Tyrosine phosphorylation of rabphilin during long-lasting long-term potentiation

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Abstract

Long-term potentiation (LTP) is a persistent increase in the strength of synaptic transmission triggered by neuronal activity. Here, we submitted hippocampal slices to a perfusion of forskolin and IBMX, which induces a long-lasting LTP (>4 h) (L-LTP). We separated the proteins of the CA1 region by two-dimensional gel electrophoresis (2-DE). We then immunoblotted them using an anti-p-Tyr antibody. We found a protein whose tyrosine phosphorylation was unchanged 10 min after LTP induction but was dramatically increased after 1 h, dropping back to its baseline after 4 h. This protein was identified as rabphilin using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). We also demonstrated that genistein, an inhibitor of tyrosine phosphorylation, prevented the development of the late phase of electrically-induced L-LTP. Our results suggest that rabphilin, a protein present in presynaptic terminals, could play a role in the late phase of L-LTP.
were then rapidly stored at −80 °C until used. Each CA1 sample intended to be submitted to further biochemical analysis was obtained by pooling the CA1 regions taken from six individual slices. Samples were homogenized in a buffer (5 μL per CA1) containing protease inhibitor cocktail (Roche, Complete Mini EDTA-free) and phosphatase inhibitor cocktails (Sigma). Samples prepared for 2-DE were homogenized in buffer 2 of a Ready Prep Sequential Extraction Kit (Bio-Rad). For samples prepared for SDS-PAGE, the homogenization buffer contained 10 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA and 1% (v/v) Triton X-100. Each individual CA1 provided 40–50 μg of protein.

Cell lysates were diluted in 8 M urea, 4% (w/v) CHAPS, 2 mM TBP, 40 mM Tris, 0.2% (w/v) Biolyte 3/10 (Bio-Rad). Proteins (100 μg/sample) were separated in the first dimension by isoelectric focusing performed at 20 °C in a Multiphor II apparatus (Amersham Biosciences) using Immobiline Dry Strips, pH 6–11 (Amersham Biosciences). After focusing, proteins in the IPG strips were reduced and alkylated as described before [18]. The strips were then loaded on top of the second dimension gel (4–15% acrylamide precast Criterion gels, Bio-Rad) by submerging them in warm agarose solution [1% (w/v) low melting agarose, 0.2% (w/v) SDS, 150 mM Tris–HCl pH 6.8 and bromophenol blue]. Electrophoresis was performed in a Criterion tank (Bio-Rad) at constant voltage (200 V) with a maximal current of 125 nA/gel. The proteins contained in the gels were either electrically transferred to a nitrocellulose membrane or stained with a mass spectrometry-compatible silver staining [16].

After the 2-DE and electrotransfer, the nitrocellulose membranes were blocked overnight at 4 °C by TTBS buffer [Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20] containing 1% (w/v) BSA and then incubated with a rabbit anti-p-Tyr polyclonal antibody [1:10000 dilution in TTBS containing 1% (w/v) BSA; Chemicon International, Temecula, CA, USA] for 2 h at room temperature. Primary antibody was visualized using horseradish peroxidase-conjugated donkey antirabbit IgG antibody [1:1000 dilution in TTBS containing 1% (w/v) BSA]. SuperSignal West Pico chemiluminescent substrate (Pierce) was used for revelation. The spots were scanned and quantified using PDquest 7.0 Software (Bio-Rad Laboratories, Hercules, CA, USA).

Stained protein spots or bands were excised, washed and digested using 10 μL of modified trypsin (10 ng/μL) (Promega, Madison, WI, USA). After incubation at 37 °C overnight, the peptides were extracted manually and purified with ZipTip C18 microcolumns (Millipore), according to the instructions provided by the manufacturer. Peptides were applied to the MALDI target plate as described previously [11]. The mass spectra were obtained automatically by MALDI-TOF MS in reflectron mode (M@LDI-TOF, Micromass, Manchester, UK). This was followed by automatic internal calibration using tryptic peptides from trypsin autodigestion. The resulting peptide mass list was compared to the NCBI non-redundant database (downloaded locally) using the search engine Mascot.

