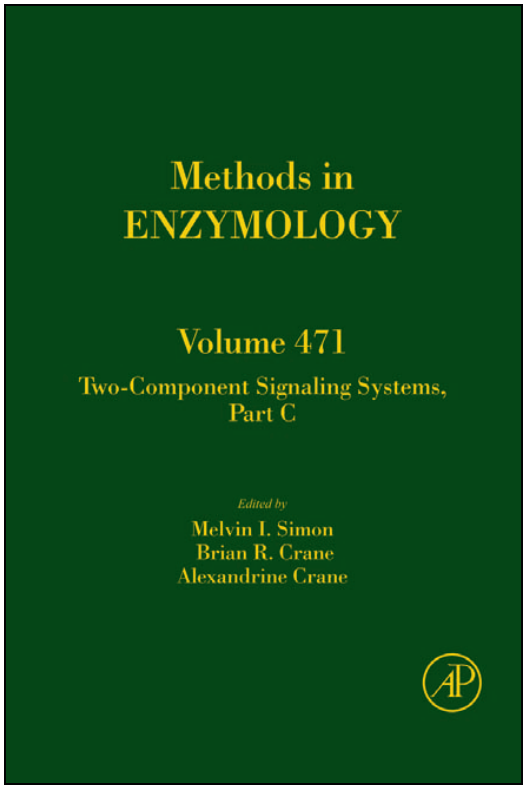

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CHAPTER TWENTY

Reversible Histidine Phosphorylation in Mammalian Cells: A Teeter-Totter Formed by Nucleoside Diphosphate Kinase and Protein Histidine Phosphatase 1

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Contents

Abstract

Regulation of protein phosphorylation by kinases and phosphatases is involved in many signaling pathways in mammalian cells. In contrast to prokaryotes and lower eukaryotes a role for the reversible phosphorylation of histidine residues

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is just emerging. The β subunit of heterotrimeric G proteins, the metabolic enzyme adenosine 5′-triphosphate-citrate lyase (ACL), and the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ have been identified as targets for nucleoside diphosphate kinase (NDPK) acting as protein histidine kinase and the so far only identified mammalian protein histidine phosphatase (PHPT-1). Herein, we describe the analysis of the phosphorylation and dephosphorylation of histidine residues by NDPK and PHPT-1. In addition, experimental protocols for studying the consequences of heterotrimeric G protein activation via NDPK/G$\beta\gamma$ mediated phosphorelay, the regulation of ACL activity and of $K_{Ca}3.1$ conductivity by histidine phosphorylation will be presented.

1. Introduction

The importance of reversible phosphorylation of proteins as a widespread, posttranslational regulatory mechanism of cellular functions is nowadays well recognized, and thus the two regulatory components of such systems, kinases and phosphatases, are interesting targets for drug development. In mammalian systems, however, kinases and phosphatases are believed to mainly target protein serine, threonine, and tyrosine residues, and such regulatory systems have been extensively studied. Nevertheless, the phosphorylation of histidine residues (P-His) was already observed in bovine liver in the early 1960's (Boyer *et al.*, 1962), but only sporadic reports during the following decades have addressed this issue in vertebrate systems. In contrast, in prokaryotes (Hess *et al.*, 1988) as well as in lower eukaryotes, like yeast, fungi, and plants (Kennelly and Potts, 1996; Swanson *et al.*, 1994) two or multicomponent signaling systems were discovered in which protein histidine kinases and histidine phosphatases are important mediators of cellular responses such as bacterial chemotaxis. Homology screens did not uncover similar systems and thus a role of P-His, besides its existence as phosphorylated intermediate in enzymatic reactions, was for quite sometime believed to be of minor importance for vertebrates.

Therefore, when Wagner and Vu (1995) reported the phosphorylation of the histidine residue 760 of the enzyme ATP-citrate lyase (ACL; EC 2.3.3.8) by another metabolic enzyme commonly known as nucleoside diphosphate kinase (NDPK; EC 2.7.4.6), the data were perceived with great skepticism. The correct biochemical name of NDPK is NTP/NDP transphosphorylase. It catalyzes the transfer of terminal phosphate groups from 5′-triphosphate- to 5′-diphosphate-nucleotides. In man 10 genes have been identified to be a part of the NDPK family. Nine genes are named NME 1–9. The 10th gene encoding a truncated NDPK is named RP2. The enzymatic activity has been unequivocally demonstrated for the NME1–4 gene products NDPK A–D which are also often called Nm23-H1–H4

(H stands for Human) with respect to the function of NDPKs as tumor suppressors (Boissan *et al.*, 2009; Steeg *et al.*, 2008). ACL has meanwhile been confirmed as a substrate of NDPK A acting as protein histidine kinase, and at least two additional proteins, the G protein β subunit (Cuello *et al.*, 2003) and the intermediate conductance potassium channel $K_{Ca}3.1$ (Srivastava *et al.*, 2006) are complexed with and phosphorylated specifically by NDPK B at residues His-266 and His-358, respectively. Therefore, at least the NDPK isoforms A and B have been proven to act as protein histidine kinases and the similarity to systems in lower organisms was further increased by the identification of the mammalian protein histidine phosphatase (PHPT-1, also named PHP) by two independent groups (Ek *et al.*, 2002; Klumpp *et al.*, 2002). The phosphorylated forms of all three afore mentioned substrates of the NDPK protein histidine kinase activity are targets of this phosphatase *in vitro* and in living cells (Klumpp *et al.*, 2003; Mäurer *et al.*, 2005; Srivastava *et al.*, 2008) and thus novel roles for reversible histidine phosphorylation in vertebrates are emerging (for review see Klumpp and Krieglstein, 2009).

In this chapter, we will therefore describe methods to analyze the phosphorylation and dephosphorylation of histidine residues by NDPK and PHPT-1 *in vitro* and provide experimental protocols for studying the consequences of heterotrimeric G protein activation via NDPK/G$\beta\gamma$ mediated phosphorelay (Hippe *et al.*, 2007), the regulation of ACL activity (Klumpp *et al.*, 2009; Krieglstein *et al.*, 2008; and of $K_{Ca}3.1$ conductivity (Srivastava *et al.*, 2008) by histidine phosphorylation and dephosphorylation.

2. Analysis of Phosphorylation and Dephosphorylation of Histidine Residues *In Vitro*

2.1. Expression and purification of NDPK and PHPT-1

NDPK A and B (Srivastava *et al.*, 2006; Wagner *et al.*, 1997) as well as PHPT-1 (Bäumer *et al.*, 2007; Srivastava *et al.*, 2008) can be expressed and purified from *Escherichia coli* and the purified enzymes can be used to study their enzymatic activity as well as the consequences of their interaction with their common target proteins *in vitro*. In the following, we will therefore describe the purification of fusion proteins from *E. coli* lysates and standardized activity assays.

2.1.1. Expression and purification of recombinant human NDPK, standardized activity assays

The coding sequence of human NDPK A or B was subcloned into the *Eco*RI site of the pGEX2T vector (GE Healthcare) and expression is performed in the *E. coli* BL21DE3 strain. A single colony of transformed

bacteria is picked from an agar plate and cultured overnight at 37 °C in a shaking incubator in 10 ml Y2T medium containing 100 μg/ml ampicillin. Two milliliters of this culture is added to 200 ml of fresh Y2T/ampicillin medium and incubated further until the OD_{600} reaches 0.5. For induction of protein expression, isopropyl-β-thiogalactoside (IPTG, final concentration 1 m*M*) is added and the incubation is continued for 1 h at room temperature. The bacteria are harvested by centrifugation (10 min at 10,000×*g* at 4 °C) and the pellet is resuspended in 3 ml ice-cold phosphate buffered saline (PBS, 137 m*M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4) containing 1 m*M* dithiothreitol and 0.1% Triton X-100. The bacteria are lyzed on ice by ultrasonic homogenization using five pulses for 30 s each, with 2 min intermittent cooling phases. The lysate is centrifuged for 15 min at 25,000×*g* to pellet cell debris and particles. The clear supernatant is added to 500 μl of a glutathione sepharose slurry (GE Healthcare) in PBS and incubated on a shaker for min at 4 °C and then transferred to disposable column. The beads are washed with 20 ml PBS and then eluted with 10 ml PBS containing 10 m*M* glutathione. The eluate is then concentrated by using Vivaspin 20 centrifugal concentrators (Satorius). Typically, 1 ml of recombinant GST-fusion protein at concentration of 500–600 μg/ml is obtained.

