figure 2B shows the map of a fusion protein corresponding to the C terminus of GluR1 phosphorylated *in vitro* with purified PKA. Note the presence of a phosphopeptide cluster that closely resembles and seems to comigrate with phosphopeptide 5 from full-length receptor (Fig. 1B). This finding suggests that phosphopeptide 5 may be contained within this fusion protein and that it may be a substrate for PKA phosphorylation. (To confirm that phosphopeptides from the two preparations actually comigrate, it is often useful to spot both on the same TLC plate and look to see whether they overlap when processed together.) Also note the presence of an additional phosphopeptide not observed in maps of the full-length receptor (indicated by an arrow). Such spurious phosphopeptides are often seen when proteins are phosphorylated *in vitro* and may represent phosphorylation at sites that are normally extracellular or otherwise inaccessible to protein kinases.

Site-Specific Mutagenesis of Phosphorylation Sites

Once phosphorylation sites have been narrowed to small domains within a channel and the identity of the phosphorylated amino acid has been determined by phosphoamino acid analysis, candidate serines, threonines, and tyrosines can be targeted for point mutation. Typically, alanines are substituted for serines and threonines whereas phenylalanines are substituted for tyrosines. The resulting mutant recombinant receptors are then expressed in transfected cells and phosphopeptide maps generated as pre-

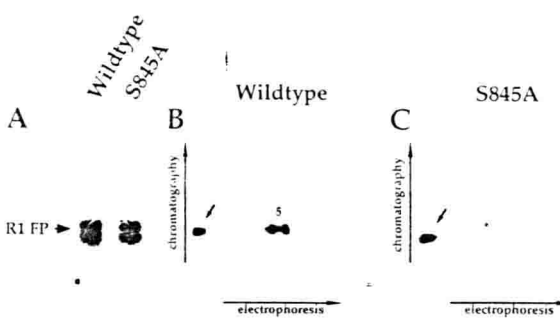

FIG. 2. *In vitro* phosphorylation and mapping of fusion proteins. Fusion proteins corresponding to the C termini of GluR1 and GluR1 S845A were phosphorylated *in vitro* with purified PKA and analyzed by SDS–PAGE (A). Two-dimensional phosphopeptide maps were generated from the PKA phosphorylated GluR1 (B) and GluR1 S845A (C) C-terminal fusion proteins.

viously described. In the case of GluR1, for example, mutating Ser-845 to Ala abolished phosphopeptide 5 (compare Figs. 1B and 1C). Mutant fusion proteins may also be created, phosphorylated *in vitro*, and screened by two-dimensional map analysis. Figure 2C shows that a phosphopeptide map of the S845A C-terminal GluR1 fusion protein phosphorylated *in vitro* with purified PKA does not include phosphopeptide 5. Taken together with the finding that phosphopeptide maps of GluR1 from cells which are not treated with forskolin do not include phosphopeptide 5 (not shown), these results suggest that PKA phosphorylates Ser-845 on GluR1. In general, the absence of wild-type phosphopeptides from maps of mutant channels is taken as strong evidence that the mutated residues are sites of protein phosphorylation.

Phosphorylation Site-Specific Antibodies

After a specific channel residue has been identified as a phosphorylation site, its phosphorylation state in a number of different preparations and under a wide variety of conditions is often of interest. To facilitate such studies, phosphorylation site-specific antibodies which recognize the channel only when the residue of interest is phosphorylated can be generated. These antibodies reduce the difficulty and expense associated with phosphopeptide mapping and allow the rapid screening of many samples by Western blot analysis. To generate such antibodies, phosphopeptides with chemically phosphorylated serine, threonine, or tyrosine residues must be synthesized and injected into rabbits. We have found that 12-mers with the phosphorylated residue at the sixth position work well. Including a lysine at the end of the phosphopeptide to facilitate coupling to the carrier thyroglobulin is also helpful (see Ref. 9 for a detailed discussion of phosphopeptide synthesis methods).

Phosphorylation site-specific antibodies can be separated from antibodies that recognize the nonphosphorylated peptide by loading the serum on an affinity column containing the nonphosphorylated equivalent of the phosphopeptide antigen. The phosphorylation site-specific antibodies should be contained in the flow-through along with other nonspecific antibodies which fail to bind the nonphosphorylated peptide. Often, the serum contains very few antibodies that recognize the nonphosphorylated peptide, and this first purification step may not be necessary. To purify the antibody of interest away from nonspecific antibodies, the flow-through from the first column (or crude serum) may be loaded onto an affinity column containing the phosphopeptide antigen. After washing the column, the

[9] W. G. Tingley, M. D. Ehlers, K. Kameyama, C. Doherty, J. B. Ptak, C. T. Riley, and R. L. Huganir, *J. Biol. Chem.* **272**, 5157 (1997).

phosphorylation site-specific antibodies can be eluted with standard methods and used for Western blot analysis.

Figure 3 demonstrates the utility of a phosphorylation site-dependent antibody that recognizes GluR1 only when Ser-845 is phosphorylated (GluR1 845-P). QT6 cells were transfected with wild-type or mutant GluR1 and treated with phorbol ester, forskolin and IBMX, or vehicle. Membrane extracts were prepared from each sample, separated by SDS–PAGE, blotted onto PVDF membrane, and analyzed by Western blot. Forskolin treatment caused an increase in GluR1 845-P labeling of wild-type channel, confirming that PKA phosphorylates GluR1 on Ser-845. However, when the blot was treated with lambda phosphatase prior to application of the antibody, GluR1 845-P did not label GluR1 from forskolin-treated cells. Furthermore, when QT6 cells were transfected with the GluR1 S845A channel and treated with forskolin, the GluR1 845-P antibody did not recognize the mutant channel. These two results demonstrate the phosphorylation state dependence of the GluR1 845-P antibody. A different antibody

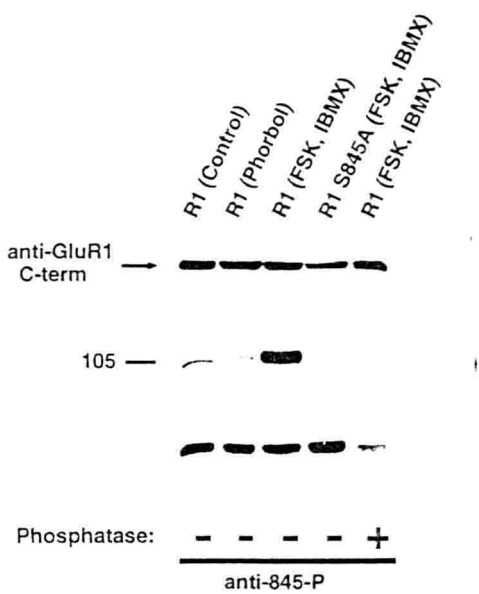

FIG. 3. Specificity of a phosphorylation site-specific antibody. QT6 cells expressing GluR1 or GluR1 S845A were treated with control solution (Control), 100 n*M* phorbol 12-myristate 13-acetate (PMA), or 10 μM forskolin and 100 μM IBMX (FSK, IBMX) for 15 min as indicated. Membranes were prepared and run on SDS–PAGE, transferred to PVDF membranes, and immunoblotted with the phosphorylation site-specific antibody GluR1 845-P or an antibody to the C terminus of GluR1.