In other experiments CA1 proteins were extracted in laemmli solubilization buffer and immunoprecipitation was carried out as described by Ohya et al. [19] with minor modifications. Ten microliter of anti-rabphilin antibodies (Santa Cruz Biotechnology) were preincubated with 30 μL of protein A Agarose (Roche Diagnostics) for 1 h at 4 °C with gentle rocking. After centrifugation at 1000 × g for 2 min and addition of 200 μg of CA1 proteins, the extract was incubated for 3 h at 4 °C with gentle rocking. The complex agarose beads were then washed once with PBS buffer containing 0.5% (v/v) Triton X-100, twice with PBS buffer containing 0.5% (v/v) Triton X-100 and 0.5 M NaCl, and finally twice in PBS buffer. The proteins bound to protein A were eluted with laemmli solubilization buffer, applied to 8% SDS-PAGE and immunoblotted using anti-p-tyrosine antibody or silver staining.

In preliminary studies, we observed that application of FSK (50 μM) and IBMX (30 μM) for 15 min produced an LTP lasting more than 4 h [3]. The slope of the fEPSP increased to 258 ± 17% (mean ± S.E.M.) of baseline 1 h after the end of the perfusion and was still as high as 204 ± 19% of baseline 3 h later (n = 15) (Fig. 1A).

Slices dedicated to further biochemical analysis were perfused with either FSK–IBMX (treated slices) or DMSO 0.02% (control slices). Six slices were submitted to the same perfusion together, the fEPSP slope of one of them being monitored. Ten min, 1 h or 4 h after the end of the perfusion, CA1 regions were cut out from the six individual slices and pooled for further biochemical analysis (Fig. 1B).

Tyrosine phosphorylation of proteins from CA1 samples was investigated using a 2-DE approach coupled with a labeling technique based on a specific anti-phosphotyrosine antibody (Fig. 1C). Phosphotyrosine 2-DE maps of CA1 proteins from control and FSK plus IBMX-treated hippocampal slices were then compared (Fig. 1D). Gels for CA1 samples were run by pairs (one control and one treated). In each of the six in-parallel experiments, a protein spot characterized by a molecular weight around 75 kDa and a pI ranging from 6.5 to 7.5 appeared much more phosphorylated in treated slices when sampling occurred 1 h after LTP induction (Fig. 1C and D). Spot density of this protein (referred to as “protein 1” in Fig. 1C) increased from 85 ± 6 d.u. in controls to 295 ± 89 d.u. One hour after LTP induction (p < 0.05) (Fig. 1E). By contrast, the sum of the spot densities of three p-Tyr proteins taken as references (referred to as “proteins 2, 3 and 4” in Fig. 1C) did not change significantly (41 ± 10 d.u. in controls and 41 ± 6 d.u. after LTP induction, p = 0.89) (Fig. 1F). The increase of tyrosine phosphorylation of “protein 1” was not yet detected 10 min after FSK plus IBMX treatment (59 ± 41 d.u. versus 79 ± 41 d.u., p = 0.75) and was no longer observed 4 h after LTP induction (99 ± 61 d.u. versus 177 ± 65, p = 0.42).

In almost all cases, a comparison between the immunoblot anti-p-Tyr and the silver-stained gel from the same experiment allowed unambiguous location of the tyrosine-phosphorylated proteins on the 2-DE gel. The corresponding spots were then
Fig. 1. Tyrosine phosphorylation of proteins from CA1 samples taken from hippocampal slices treated either with DMSO (sham experiments) or with FSK–IBMX dissolved in DMSO. (A) Induction of LTP by a 15-min perfusion of forskolin (FSK, 50 μM) and IBMX (30 μM). The strength of the synapses between Schaffer collaterals of the CA3 neurons and CA1 pyramidal neurons was assessed by stimulating the Schaffer collaterals and recording the field excitatory synaptic potential (fEPSP) in the CA1 region. The slope of the fEPSP is shown in function of time. After application of FSK/IBMX, the slope of the fEPSP increased for at least 4 h. (B) Sketch showing how the CA1 region was removed for subsequent biochemical analysis. Thick lines represent the cuts made with a scalpel blade. (C) Immunoblot with anti-p-Tyr antibodies from a map of proteins separated by 2-DE. The spots highlighted in the box correspond to a single protein, called “protein 1”. Three spots corresponding to three distinct proteins, which are circled, were considered as reference proteins and called “proteins 2, 3 and 4”. (D) Illustration of the spots of the protein boxed in C (“protein 1”) in 3 CA1 samples from DMSO-treated slices and in 3 CA1 samples from FSK/IBMX-treated slices when samples were taken 1 h after the treatment. In each experiment, a sham sample and a FSK/IBMX-treated sample were processed in parallel. (E) Comparison of the mean densities of the spot of interest in control gels (white columns) and in gels prepared from samples treated with FSK/IBMX (black columns) 10 min, 1 h and 4 h after LTP induction. Data are shown as mean ± S.E.M. A significant difference (indicated with an asterisk) was observed 1 h, but not 10 min or 4 h after LTP induction. (F) Comparison of the mean densities (in control and in treated gels) of the other three spots (“proteins 2, 3, 4”) that have been pooled. No significant difference was observed.