2.1.2. Assay for NDPK activity

For determination of the enzymatic NDPK activity, 3–10 ng protein of recombinant protein is used in reaction buffer (100 μl) consisting of 50 m*M* triethanolamine, pH 7.5, 0.2 m*M* cAMP, 0.2 m*M* ATP, 6 m*M* $MgCl_2$, 2 m*M* EDTA, 2 m*M* DTT, 0.2 m*M* GDP, and 1 μCi of [8-^{3}H]GDP. The samples are incubated for 10 min at 37 °C in a water bath and the reaction is terminated by adding 10 μl of a 10% (w/v) SDS solution. The guanine nucleotides are separated by thin layer chromatography. First, for each sample 2 μl of a GMP–GDP–GTP solution (each 3 m*M*) is spotted as a carrier near the bottom of a PEI cellulose plate, then 10 μl of the sample is spotted in four steps (2.5 μl each) onto the carrier. The plate is developed in a reservoir with a freshly prepared soluble phase consisting of equal parts of 1 *M* LiCl and 2 *M* formic acid. The separated guanine nucleotides are visualized with UV light at 254 nm, the areas containing GTP, GDP, and GMP are cut off and 4 ml scintillation liquid is added. To elute the nucleotides the samples are shaken vigorously for 2 h. The radioactivity is determined in a liquid scintillation spectrometer. As described in earlier *Methods in Enzymology* chapter (Lutz *et al.*, 2004), this assay can also be used to quantify NDPK activity in cell lysates or homogenates, membranes or cytosol. For such applications protein amounts of 0.3–1 μg is good point to start with.

2.1.3. Expression and purification of human recombinant PHPT-1

The coding sequence of human PHPT-1 was subcloned into the expression vector pET16b (Novagen) providing an N-terminal 10× His-tag extension (Bäumer *et al.*, 2007). For expression of PHPT-1, transformed *E. coli* BL21 (DE3) is inoculated in 50 ml of LB medium supplemented with 50 μg/ml ampicillin overnight at 37 °C in a shaking incubator. 5 ml of this culture are added to 1 l of LB/ampicillin medium and grown further at 37 °C until OD_{600} reaches 0.6. For induction of protein expression, IPTG (final concentration 10 μ*M*) is added and the cells are further incubated for 5 h at 37 °C in the shaking incubator before being harvested by centrifugation (15 min, 3000×*g*, 4 °C). The pellet is resuspended in 10 ml of a buffer 1 consisting of 20 m*M* Tris–HCl, pH 7.9, 5 m*M* imidazole, and 250 m*M* NaCl. Cells are lyzed on ice by sonification using 10 × 20 s pulses with 10 s cooling phases in between. The lysate is centrifuged (30 min, 20,000×*g*, 4 °C) and the supernatant is used for isolation of PHPT-1. 1.5 ml Ni-NTA agarose (Qiagen) slurry is poured into a Bio-Rad column (diameter 1.5 cm, length 15 cm). The resin is allowed to settle and equilibrated with 10 column volumes of buffer 1. The supernatant is loaded onto the column a with a flow rate of approximately 1 ml/min. The column is extensively washed with about 40 column volumes buffer 2 (20 m*M* Tris–HCl, pH 7.9, 50 m*M* imidazole, 500 m*M* NaCl). PHPT-1 is eluted with buffer 3 (20 m*M* Tris–HCl, pH 7.9, 500 m*M* imidazole) in 1 ml fractions. Those containing the highest amount of protein are pooled, then dialyzed against 2 l of buffer 4 (25 m*M* Tris–HCl, pH 7.9, 2 m*M* β-mercaptoethanol) for 1 h and for a second time against 5 l overnight at 4 °C. The eluate is concentrated using Amicon Ultra centrifugation devices (Millipore) up to a concentration of 5–10 mg/ml.

2.1.4. Measurement of PHPT-1 activity

Recombinant bacterial CheA, which autophosphorylates on His48, is expressed His-tagged protein in *E. coli* and purified exactly as described by Swanson *et al.* (1993). The phosphorylation of 8.5 μg CheA is performed a reaction mixture (50 μl) consisting of 50 m*M* HEPES, pH 8.0, 1 m*M* $MgCl_2$, 1 μ*M* ATP containing 100 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Perkin-Elmer), for 3 h at 37 °C. After removal of unincorporated [γ-^{32}P]ATP by Centrisep spin columns (Applied Biosystems) [^{32}P-His48]CheA is stable for several days at 4 °C and can be used as standard protein to determine P-His-phosphatase activity of PHPT-1 and other phosphatases.

Dephosphorylation is carried out in a volume of 40 μl for 30 min at 37 °C with 0.2 μg purified PHPT-1, 200 pmol [^{32}P-His48]CheA, in a reaction mix containing 25 m*M* triethanolamine–HCl, pH 7.5, 10 m*M* $MgCl_2$, and 0.1% β-mercaptoethanol. The incubation is terminated by adding 10 μl of 0.5 *M* EDTA immediately followed by addition of 150 μl

methanol/acetone (1:1) to precipitate the proteins. Note, that protein precipitation by strong acids such as trichloroacetic acid has to be avoided due to the acid lability of P-His. Samples are centrifuged (5 min, 14,000×*g*, room temperature). The supernatant is quantified for $^{32}P_i$ content by liquid scintillation counting after addition of a suitable scintillation cocktail.

Alternatively, PHPT-1 activity can be determined by autoradiography which does not require precipitation of not-hydrolyzed [^{32}P-His48]CheA and thus is performed with a smaller amount of the substrate (0.4 μg) in a reduced volume of 15 μl. In this case, the reaction is terminated by addition of 5 μl of fourfold concentrated sample buffer (Laemmli, 1970) As the phosphoamidate bond of P-His is heat sensitive, boiling of the samples should be avoided. Proteins are separated by discontinuous SDS–PAGE on gels containing 10% (w/v) acrylamide. The dye front containing free nucleotides is cut off from the gel. The gel is rinsed for 5 min in a 10% (v/v) glycerol/water mixture, dried, and autoradiographed. As phosphoamidate bonds are sensitive to acid, staining of the proteins by methods using an acidic fixation, for example, Coomassie Brilliant Blue staining, should be avoided. The use of prestained molecular weight markers can be recommended.

