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ERK and RSK are necessary for TRH-induced inhibition of r-ERG potassium currents in rat pituitary GH_3 cells

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ABSTRACT

The transduction pathway mediating the inhibitory effect that TRH exerts on r-ERG channels has been thoroughly studied in GH_3 rat pituitary cells but some elements have yet to be discovered, including those involved in a phosphorylation event(s). Using a quantitative phosphoproteomic approach we studied the changes in phosphorylation caused by treatment with 1 μM TRH for 5 min in GH_3 cells. The activating residues of Erk2 and Erk1 undergo phosphorylation increases of 5.26 and 4.87 fold, respectively, in agreement with previous reports of ERK activation by TRH in GH_3 cells. Thus, we studied the possible involvement of ERK pathway in the signal transduction from TRH receptor to r-ERG channels. The MEK inhibitor U0126 at 0.5 μM caused no major blockade of the basal r-ERG current, but impaired the TRH inhibitory effect on r-ERG. Indeed, the TRH effect on r-ERG was also reduced when GH_3 cells were transfected with siRNAs against either Erk1 or Erk2. Using antibodies, we found that TRH treatment also causes activating phosphorylation of Rsk. The TRH effect on r-ERG current was also impaired when cells were transfected with any of two different siRNAs mixtures against Rsk1. However, treatment of GH_3 cells with 20 nM EGF for 5 min, which causes ERK and RSK activation, had no effect on the r-ERG currents. Therefore, we conclude that in the native GH_3 cell system, ERK and RSK are involved in the pathway linking TRH receptor to r-ERG channel inhibition, but additional components must participate to cause such inhibition.

1. Introduction

The *ether-a-go-go* related gene channel erg-1, also known as KCNH2 or Kv11.1 (human isoform hERG, rat isoform r-ERG), belong to the ERG subfamily of the *eag* group of voltage gated potassium channels [1,2], and is expressed in a variety of normal tissues and in some cancer cells [3,4]. Like other Kv channels, they are involved in crucial cellular and biological events such as excitability, proliferation and apoptosis [4,5]. Indeed, ERG channels are essential for setting the electrical behaviour of many cell types and, due to their special gating kinetics, they display a key role in regulating spiking in the pituitary and heart cells, thus respectively controlling hormone secretion and cardiac rhythm [5,6]. The activity of ERG channels can be modulated by hormones, and several intracellular second messengers regulate them and control their physiological functions in different cells [3,7]. While some of the pathways involved in the control of ERG by different hormones have been elucidated, some other remain partly unresolved.

The rat pituitary GH_3 cell line endogenously expresses r-ERG channels, for which they have become a widely used system to study ERG currents and their regulation, specially by thyrotropin releasing hormone (TRH) [8,9]. In GH_3 cells binding of TRH to its receptor (TRH-R) promotes its coupling to $G_{q/11}$ and stimulates PLCβ activity, leading to activation of PKC and release of Ca^{2+} from intracellular storage sites [10,11]. In these cells TRH also inhibits r-ERG currents through a pathway involving TRH-R coupling to G_s and/or G_{13} and βγ complexes [8,12,13], resulting in a membrane depolarization that increases the action potential firing rate, which in turn leads to a second phase of Ca^{2+} influx

Abbreviations: Akt/PKB, protein kinase B; CaM kinase-II, calcium/calmodulin-dependent protein kinase II; EGF, epidermal growth factor; ERK/Erk, extracellular-signal-regulated kinase activity/protein; GEF, guanine nucleotide-exchange factor; Itsn1, intersectin-1; MAPK/Mapk, mitogen-activated protein kinase activity/protein; MEK, MAPK/ERK kinase; PDK1, phosphoinositide-dependent kinase 1; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PRKD1, protein kinase D1; RSK/Rsk, p90 ribosomal protein S6 kinase activity/protein; TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; XIC, extracted ion chromatograms.