punched out for further analysis using MALDI-TOF MS. MALDI-TOF MS analysis confirmed that the spots labeled “proteins 2, 3, and 4” corresponded to distinct proteins, whereas the pooled spots labeled “protein 1” corresponded to a single protein (see below).

Identification of the protein showing a major change in tyrosine phosphorylation (“protein 1” in Fig. 1C) was in fact more difficult. Despite the fact that this protein was detected by its large spot on the phosphotyrosine 2-DE map, there was no corresponding large spot on the silver-stained gel, because it corresponded to a negatively (and not to a positively) stained spot [7]. To get around this problem, the identification of “protein 1” was achieved using protein spots corresponding to NSF as landmarks. In this context, proteins from a same CA1 sample taken from slices treated with FSK–IBMX were separated on two large 2-DE gels (pH 6–11, 16 cm × 14 cm). Six hundred
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microgram of proteins were placed on the gel intended for silver staining (according to the Vorum protocol [16]), whereas 300 µg were placed on the gel from which blotting was to be done. Immunoblotting with anti-p-Tyr antibodies revealed the position of “protein 1”. The nitrocellulose membrane was then stripped and restained with a rabbit anti-NSF antibodies (30 µg/100 ml, Lake Placid, NY, USA). Superimposition of the two corresponding autoradiographs taken from the same membrane showed that the NSF spot consisted in a long strip located just under “protein 1”. After comparing the silver-stained gel with the two autoradiographs, “protein 1” was identified as rabphilin thanks to peptide mass fingerprinting (Fig. 2).

To ascertain that “protein 1” was rabphilin, we used immunoprecipitation. Proteins from a new CA1 sample (taken from six individual slices submitted to FSK–IBMX) were immunoprecipitated using anti-rabphilin antibodies. The resulting immunoprecipitate was divided into two parts, which were run along two lanes of 8% SDS-PAGE gel. The two lanes were

![Graph showing relative intensity of protein bands](image)

Fig. 2. Identification of the protein of interest by peptide mass fingerprinting. (A) MS spectrum of the protein spot corresponding to “protein 1” after trypsin digestion. \( m/z \) = mass divided by electric charge. (B) Identification of the peptides corresponding to rabphilin. These peptides are tagged on the spectrum by an (*). (C) Sequence of rabphilin (mus musculus). Areas in gray correspond to matches with experimentally identified peptides.
then cut apart. One was silver stained according to the Vorum protocol, whereas the other was the starting point for anti-p-Tyr immunoblotting (Fig. 3). Analysis of the 75 kDa band on the silver-stained gel using MALDI-TOF MS confirmed that this band was rabphilin. Labeling with anti-p-Tyr antibody confirmed that this protein was indeed phosphorylated at a tyrosine residue.

Finally, we had to rule out the possibility that the observed increase in immunoreactivity of the rabphilin spot to anti-p-Tyr antibody was due to an increased synthesis of that protein under the action of FSK–IBMX. Proteins from four control CA1 samples and four treated CA1 samples (30 μg each) were separated on an 8% SDS-PAGE gel and subsequently immunoblotted using anti-rabphilin antibodies (1:200 dilution). There was no difference between the two groups. Band density of rabphilin using anti-rabphilin antibodies (1:200 dilution). There was no