2.2. Analysis of P-His-phosphorylation and dephosphorylation in NDPK and PHPT-1 substrate proteins

2.2.1. Phosphorylation and dephosphorylation of Gβ

Apparently, phosphorylation of Gβ on His-266 by NDPK B is more complex than the phosphorylation of the other known protein substrates. As mentioned before (Cuello *et al.*, 2003), a reconstitution of Gβ phosphorylation cannot be achieved by an interaction of purified recombinant proteins as a so far unknown scaffold protein is involved. Therefore, Gβ phosphorylation is usually detected in cell membranes or in a NDPK B/G$\beta\gamma$ complex enriched, for example, from retinal rod outer segments, the preparation of which has been described in detail in a previous volume of *Methods in Enzymology* (Lutz *et al.*, 2004). [γ-^{32}P]GTP (6000 Ci/mmol, Perkin-Elmer) is the best substrate for the phosphorylation of Gβ. It offers the advantage that it is a poor substrate for many protein kinases using ATP and therefore offers a simple method to visualize phospho-NDPK and phospho-Gβ with low background phosphorylation (Fig. 20.1). For that reason the assay described herein can also be used to visualize the phosphorylation and dephosphorylation of $K_{Ca}3.1$ by NDPK and PHPT-1, respectively (Edward Skolnik, New York, personal communication). The assay is usually performed in Eppendorf tubes in a total volume of 10–20 μl in a reaction mixture containing 50 m*M* triethanolamine–HCl, pH 7.4, 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* EDTA, and 1 m*M* dithiothreitol,

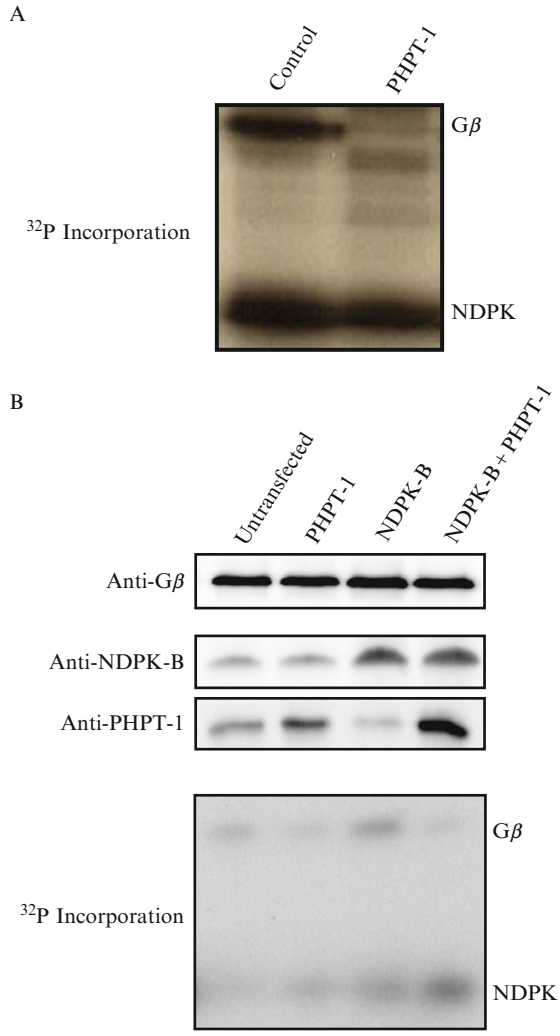

Figure 20.1 Dephosphorylation of His-P on the Gβ subunit by PHPT-1. (A) A complex formed by G$\beta_1\gamma_1$-dimers of transducin and NDPK B was purified as described (Lutz *et al.*, 2004) and 10 μg of protein were phosphorylated with [γ-^{32}P]GTP for 10 min at 37 °C. The phosphorylated proteins were subsequently treated with solvent (Control) or 2.5 μg PHPT-1 for 30 min. Proteins were separated on 15% SDS–PAGE and autoradiographed. (B) Five micrograms of membrane of H10 cells (Lutz *et al.*, 2004) obtained from control cells (Untransfected), cells sevenfold overexpressing PHPT-1 (PHPT-1), threefold overexpressing human NDPK B (NDPK B) or cells threefold overexpressing NDPK B, and sevenfold PHP (NDPK B + PHPT-1) were subjected to SDS–PAGE and the content of Gβ, NDPK B, and PHPT-1 was visualized by immunoblot analysis (upper panels) using antibodies against Gβ (T-20, Santa Cruz), NDPK B (MC-381, Kamya), or PHPT-1 (Klumpp *et al.*, 2002). The same membranes (5 μg) were phosphorylated with [γ-^{32}P]GTP for 5 min. An autoradiogram after SDS–PAGE is shown in the lower panel. Phosphorylated Gβ and phosphorylated NDPK are indicated.

10 n*M* [γ-^{32}P]GTP (Perkin-Elmer). Usually 5 μl of the fourfold concentrated reaction mixture is given into a tube on ice. Before incubation the tubes are warmed for 5 min at 30 °C and the protein, for example, 10 μg NDPK B/G$\beta\gamma$ enriched fraction from transducin or 5 μg of a membrane suspension in 10 m*M* triethanolamine–HCl, pH 7.4, is added and incubated for the desired periods of time (see Fig. 20.1). Note that especially in membranes the phosphorylation of Gβ is transient due to the transfer of high energetic P-His onto GDP and the consumption of the formed GTP by GTPases and thus incubation times in range 1–5 min can be recommended (Fig. 20.1B). The optimal conditions for obtaining good phosphorylation results have to be adjusted in each system by varying incubation times and protein amount (see Lutz *et al.*, 2004). If a subsequent analysis of the dephosphorylation by PHPT-1 is desired (Fig. 20.1A) the reaction should be terminated by the addition EDTA (5 m*M* final concentration). As both the kinase activity of the NDPK and the retransfer of p-His onto GDP require the presence of divalent cations, this preserves the amount of incorporated phosphate in absence of the PHPT-1, which do not require the presence of divalent cations. Addition of 2.5 μg of recombinant PHPT-1 per sample is sufficient to obtain complete dephosphorylation of the PHPT-1 substrate (Fig. 20.1A) after an incubation time of 30 min at 37 °C.

The reaction is terminated by the addition of 10 μl of threefold concentrated sample buffer (Laemmli, 1970), followed by a 1 h incubation at room temperature. Analysis is performed by SDS–PAGE as described above.

2.2.2. Phosphorylation and dephosphorylation of ATP-citrate lyase

ACL is an enzyme involved in acetylcholine and cholesterol synthesis, fatty acid and energy metabolism, and was the first vertebrate protein substrate of NDPK and PHPT-1 to be identified (Klumpp *et al.*, 2003; Wagner and Vu, 1995). The protein is a homotetramer formed of 110 kDa subunits, and autophosphorylates at His-760 in the catalytic domain using ATP as substrate to form an enzymatic intermediate (Cottam and Srere, 1969; Elshourbagy *et al.*, 1990). Noiman and Shaul (1995) reported that phosphorylation by ATP in the presence of an excess of EDTA (e.g., 5 m*M* final concentration) results in an *exclusive* labeling of P-His-containing proteins as kinases acting on serine, threonine, or tyrosine residues essentially require the presence of divalent cations such as Mg^{2+}. The method used for searching PHPT-1 substrates took advantage of this selective labeling: Phosphorylation of soluble rabbit liver extract with [γ-^{32}P]ATP in the presence of EDTA resulted only in three labeled proteins, one of them being dephosphorylated after addition of PHPT-1. This protein was identified as ACL. Note, that in contrast to soluble extracts of tissues or cell culture lysates, autophosphorylation of purified ACL can only be observed

in the presence of Mg^{2+}. Autophosphorylation of ACL is carried out in Eppendorf tubes in a total volume of 10 μl in a reaction mixture consisting of 25 m*M* Tris–HCl, pH 7.5, and 1 μ*M* ATP including 2 μCi [γ-^{32}P]ATP at 37 °C for 15 min. The use of 0.1 μg purified ACL in the presence of 5 m*M* $MgCl_2$, 100 μg soluble liver extract or 15–25 μg of extract of cultured cells, both in the presence of 5 m*M* EDTA, can be recommended. To study dephosphorylation of [^{32}P-His]ACL 0,6 μg recombinant PHPT-1 is added in a volume of 5 μl containing 25 m*M* Tris–HCl, pH 7.5, and 5 m*M* EDTA. Samples are incubated at 37 °C for 30 min and the reaction is terminated by addition of 5 μl fourfold concentrated sample buffer without heating. Analysis is performed by SDS–PAGE (12.5%, w/v, acrylamide) as described above and labeled ACL is detected by autoradiography.