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and secretion of prolactin [5]. Additionally, TRH-induced signaling events activate several kinases in GH_3 cells, including PKC and CaM kinase-II [14,15]. Early studies regarding the signaling pathways involved in r-ERG current inhibition by TRH in GH_3 cells pointed to a yet undetermined phosphorylation event as a key element in the transduction of the hormonal response [16]. It is unclear which kinase(s) is(are) responsible for such an event, but it has been indicated that the effect is not dependent on PKC, PKA, Rho kinase or Akt/PKB [17–20]. It is also known that in GH_3 cells TRH is able to activate a double route that converges at Raf activation, and causes a rapid Erk/Mapk phosphorylation and activation [21]. However, the possible implication of the ERK pathway in the inhibition of r-ERG currents remains to be thoroughly studied. The ERK pathway is activated by many extracellular stimuli, such that distinct pathways converge at the main core formed by Ras/Raf/MEK/ERK to transduce signals towards gene transcription, differentiation, or proliferation [22–24]. Three main families of kinases act just downstream of ERK: RSK, MSK and MNK, with the ubiquitous RSK kinases showing the widest substrate specificity, especially in non-nuclear events [24,25].

It is well known that phosphorylation of proteins can affect their function, and that the combined action of protein kinases and phosphatases controls many biological responses including most signaling pathways [26]. As for other members of the Kv family, reversible phosphorylation allows ERG channels to be directly or indirectly modulated by hormones and signal transduction pathways [7,16,19,27]. Evidence for the modulation of ion channels by phosphorylation has classically been based on the response to pharmacological inhibitors of kinases and phosphatases [17–19]. However, their use is a frequent cause of technical problems when measuring ERG currents, which are often inhibited due to the extraordinary ability of the ERG channels to bind drugs and chemicals [3,6,28].

The identification of phosphorylated proteins and the study of their changes have recently been improved by the advance of the phosphoproteomic techniques [29], that have also been used to study ion channels regulation since their activity is often regulated by the combined action of protein kinases and phosphatases [30,31]. In this work we have used a phosphoproteomic approach to analyze the changes in protein phosphorylation observed in TRH-treated GH_3 cells, resulting in a dramatic increase of phosphorylated Erk1 and Erk2 levels. We found that blocking or silencing ERK action by using a MEK inhibitor or siRNAs against Erk1 or Erk2 impair the TRH-induced inhibition of r-ERG currents. Indeed, RSK is also activated by TRH, and silencing Rsk1 expression also impairs such regulation. Based on the differential effect of TRH and EGF, which also causes Erk and Rsk phosphorylation in GH_3 cells, we conclude that the activation of both Erk1/2 and Rsk1 is necessary but not sufficient for TRH inhibition of the endogenous r-ERG channels in these cells.

2. Materials and methods

2.1. Chemicals and reactives

MEK inhibitors PD98059, PD184352 and U0126 were obtained from Calbiochem, Sigma-Aldrich and Cell Signaling, respectively. RSK inhibitors BI-D1870 and SL0101 were from Enzo Life Sciences and Calbiochem. Anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and anti-phospho-p90RSK (Ser380) antibodies were from Cell Signaling. Anti-Rsk1 (C-21) was from Santa Cruz (sc-231) and anti-β-actin was from Sigma-Aldrich (A5441). Anti-mouse and anti-rabbit secondary antibodies were either HRP-linked from Cell Signaling or fluorophore-linked from LI-COR Biosciences. TRH, EGF, nystatin and RIPA buffer were from Sigma-Aldrich, Lipofectamine 2000 was from Invitrogen, and E-4031 was from Alomone Labs. Plasmid pEGFP-C1 used as a transfection reporter was from Clontech.