Here, using a perfusion of forskolin and IBMX, we induced a phosphorylation of rabphilin in the CA1 area (1) which was located at a tyrosine and (2) which was late. This result is not in contradiction with previous works about rabphilin phosphorylation or with theoretical predictions. Lonart and Südhof [13] found out that when CA1 and CA3 synaptosomes were treated with forskolin or high K+-induced Ca++ influx, rabphilin phosphorylation increased selectively in CA3 synaptosomes but not at all in CA1 synaptosomes. Although they assessed rabphilin phosphorylation using 32P-orthophosphate labeling – and therefore explored tyrosine phosphorylation as well as serine/threonine phosphorylation – their result is not in contradiction with ours. Indeed, their measurements were made in the minutes following the treatment and not 1 h later, the point in time where we observed a phosphorylation of rabphilin at a tyrosine residue. In brain slices, incubation with either high K+ Ringer’s solution or a combination of a membrane-permeable analog of cAMP and IBMX triggers a rapid (within min) but transient phosphorylation of rabphilin at serine-234 and at serine-274 [4]. In the work of Foletti et al. [4], phosphorylation was detected using antibodies specific for rabphilin phosphorylated at S234 and S274; tyrosine phosphorylation was not investigated. Moreover, phosphorylation sites at tyrosine can be predicted by computer programs. NetPhos 2.0, an artificial neural network method available on the Internet [2], predicts seven sites of the kind on rabphilin: Tyr 322, Tyr 372, Tyr 418, Tyr 572, Tyr 610, Tyr 629 and Tyr 637.

A direct piece of evidence for a role played by rabphilin in L-LTP would be provided by a defect of L-LTP in rabphilin knockout mice. Unfortunately, this has not been tested yet. Rabphilin is an effector of Rab3A. Rab3A is not necessary for the S-LTP but is required for the expression of the late phase of CA3-CA1 L-LTP [8]. The question of a potential involvement of rabphilin in synaptic plasticity was investigated directly in rabphilin knockout mice. No abnormalities were found in the S-LTP induced by cAMP-dependent protein kinase (PKA) in activation of its substrate, the activator of guanylyl cyclase, cGMP-PKG, and PKG was shown to be activated by the inhibitor of PKA, which was caused by a reduction in PKA activity [21]. In this case, the role of PKG would be to promote the activation of PKA and thereby to enhance the cAMP-dependent activation of PKA. The mechanism by which PKG and PKA interact is still unknown. However, it is possible that PKG can phosphorylate PKA on serine residues, leading to the activation of PKA.

Here, we found that a perfusion of FSK/IBMX, which triggered a long-lasting LTP, also induced a tyrosine phosphorylation of rabphilin in the CA1 region, which was maximal 1 h after the end of the stimulation. This suggested that this late phosphorylation could play a role in L-LTP. Interestingly, using electrical LTP, we found that tyrosine phosphorylation played a role in the late phase of L-LTP (Fig. 4). LTP was triggered by four trains of high frequency stimulation (100 Hz, 1 s) delivered 5 min apart to the Schaffer collaterals of hippocampal slices either in a control situation or in presence of genistein (100 μM), an inhibitor of tyrosine phosphorylation. One hour after induction, the increase in the slope of fEPSP was not significantly lower in presence of genistein (206 ± 20%, n = 5) than in its absence (242 ± 45%, n = 9, p = 0.18). In contrast, 4 h after induction, the increase in the slope of fEPSP was dramatically reduced under the influence of genistein (112 ± 15% versus 248 ± 29% in the control state, p < 0.05).

Rabphilin was discovered as a protein interacting with the GTP-binding protein Rab3A, a protein present on synaptic vesicles [21]. Rabphilin as well as Rab3A are involved in the synaptic vesicle life cycle (for reviews, see [5,9,12,22,23]).

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a single train (100 Hz, 1 s) of high-frequency stimulation [20]. Unfortunately, L-LTP, the only type of LTP which was found to be impaired in Rab3A knock-out mice, was not tested in the study by Schlüter et al. [20].

The fact that the late phase of L-LTP is much more hindered than its early phase by genistein is a piece of evidence that tyrosine phosphorylation is important for the maintenance of L-LTP. It remains to demonstrate that, among the proteins phosphorylated at the tyrosine level, rabphilin plays a significant role. The most stringent approach would be to document in rabphilin knock-out mice both a defect in the late phase of L-LTP and the absence of pTyr-“protein1” signal.

In conclusion, using a perfusion of FSK and IBMX, we induced phosphorylation of rabphilin (1) which was located at a tyrosine residue and (2) which was late. Using genistein, we demonstrated that tyrosine phosphorylation played an important role in the late phase of L-LTP. These results suggest that rabphilin, a presynaptic protein, could play a role in the late phase of long-lasting LTP.

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