3. Functional Analysis of NDPK/PHPT-1 Regulated Systems in Living Cells

3.1. Regulation of basal cardiac cAMP synthesis by the NDPK B/G$\beta\gamma$ phosphorelay

It has been demonstrated that the NDPK B/G$\beta\gamma$ mediated phosphorelay contributes to basal, G protein coupled receptor (GPCR)-independent cAMP formation in cardiac myocytes and thereby regulates contractility (Hippe *et al.*, 2003, 2007). We therefore describe here two experimental settings which allow the quantification of the contribution of this phosphorelay to cAMP formation. The first is the overexpression of a mutant of the G protein β_1-subunit, in which the intermediately phosphorylated His-266 is substituted by leucine (Gβ_1H266L), and thus is no substrate for the NDPK B mediated phosphorylation. The second is the siRNA-induced knockdown of NDPK B. The loss in cAMP formation in cultured cardiomyocytes in both settings reflects the reduced Gα_s activation via the NDPK B/G$\beta\gamma$ phosphorelay. Note however, that the siRNA-mediated depletion might effect other cellular functions of NDPK B as well, whereas the overexpression of Gβ_1H266Lγ_2-dimers selectively disturbs the NDPK B-induced phosphorelay without interfering with the GPCR-induced G protein activation (Hippe *et al.*, 2007).

3.1.1. Generation and amplification of recombinant adenoviruses

To allow for formation of recombinant G$\beta_1\gamma_2$-dimers in the target cells, biscistronic adenoviral shuttle vectors can be generated. These vectors encode wild-type (WT) Gβ_1 (Gβ_1WTγ_2) or H266L-mutated Gβ_1 (Gβ_1H266Lγ_2) in conjunction with Gγ_2. First, the cDNA encoding WT Gβ_1 or the H266L mutant (Hippe *et al.*, 2007) is subcloned into pShuttle-IRES-hrGFP-1 vector (Stratagene). Second, Gβ_1 in conjunction with the

internal ribosome entry site (IRES) is isolated from pShuttle by *Bst*XI/*Nhe*I digest and then subcloned into the pAdTrack-CMV shuttle vector containing a GFP reporter driven by an additional CMV promoter. Finally, $G\gamma_2$ was inserted into this vector construct downstream the IRES, allowing the expression of two proteins from the same promoter. A shuttle vector encoding PHPT-1 is obtained by subcloning the PHPT-1 cDNA into the *Not*I/*Hin*dIII sites of pAdTrack-CMV vector. All constructs have to be confirmed by restriction digest analysis and DNA sequencing. As control vector the use of pAdTrack-CMV vector, which will produce an adenovirus encoding for EGFP (AdGFP) only, can be recommended. Homologous recombination between the shuttle vectors and the virus genome (pAdEasy) is performed in *E. coli* strain BJ5183 (Luo *et al.*, 2007). Detailed protocols for this procedure as well as the amplification and purification are published (Luo *et al.*, 2007) and can be obtained at www.coloncancer.org/adeasy.htm. Thus, they are described here only briefly: Linearized recombinant viral DNA is transfected into HEK293 cells at 50–70% confluency in a culture dish (ø 60 mm) using Lipofectamine 2000 Reagent (Invitrogen) or Polyfect (Qiagen). After 7–14 days the cells are harvested and cell extracts are prepared by three cycles of freezing (in liquid nitrogen) and thawing (in a water bath of 37 °C) to achieve total disruption of infected cells. The cell debris is removed by centrifugation (730×*g*, 7 min, at room temperature) and the supernatant is dispensed on 70–80% confluent culture dishes (ø 15 cm) with HEK293 cells. After 2 days cells are collected again using the procedure described before and the supernatant is distributed to five 70–80% confluent dishes with HEK293 cells. In the next step, the supernatant of these cells obtained again after 48 h is distributed to 25 culture dishes. In the final step, the cells are harvested by centrifugation (730×*g*, 7 min, room temperature) just before the general cell lyzes is about to start (usually 36–48 h after infection). The resulting pellet is resuspended in 2 ml of the supernatant and the cell suspension is freezed and thawed (see above) three times and is incubated with 35 m*M* $MgCl_2$, 6 μg/ml *DNaseI* and 100 μg/ml *RNaseA* for 30 min at 37 °C, followed by a second centrifugation (1500×*g*, 30 min, 4 °C). The supernatant is used for purification of the viral particles by a CsCl gradient centrifugation (100,000×*g*, 20 h, 4 °C). The virus suspension is dialyzed twice against PBS (3 h, 20 h), and then against virus storage buffer (140 m*M* NaCl, 1 m*M* KCl, 10 m*M* Tris–HCl, pH 7.4, 2 m*M* $MgCl_2$) for another 4 h. The virus suspension is diluted 1:1 with glycerol and stored at −20 °C. Viral stocks (about 10^9 biological active viral particles per μl) can be used for at least 6 months. Note that the amount of viruses (MOI, *m*ultiplicity *o*f *i*nfection, ratio of the number of biological active viruses to number of cells) required to obtain sufficient infection rates has to be optimized for each target cell type.

3.1.2. Isolation and culture of neonatal rat cardiomyocytes

Neonatal rat cardiac myocytes (NRCM) were isolated from 1- to 3-day-old Sprague–Dawley neonates by serial collagenase/pancreatin digestion. Rat hearts from 30 to 50 pups were minced and subjected to a serial collagenase digestion to release single cells. The digestion step in the digestion buffer (116 m*M* NaCl, 20 m*M* HEPES, 0.8 m*M* Na_2HPO, 5.6 m*M* glucose, 5.4 m*M* KCl, 0.8 m*M* $MgSO_4$, pH 7.35, 0.6 mg/ml of pancreatin, and 0.53 mg/ml collagenase type II) is repeated five times. The cell suspensions from each digestion are combined and centrifuged at $730\times g$ for 10 min. The pellet of cells is resuspended in complete culture medium (Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin). The cells are filtered through 45 μm mesh and preplated for 1.5–2 h to allow the differential attachment of nonmyocyte (fibroblasts, endothelial, and vascular smooth muscle) cells. The nonattached cells, that is, the cardiac myocyte fraction, are pelleted and replated in 12 well-tissue culture dishes at a density at 250,000 cells per well in complete culture medium with 0.1 m*M* bromodeoxyuridine (BrdU) to prevent overgrowth by the remaining fibroblasts. NRCM are used for experiments 4 days after isolation, when they spontaneously and synchronously contract at approximately 300 beats/min.

For immunoblot analysis, cardiac myocytes were lyzed in lysis buffer (10 m*M* Tris–HCl, pH 7.45, 0.1 m*M* EDTA, 1 m*M* PMSF, 1% Triton X-100, 0.5% sodium deoxycholate, and protease inhibitors (protease inhibitor cocktail, Sigma)).

3.1.3. Adenoviral overexpression of WT or phosphorylation-deficient $G\beta_1\gamma_2$-dimers in cardiomyocytes

To analyze the impact of the phosphorylation-deficient $G\beta_1$-mutant ($G\beta_1$H266L) on the activation of $G\alpha_s$ in intact cardiac myocytes cAMP production is measured following overexpression of $G\beta_1WT\gamma_2$ or $G\beta_1H266L\gamma_2$ together with $G\alpha_s$ by adenoviral gene transfer. Spontaneously beating NRCM are infected 4 days after isolation in 12 well-tissue culture dishes at a density of 250,000 cells per well cultured in complete culture medium. NRCM were washed once with serum-free DMEM. Viral stocks are diluted in 300 μl serum-free DMEM per well to obtain the desired MOI. NRCM are incubated with the virus-containing medium for 20 min at 37 °C. Then 700 μl DMEM containing 4% FCS is added per well, and the cells are analyzed after additional 48 h in culture. Figure 20.2A shows the overexpression of WT and mutant $G\beta_1$ with or without overexpression of $G\alpha_s$ at MOI of 10 bav per cell and the suppression of basal, receptor-independent cAMP content induced by $G\beta_1H266L\gamma_2$ compared to $G\beta_1WT\gamma_2$.