2.2. Cell culture, transfection and RNAi

When intended for electrophysiological measurements, GH_3 rat anterior pituitary cells (ATCC-CCL 82.1) were grown on poly-L-lysine coated coverslips as previously described [13,17]. For RNAi experiments, cells trypsinized 24 h prior to transfection were transfected with 150 nM (for the SMARTpools) or 200 nM (for the RSK siRNAs mix) of the corresponding siRNAs, plus 0.5 μg of pEGFP-C1 plasmid, mixed with 3 μl of Lipofectamine 2000 (Invitrogen) in 300 μl of serum-free medium. This mixture was incubated for 20 min and added to the 35 mm dishes containing the cells in serum-containing medium without antibiotics. The amount of transfection reagent was optimized to keep the cell membranes suitable for electrophysiological measurements. Alternatively, when checking by Western analysis (see Section 2.4) the effectiveness of the siRNAs in silencing their targets, cells were grown on poly-L-lysine coated plates without coverslips and 6 μl of Lipofectamine 2000 was added to the transfection mix.

In order to silence rat Erk expression we used the specific siRNA siGENOME SMARTpools (Dharmacon) against rat Mapk1/Erk2 (M-096054-00-0010) or Mapk3/Erk1 (M-100592-00-0010). Additionally, to silence rat Rsk1 expression we used the siGENOME SMARTpool against rat Rps6ka1 (M-095800-01-0005), as well as a siRNAs mix prepared against rat Rp6ska1 (NM_031107.1) as follows. Total RNA from GH_3 cells was retrotranscribed from position 2964 using a rat Rsk1 primer (5′-AGGAAGTCTCCCAAAAAG). Then, Rsk1 cDNA was amplified by PCR from positions 2054 to 2677 using specific primers with a T7 promoter sequence (5′-CGCTAATACGACTCACTATAGGG) attached to their 5′ end, (fwd: 5′-T7-GGGAAGTTCACCCTCAGTGG; rev: 5′-T7-GGGCGGAGTCCCAAAGCTAT). After amplification, PCR products were purified, quantified and sequenced. Next, rat Rsk1 cDNA fragments were in vitro transcribed from the T7 promoter with MEGAscript® T7 High Yield Transcription Kit (Ambion), and the dsRNAs were purified and quantified. Finally, dsRNAs were treated with BLOCK-iT™ Dicer Enzyme (Invitrogen), and the anti-Rsk1 siRNA mix was purified using BLOCK-iT™ RNAi Purification Kits (Invitrogen), quantified and checked for the correct size. Control cells for RNAi experiments were transfected with 150 nM of ON-TARGETplus non-targeting pool also from Dharmacon (D-001810-10-05) containing a mix of four non-targeting siRNAs. Electrophysiological measurements (Section 2.5) or cell extract preparations (Section 2.4) were performed 48 h after transfection. Before the treatments with TRH, EGF or inhibitors, cells were maintained for 1 h in serum-free medium.

2.3. Quantitative phosphoproteomics

Phosphopeptides were obtained from three independent cultures of GH_3 cells untreated or treated with TRH in triplicate dishes. Cells were switched to serum-free medium and maintained for 1 h before treatment with 1 μM TRH for 5 min. Cells were then lysed and 500 μg of each protein extract was digested with trypsin and desalted with C-18 Spin Columns. Finally, phosphopeptides were enriched using TiO_2 as described in [32,33]. Pellets were diluted in 10 μl of 0.1% trifluoroacetic acid and 4 μl were run in an LC-MS/MS system consisting on a Ultra Performance Liquid Chromatography (nanoAcquity, Waters) directly connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using a 100 min gradient as described in [34]. Peptide identification was automated using Mascot Daemon (version 2.2.2). Mascot Distiller was used to smooth and centroid MS/MS data and Mascot search engine was used to search processed data against the SwissProt database. For these searches, trypsin was used as digestion enzyme and 2 miss cleavages were allowed. Carbamidomethyl (C) was selected as a fixed modification and Pyro-glu (N-term), Oxidation (M) and Phospho (STY) as variable modifications. Rat taxonomy restriction was applied. Mass tolerance of ± 7 ppm for the precursor ions and 600 mmu for fragment ions were accepted. Hits were considered significant when they had an Expectation value < 0.05 (as returned by Mascot). False discovery rates were ~2% as determined by decoy database