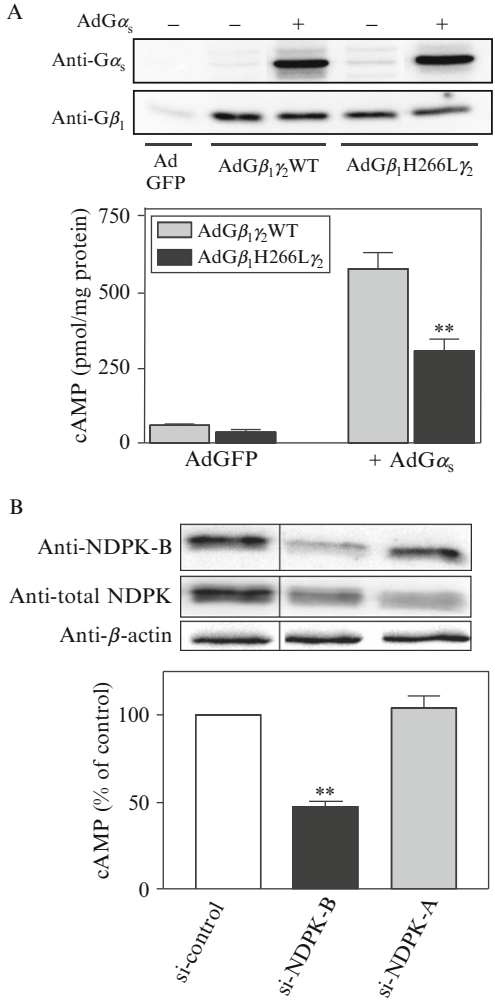

Figure 20.2 Suppression of basal cAMP production in cardiac myocytes by expression of $G\beta_1H266L\gamma_2$ or depletion of NDPK B. (A) NRCM were coinfected with $AdG\alpha_s$ and AdGFP or $AdG\beta_1\gamma_2$, either WT or H266L-mutant at MOI 10. Representative immunoblots of the expression of $G\beta_1$ and $G\alpha_s$ 48 h after infection are shown. (B) NRCM were sequentially, double transfected with scrambled siRNA (si-Control) or specific siRNA (si-) against NDPK A or B as indicated. Representative immunoblots of total NDPK and specific NDPK B expression in NRCM 72 h after the second transfection are shown. Expression levels of β-actin served as loading controls. Basal cAMP content in NRCM was measured in the presence of 1 m*M* IBMX and 1 μ*M* propranolol. Data are means $\pm$ S.E.M., $n > 4$, **, $P < 0.01$. Both, inhibition of the phosphorelay by expressing $G\beta_1H266L\gamma_2$ and depletion of NDPK B reduced the basal cAMP content by about 50%.

3.1.4. siRNA-induced depletion of NDPK B in NRCM

Knockdown of NDPK A or B in NRCM is performed by a sequential, double transfection with siRNA duplexes. siRNAs are directed against the following target sequences: NDPK A: GGA TTC CGC CTG GTT GGT T (Fan *et al.*, 2003), NDPK B#1: GGG GTT CCG CCT GGT GGC C, NDPK B: AACTGATTGACT ATA AGT CTT (Kapetanovich *et al.*, 2005). Control siRNA (si-Control) was a scrambled siRNA (Ambion). Spontaneously beating NRCM were transfected 2–3 days after isolation in 12 well-plates at a density of 250,000 cells/well cultured in complete culture medium without antibiotics using Lipofectamine 2000 reagent (Invitrogen). For transfection, 100 pmol siRNA is diluted in 100 μl Opti-MEM I (Invitrogen) and combined with 2 μl Lipofectamine 2000 (Invitrogen) prediluted in 100 μl Opti-MEM I (Invitrogen). After gently mixing and incubation for 20 min, the siRNA-Lipofectamine complexes (200 μl) are added to each well containing myocytes in 1 ml culture medium without antibiotics and incubated at 37 °C and 5% CO_2. To achieve maximally effective knockdown, a second transfection is performed after 24 h precisely as described for the first transfection. The following day, the medium is changed to fresh culture medium including antibiotics, and the cells are analyzed 72 h after the second transfection. Figure 20.2B shows the specifically reduced expression levels of the targeted NDPK isoform. Depletion of NDPK B, but not of NDPK A, results in significantly diminished cAMP levels which correlate to the reduction in NDPK B expression levels.

3.1.5. Determination of basal cAMP synthesis in NRCM

To quantify $G\alpha_s$ activation by the NDPK B/$G\beta\gamma$ phosphorelay cAMP accumulation in intact NRCM is determined a competitive enzyme immunoassay. Myocytes are cultured in 12 well-tissue culture dishes containing complete culture medium as described above. Twelve hours before starting the cAMP accumulation assay (i.e., 36 h after adenoviral infection or 72 h after the second siRNA transfection) the medium is switched to serum free medium. The accumulation of cAMP is determined in 1 ml per well of an assay buffer containing serum-free DMEM, 20 m*M* HEPES, pH 7.4, 1 m*M* isobutylmethylxanthine (IBMX) and 100 μM propranolol, an inverse agonist of β-adrenoceptors, which inhibits constitutive receptor activity and blocks the binding of eventually remaining catecholamines. Myocytes are allowed to equilibrate in the assay buffer for 30 min at 37 °C. Thereafter, the assay medium is removed, cells are lyzed by addition of 400 μl 0.1 *M* HCl per well, scraped off and transferred into an Eppendorf tube. After centrifugation at 20,000×*g* for 15 min at 4 °C, 100 μl of the supernatant is used for the competitive enzyme immunoassay for cAMP (R&D System, Wiesbaden, Germany) precisely following to the manufacturer's

instructions. The acid-insoluble cell pellets are neutralized with 0.1 *M* NaOH and the protein content is determined according to Bradford (1976). Production of cAMP is normalized to the protein content.

3.2. Regulation of ACL activity by PHPT-1

It has been hypothesized that an increased activity of PHPT-1 accelerates the dephosphorylation of ACL at His-760 in the active site and, subsequently, reduces its cellular activity. As ACL is crucial for fat and cellular energy metabolism, a loss of ACL activity, for example, by siRNA-mediated knockdown is associated with reduced viability of, for example, neuronal cells due to enhanced apoptosis (Krieglstein *et al.*, 2008). Similarly, the reduction of ACL activity by PHPT-1 overexpression in primary cultures of hippocampal or cortical neurons from embryonic rats, in the human neuroblastoma cell line (SH-SY5Y) as well as in the cholinergic murine neuroblastoma cell line SN56.B5 (SN56) causes a reduction of ACL activity and impairs cell viability (Klumpp *et al.*, 2009; Krieglstein *et al.*, 2008). We therefore describe herein the culture of SN56 cells, the adenoviral overexpression of PHPT-1 and assays to detect the phosphorylation status of ACL (see above) as well as ACL activity in SN56 cell extracts.

3.2.1. Culture of SN56 neuroblastoma cells and adenoviral infection

The SN56 cells are cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% (v/v) FCS and 50 μg/ml gentamycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium is changed every 2–3 days and the cells are splitted when confluent. Therefore, the medium is removed and the cells are washed once with prewarmed sterile PBS before 2 ml of trypsin–EDTA (0.05% trypsin, 0.02% EDTA) is added for 5 min at 37 °C. To stop trypsinization, 4 ml of culture medium is added to the flask. Cells are collected and centrifuged (130×*g*, 7 min, room temperature). The pellet is resuspended in 5 ml culture medium and 1 ml is transferred to a new culture flask with 10 ml culture medium. Passages 25–36 are used for experiments. For adenoviral infection, 600,000 SN56 cells were plated on culture dishes (ø 60 mm). After 1 day in culture the medium is replaced by Opti-MEM containing active viral particles (AdGFP, AdPHPT-1) at an MOI of 1.

3.2.2. Preparation of cell extracts and analysis of PHPT-1 expression and ACL activity

Cell extracts are prepared after washing cells with ice-cold PBS first. Thereafter, the cells are resuspended in homogenization buffer containing, 130 m*M* Tris–HCl, pH 6.8, 10% glycerol 1 m*M* PMSF, 7 μg/ml trypsin

inhibitor (Sigma), 1 μM calpain inhibitor (Sigma), and are briefly sonificated. The homogenate is separated by centrifugation (13,000×*g*, 10 min, 4 °C), and the supernatant containing soluble ACL as well as PHPT-1 is used for further analysis. For immunoblot detection of PHPT-1 expression, 20–40 μg of extract protein are separated by a SDS–PAGE (15%, w/v, arylamide) and later transferred electrophoretically to a nitrocellulose membrane. A purified polyclonal antibody (Klumpp *et al.*, 2002) is used at 1:400 dilution in 10 m*M* Tris–HCl, pH 7.6, 150 m*M* NaCl, 0.1% Tween, 0.1% bovine serum albumin overnight at 4 °C. The detection of the primary antibody by a secondary horseradish–peroxidase-conjugated antirabbit antibody and the use of an enhanced chemiluminescence reagent can be recommended. The phosphorylation of ACL (15 μg extract protein/10 μl assay volume) is detected as described above (*Phosphorylation and Dephosphorylation of ATP-citrate lyase*).

ACL activity is monitored using a method introduced by Linn and Srere (1979) in which a malate dehydrogenase (EC 1.1.1.37) coupled reaction is used to assay ACL activity via the oxidation of NADH and the thereby caused decrease in absorbance at 340 nm. The reaction mixture A containing 100 m*M* Tris–HCl, pH 7.4, 1.5 m*M* ATP, and 5–15 μl cell extract is incubated for 10 min at 30 °C in an Eppendorf tube. Reaction mixture B containing 16 m*M* potassium citrate, 1 m*M* dithiothreitol, 10 m*M* $MgCl_2$, 2.5 U malate dehydrogenase, 0.2 m*M* coenzyme A, and 0.14 m*M* NADH is warmed in parallel also for 10 min at 30 °C. Mixture B is given into a quartz cuvette, H_2O and the preincubated mixture A is added to give final volume of 150 μl and mixed. The absorbance at 340 nm is read every 10 s in a suitable spectrophotometer for a period of 10 min. The decrease in absorbance is plotted against the incubation time. The slope in the linear range of the resulting curve is directly correlated to ACL activity.

As shown in Fig. 20.3, the adenoviral overexpression of PHPT-1 in SN56 cells largely reduces the phosphorylation level of ACL which correlates to a loss of about 40% of ACL activity in the cell extract.

3.3. Regulation of $K_{Ca}3.1$ activity by NDPK B and PHPT-1

$K_{Ca}3.1$ channels belong to a subfamily of Ca^{2+}-activated K^+ channels with an intermediate conductance (syn. SK4, $K_{Ca}4$, IK_1, IK_{Ca} KCNN4, ''Gardos channels''). They are expressed predominantly in peripheral cells, including those of the hematopoietic system, colon, lung, placenta, pancreas, salivary glands, and proliferating vascular smooth muscle (Chou *et al.*, 2008; Wulff *et al.*, 2007). Activation of the channels mediates K^+ efflux setting up a negative membrane potential, which is required to establish a favorable electrochemical gradient for Ca^{2+} influx. $K_{Ca}3.1$ channels are important for diverse physiological responses in a variety of cell types, including osmotic and volume regulation in red blood cells, mitogen-dependent

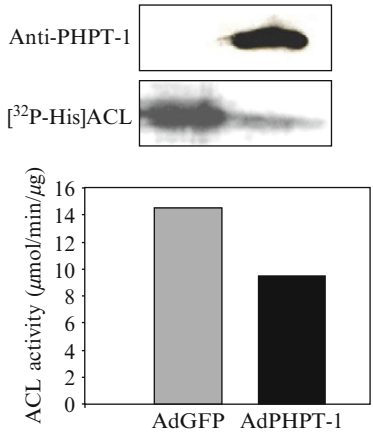

Figure 20.3 **Overexpression of PHPT-1 in SN56 cells reduced ACL phosphorylation and activity. SN56 cells were infected with AdGFP or AdPHPT-1 and cell extracts were prepared 72 h later. PHPT-1 was visualized by immunoblot analysis of 30 μg of cell extracts (upper panel). Phosphorylation of ACL was visualized by incubation of 15 μg cell extracts with [γ-^{32}P]ATP, 10% SDS–PAGE and autoradiography (middle panel). ACL activity was quantified by the malate dehydrogenase-dependent NADH oxidation (lower panel). The overexpression of PHPT-1 resulted in decreased phosphorylation levels of ACL which correlated with its reduced activity.**

activation of T-lymphocytes, Cl^- secretion of exocrine epithelial cells, and control of proliferation of T- and B-lymphocytes, vascular smooth muscle cells, and some cancer cell lines (Chou *et al.*, 2008; Wulff *et al.*, 2007). Intermediate conductance $K_{Ca}3.1$ channels can be distinguished pharmacologically from calcium-activated K^+ channels with small (SK channels) or big (BK channels) conductance by their sensitivity to blockade by the clotrimazole-derivative 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) and by their insensitivity to apamin and iberiotoxin. $K_{Ca}3.1$ channels associate with calmodulin and the channels open only after Ca^{2+} has bound to this regulatory protein. The intracellular Ca^{2+} concentration is the most evident regulator of $K_{Ca}3.1$ channels, but other intracellular factors like protein kinases can also regulate the activity of $K_{Ca}3.1$ channels. Protein kinase A (Pellegrino and Pellegrini, 1998) and protein kinase C (Wulf and Schwab, 2002) have been shown to activate $K_{Ca}3.1$ channels. As mentioned in Section 1, it was recently reported that specifically NDPK B is complexed with the channel and is able to activate the channel by phosphorylation of His-358 located in the C-terminal tail (Srivastava *et al.*, 2006). Moreover, PHPT-1 is apparently also part of the complex and can reverse the channel activation by P-His-dephosphorylation (Srivastava *et al.*, 2008).

To demonstrate this regulation of $K_{Ca}3.1$ channels by NDPK B and PHPT-1, we used the heterologous expression system of HEK293 cells,

which are devoid of endogenous $K_{Ca}3.1$, and therefore do not display a TRAM-34-sensitive K^+ current before transfection with an eukaryotic expression vector in which the cDNA encoding $K_{Ca}3.1$ (pCMV-Sport/$K_{Ca}3.1$) was subcloned. Electrophysiological measurements are carried out in $K_{Ca}3.1$ expressing HEK293 cells either in the whole-cell configuration or in inside-out patches (Figs. 20.4 and 20.5). In the whole-cell configuration the regulation of the KCa3.1 by NDPK B and PHPT-1 is studied after cotransfection with the eukaryotic expression vectors pAdTrack/NDPK B and pAdTrack/PHPT-1 (Fig. 20.4). In the cell-free inside-out patches the purified recombinant proteins are applied directly to the intracellular side of the cell membrane (Fig. 20.5).

3.3.1. Culture and transfection of HEK293 cells

HEK293 cells are cultured in MEM-EARLE medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum, 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, and 6% CO_2. The cells are passaged every 3–4 days, up to the 50th passage. Briefly, when the cells reach 70–80% confluency, the medium is removed and the cells are washed once with 10 ml (for T75 flask) prewarmed sterile PBS. Thereafter, 2 ml of a trypsin–EDTA (0.05% trypsin with 0.02% EDTA) solution is added for a few minutes until cells detach. To stop trypsinization, 10 ml of culture medium is added to the flask. The cells are gently but thoroughly resuspended by pipetting up and down. Two milliliters of the cell suspension is transferred to a new culture flask with 20 ml culture medium, and kept in a humidified incubator at 37 °C and 6% CO_2. One day prior to transfection, 10^5 cells are plated in a 35-mm tissue culture dish. The cells are transiently transfected with a mixture of DNA plasmids by calcium phosphate precipitation. One microgram of each plasmid cDNA is diluted in water in a total volume of 90 μl. Ten microliters of 2.5 *M* $CaCl_2$ solution is added to the DNA and mixed by pipetting up and down twice. Then 100 μl 2× BES-buffered solution (BBS: 50 m*M* *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 1.5 m*M* Na_2HPO_4, 280 m*M* NaCl) is added and vortexed for 10 s. The sample is then incubated for 17 min at room temperature to allow for transfection complex formation. Thereafter, the sample (200 μl) is transferred to the culture dish (2 ml medium) in which the cells are growing at 40–60% confluence. Immediately before, the cells are washed once with culture medium. The cells are then cultured at 35 °C and 3% CO_2. For transfection of the cells, the following four combinations of DNA plasmids are used: (1) pCMV-Sport/$K_{Ca}3.1$ + pcDNA3/EGFP, (2) pCMV-Sport/$K_{Ca}3.1$ + pcDNA3/EGFP + pAdTrack/NDPK B, (3) pCMV-Sport/$K_{Ca}3.1$ + pcDNA3/EGFP + pAdTrack/NDPK B(H118N), and (4), pCMV-Sport/$K_{Ca}3.1$ + pcDNA3/EGFP + pAdTrack/NDPK B + pAdTrack/PHPT-1. 18 h after the transfection, the cells are washed twice with PBS, then 2 ml culture medium is added and the cells are cultivated for another 48–72 h

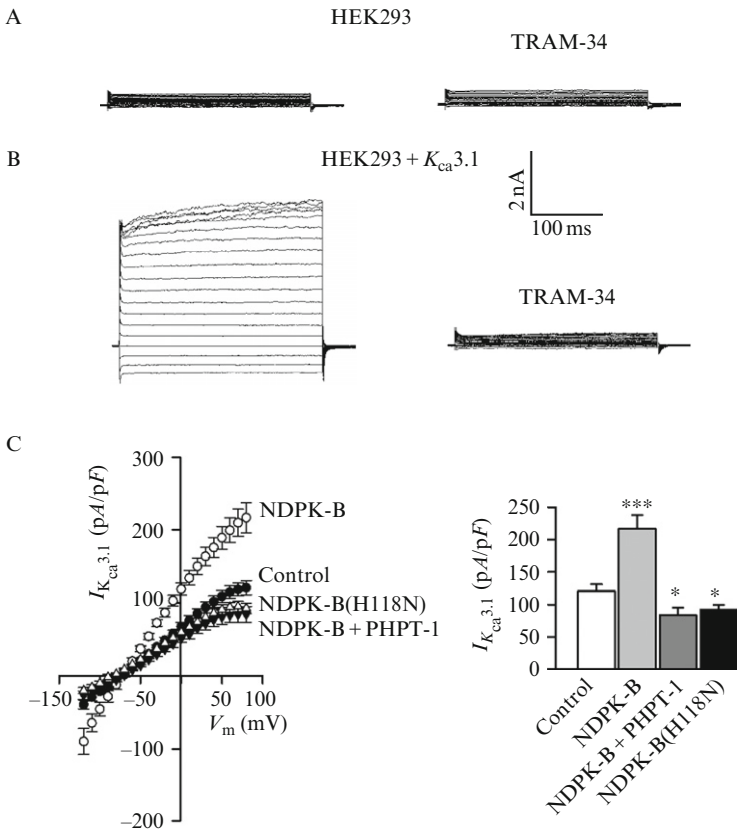

Figure 20.4 NDPK B stimulates and PHPT-1 inhibits $K_{Ca}3.1$ channel currents in HEK293 cells. Whole-cell currents were elicited from a holding potential of −70 mV by depolarizing the cells every 5 s for 300 ms from −120 to +80 mV in 10 mV increments. (A) Original current traces before and after application of 100 n*M* TRAM-34, recorded in nontransfected HEK293 cells. Note, that HEK293 cells do not exhibit endogenous TRAM-34-sensitive currents. (B) Original current traces recorded in HEK293 cells transiently transfected with $K_{Ca}3.1$ channels and inhibition of the currents by 100 n*M* TRAM-34. (C) HEK293 cells were transiently transfected with $K_{Ca}3.1$ channels alone (control) or cotransfected with NDPK B, NDPK B (H118N), or NDPK B + PHPT-1 encoding plasmids. The *I/V* curves (left) were obtained by plotting current densities (*pA/pF*) against the corresponding voltages (from −120 to +80 mV). Means ± S.E.M. of 39 cells (control), 31 cells (NDPK B), 42 cells (PHPT-1), and 45 cells (NDPK B (H118N)) are shown. Note that all currents represent TRAM-34-sensitive currents ($IK_{Ca}3.1$). Bars (right) represent mean current densities ± S.E.M. at a membrane potential of +80 mV (data from the *I/V* curve). The pipette solution contained 1 μM free Ca^{2+}. $^{\star}P < 0.05$, $^{\star\star\star}P < 0.001$ versus control.

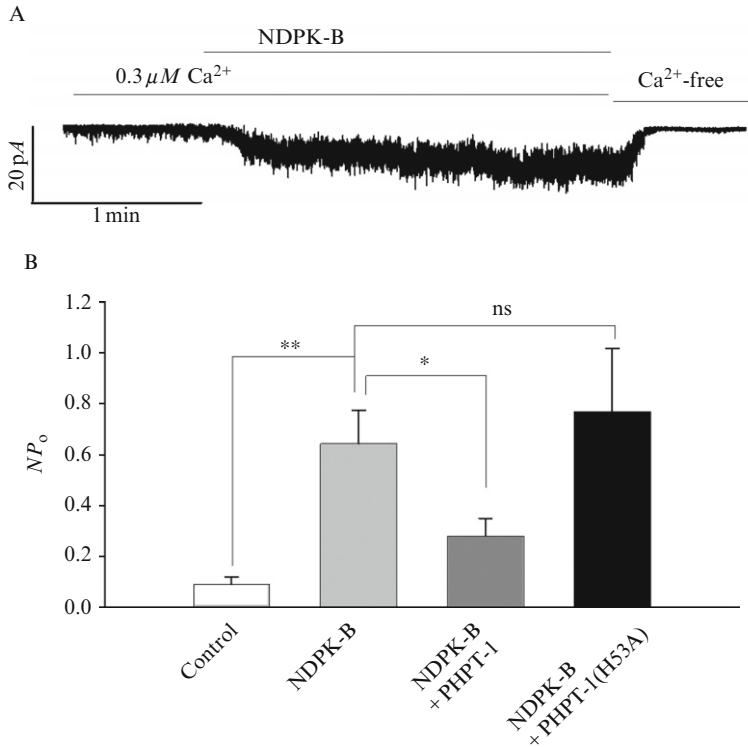

Figure 20.5 NDPK B enhances and PHPT-1 reverses the $K_{Ca}3.1$ channel activity in cell-free membrane patches. Channel open probabilities (NP_o) were recorded in inside-out patches excised from HEK293 cells transiently transfected with $K_{Ca}3.1$ channels in symmetrical K^+ (145 m*M*) solution. The membrane potential is constantly kept at -40 mV. $[Ca^{2+}]_i > 0.3$ μM. Recombinant proteins of NDPK B, PHPT-1, or PHPT-1 (H53A) were applied directly to the cytosolic surface of a single patch. (A) Original single-channel recording showing the activation of $K_{Ca}3.1$ channel activity by 10 μg ml^{-1} NDPK B and the calcium sensitivity of the channels in an inside-out patch. (B) the patches were superfused with 10 μg/ml NDPK B followed by 10 μg/ml either active PHPT-1 or the inactive PHPT-1(H53A) mutant. Bars represent mean NP_o values $\pm$ S.E.M. of 10 patches (control and NDPK B), and of five patches treated with NDPK B + PHPT-1 and NDPK B + PHPT-1(H53A), respectively. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ versus control; ns, not significant.

at 37 °C and 6% CO_2. On the day of the experiment, the cells are first washed several times with physiological saline solution (PSS; 10 m*M* HEPES, 127 m*M* NaCl, 5.9 m*M* KCl, 2.4 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 11 m*M* glucose, pH 7.4), and then transferred in the culture dish to the stage of an inverted microscope (Zeiss Axiovert, 200) for electrophysiological measurements. The transfection efficiency varies between 40% and 70% as judged by the expression of EGFP in transfected cells.

3.3.2. Recording techniques

Standard patch-clamp recording techniques are used to measure currents in the inside-out, or the whole-cell patch-clamp configuration (Hamill *et al.*, 1981). Patch electrodes are pulled from borosilicate glass capillaries (MTW 150F; world Precision Instruments, Inc., Sarasota, FL) using a DMZ-Universal Puller (Zeitz-Instrumente Vertriebs GmbH, Martinsried, Germany) and filled with prefiltered solutions of different composition (see below). Currents are recorded at room temperature with an EPC-7 amplifier (HEKA Elektronik, Lambrecht, Germany) connected via a 16-bit A/D interface to a pentium IBM clone computer. The signals are low-pass filtered (1 kHz) before 5 kHz digitization. Data acquisition and analysis are performed with an ISO-3 multitasking patch-clamp program (MFK M. Friedrich, Niedernhausen, Germany). Pipette resistance range from 2 to 3 MΩ in whole-cell, and 8–9 MΩ in the excised-patch experiments. Electrode offset potentials are always zero-adjusted before a Giga-seals is formed. To avoid dirt on the pipette tip, a slight positive pressure is applied to the pipette interior before the pipette is moved into the bath solution, but pressure is released shortly before the pipette reaches the cell surface. After the pipette tip is gently pressed against the cell membrane, Giga-seals are obtained within several seconds when a negative pressure is applied to the pipette interior. Sometimes Giga-seals develop spontaneously without suction. To reduce the difficulty of obtaining high resistance Giga-seals, only freshly pulled and clean pipettes are used. To reduce electrical noise to a minimum, the experiment is carried out only when the seal resistance is higher than 10 GΩ.

3.3.3. Recording of whole-cell currents

For recording whole-cell currents, the membrane under the pipette tip is disrupted by negative pressure and as a consequence, transient capacitance currents occur at the beginning, and at the end of small test-pulses. After establishment of the whole-cell configuration, the membrane capacitance and series resistance are compensated (60–80%). Whole-cell currents are elicited by applying 300-ms step depolarizations to potentials ranging from −120 to +80 mV in 10-mV increments from a holding potential of −70 mV. Currents are sampled by ISO-3 and saved in the computer for later data analysis. Current–voltage (I/V) relationships are obtained by plotting current density (pA/pF) measured at the end of a depolarizing pulse against the corresponding voltage. For whole-cell recordings, the bath is superfused with PSS. The pipette solution contains 10 mM HEPES, 100 mM K^+-gluconate, 24 mM KCl, 1.2 mM $MgCl_2$, 5 mM EGTA, 11 mM glucose, and 3 mM ATP, pH 7.4. The free Ca^{2+} concentration is adjusted to 1 μM by adding 4.75 mM $CaCl_2$ (Fabiato, 1988; Mermi *et al.*, 1991).

To dissect K^+ currents conducted by $K_{Ca}3.1$ channels from total outward currents, transfected HEK293 are superfused with 100 n*M* TRAM-34, a specific $K_{Ca}3.1$ channel blocker. $K_{Ca}3.1$ channel currents are obtained by subtracting the residual currents after inhibition by TRAM-34 from whole-cell outward currents (Fig. 20.4B and *I/V* curves in C). Overexpression of NDPK B enhances $K_{Ca}3.1$ channel activity whereas expression of the inactive form (NDPK B(H118N)) or of PHPT-1 leads to a significant suppression of the current (Fig. 20.4C). In accordance with previously published data in CHO and human CD4 T-lymphocytes (Srivastava *et al.*, 2006, 2008), these data demonstrate that $K_{Ca}3.1$ channel activity is controlled by NDPK B and PHPT-1 at endogenous expression levels of these proteins.

3.3.4. Single-channel recording

To obtain single-channel recordings from inside-out patches of HEK293 cells expressing $K_{Ca}3.1$ channels, a Giga-seal is formed first. Then the pipette tip is slowly withdrawn from the cell surface until the bridge between pipette tip and cell surface ruptures, leaving a small vesicle protruding from the pipette tip. The pipette tip is then passed briefly (1–2 s) through the air–water interface, which disrupts the protruding membrane of the vesicle and forms spontaneously an inside-out membrane patch. After establishment of the inside-out configuration, the membrane potential is constantly clamped at −40 mV and single-channel currents generated by spontaneous openings of $K_{Ca}3.1$ channels are recorded. To verify that single-channel currents are due to the opening of $K_{Ca}3.1$ channels, the bath solution (see below) is changed to a Ca^{2+}-free solution which immediately stops channel activity (Fig. 20.5A). Ca^{2+}-activated K^+ channels other than $K_{Ca}3.1$ are apparently functionally not expressed in HEK293 cell plasma membranes. The average channel open probability (NP_o) in inside-out patches is determined by the following equation:

$$NP_o = \frac{(\sum_{j=1}^{N} t_j j)}{T}$$

where P_o is the open probability, T is the duration of the measurement, t_j is the time spent with $j > 1, 2, \ldots, N$ channels open, and N is the maximum number of channels seen. To obtain mean NP_o values, membrane patches are equilibrated for at least 3–5 min before the recombinant proteins are applied for another 3–5 min. A multibarreled perfusion pipette placed by a hydraulic micromanipulator 200 μm away from the patch is used to switch between superfusion solutions. The flow rate of 3 μl/min generated by a syringe pump (TSE 200, Bad Homburg, Germany) resulted in a flow velocity of 1 cm/s through an 80-μm orifice. For inside-out recordings, the extracellular (pipette) solution contains 5 m*M* HEPES, 145 m*M*

KCl, 1.2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, pH 7.4, and the bath solution (cytosolic surface of the patch) contains 5 m*M* HEPES, 139 m*M* KCl, 1.2 m*M* $MgCl_2$, 5 m*M* EGTA, 11 m*M* glucose, 3 m*M* ATP, pH 7.3. The free Ca^{2+} concentration is adjusted to 0.3 μM by adding 3.8 m*M* $CaCl_2$ (Fabiato, 1988; Mermi *et al.*, 1991). Superfusion of the cytosolic side of the patch with recombinant NDPK B (10 μg/ml) enhances NP_o several-fold, whereas the subsequent application of NDPK B together with PHPT-1 (10 μg/ml) strongly attenuates the response. Application of NDPK B plus the inactive mutant phosphatase PHPT-1(H53A) (10 μg/ml) enhances NP_o comparable to NDPK B alone (Fig. 20.5B).

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