Inhibition of Voltage-gated K⁺ Channel Gene Expression by the Neuropeptide Thyrotropin-releasing Hormone

Koichi Takimoto,¹ Robert Gealy,¹ Alla F. Fomina,^{1,a} James S. Trimmer,² and Edwin S. Levitan¹

¹Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and ²Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794

Many neurotransmitters regulate action potential activity in neuronal, endocrine, and cardiac cells by rapidly modulating the gating of K⁺ channels. Neurotransmitters might also produce prolonged effects on excitability by regulating the expression of K⁺ channel genes. Here we show that the neuropeptide thyrotropin-releasing hormone (TRH) down-regulates Kv1.5 and Kv2.1 K⁺ channel mRNAs in clonal pituitary cells. The effect on Kv1.5 mRNA expression does not require protein synthesis and is due to decreased transcription. Immunoblots demonstrate that Kv1.5 and Kv2.1 immunoreactivities are significantly reduced by TRH within 12 hr. The change in channel protein expression is associated with a decrease in voltage-gated K⁺ currents. Thus, TRH enhances excitability by inhibiting K⁺ channel gene expression. Neuropeptide regulation of K⁺ channel gene expression may produce long-term changes in neuronal action potential activity and synaptic transmission.

[Key words: voltage-gated K⁺ channel, gene expression, neuropeptide, GH₃ cells, pituitary, membrane excitability]

Voltage-gated K⁺ channels are major determinants of the shape and frequency of action potential in excitable cells. Molecular genetic studies have identified a large family of mammalian genes encoding voltage-gated K⁺ channels (Pongs, 1992; Salkoff et al., 1992; Gutman and Chandy, 1993). In addition to encoding distinct electrical properties, the structural diversity of K⁺ channels may be important for differential modulation by phosphorylation and G-proteins, and for differential subcellular targeting (Trimmer, 1991; Sheng et al., 1992; Hwang et al., 1993). A large number of K⁺ channel genes also allows for cell type-specific expression and developmental regulation (Beckh and Pongs, 1990; Ribera, 1990; Roberds and Tamkun, 1991a,b; Drewe et al., 1992; Hwang et al., 1992; Lesage et al., 1992; Rudy et al., 1992). Furthermore, a variety of stimuli specifically control expression of K⁺ channel mRNAs in excitable cells. For example, chronic use followed by withdrawal of opioids decreases Kv1.5 and Kv1.6 mRNA levels in rat striatum and

NG108-15 cells (Mackler and Eberwine, 1994). Likewise, expression of Kv1.2 and Kv4.2 mRNAs is temporarily reduced in rat hippocampal neurons following drug-induced seizure activity (Tsaur et al., 1992). Finally, we have found that glucocorticoid hormones specifically upregulate pituitary Kv1.5 mRNA *in vitro* and *in vivo* (Levitan et al., 1991; Attardi et al., 1993; Takimoto et al., 1993). This effect is due to elevated transcription of the channel gene and is associated with an increase in the channel immunoreactive protein and K⁺ current density (Takimoto et al., 1993). Hence, expression of different K⁺ channel genes produces functional diversity that is cell type specific and is influenced by drugs, seizure activity, and steroid hormones.

Many neurotransmitters produce short-term effects on excitability by acutely modulating the gating of ion channels. However, it has been shown that stimulation of *Aplysia* sensory neurons with serotonin produces both short- and long-term changes in excitability (Goelet et al., 1986; Montarolo et al., 1986; Dale et al., 1987; Sholz and Byrne, 1987). In contrast to the short-term effect, the acquisition of the long-term change requires new protein synthesis. Moreover, the long-term enhancement of excitability that occurs with learning is associated with a persistent decrease in an outward K⁺ current (Sholtz and Byrne, 1987). These findings raise the possibility that neurotransmitters might enhance electrical activity by decreasing the expression of K⁺ channel genes.

Testing this hypothesis with CNS neurons is difficult because of their great heterogeneity, interconnectivity, and complex morphology. Therefore, we used GH₃ clonal pituitary cells to test directly if neurotransmitters might influence expression of K⁺ channel genes. PCR analysis indicates that GH₃ cells express three brain voltage-gated K⁺ channel genes (Kv1.4, Kv1.5, Kv2.1) and a homolog of a mouse *shal* gene (Meyerhof et al., 1992). We have detected significant levels of transcripts for the first three voltage-gated K⁺ channels (Levitan et al., 1991; Attardi et al., 1993; Takimoto et al., 1993). Furthermore, we have demonstrated that glucocorticoids specifically induce Kv1.5 gene expression in GH₃ cells and normal pituitary cells (Attardi et al., 1993). Thus, normal regulation of channel expression is intact in these cells. GH₃ cells also possess specific receptors for the neuropeptide thyrotropin-releasing hormone (TRH), which, upon activation, can induce secretion and synthesis of prolactin (Gershengorn, 1986; Hinkle, 1989). Although the neuropeptide was originally discovered in hypothalamus, it is widely distributed throughout the brain and exerts a wide variety of neuropharmacological effects including behavioral changes (Metcalf and Jackson, 1989). Therefore, we tested for regulation of volt-

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Correspondence should be addressed to Edwin S. Levitan at the above address.
*Permanent address: Bogomoletz Institute of Physiology, Kiev, Ukraine.

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age-gated K⁺ channel gene expression by applying TRH to GH₃ cells. In this report, we show that TRH specifically inhibits expression of two voltage-gated K⁺ channel genes and that this inhibition is associated with a decrease in K⁺ current density.

Materials and Methods

Cell culture and treatments. GH₃ clonal pituitary cells (American Type Culture Collection, Rockville, MD) were cultured in Ham's F-10 supplemented with 15% horse serum and 2.5% fetal calf serum in an atmosphere of 5% CO₂ and 95% air. Thyrotropin-releasing hormone and cycloheximide stock solutions were made with water, and dexamethasone stock was made with ethanol at 1000-fold concentrations. For time course experiments, the hormone stock solution was added to the culture medium various time before harvesting the cells such that the cell numbers in each plate were approximately equal at the time of harvesting.

RNA blot hybridization. Total RNAs were isolated by acid phenol chloroform extraction (Chomczynski and Sacchi, 1986). Five micrograms of total RNA were separated on 1% formaldehyde agarose gel and transferred to nylon membrane by capillary action. Prehybridization and hybridization were performed with random prime-labeled probes, as described previously (Takimoto et al., 1993). Rat channel cDNA clones were digested with restriction enzymes and the DNA fragments were isolated for probe preparations: *EcoRI-HindIII* fragment of Kv1 corresponding to nucleotides 1024–2142 for Kv1.5 (Swanson et al., 1990), *XhoI-BglII* fragment of RK3 corresponding to nucleotides 609–2693 for Kv1.4 (Roberds and Tamkun, 1991a), *HindIII-SacI* fragment of *drk1* corresponding to nucleotides 184–2404 for Kv2.1 (Frech et al., 1989), *XhoI-EcoRI* fragment of rbd55 corresponding to the 3' end of the coding region (~2.4 kb) for α_1 subunit of the calcium channel (Snutch et al., 1990). Rat cyclophilin (Danielson et al., 1988) and glutaraldehyde phosphate dehydrogenase (Fort et al., 1985) cDNA clones were digested with a unique enzyme in the polylinker region and used for probe preparation. Intensity of the hybridization signals was quantified by densitometry using enhanced laser system (LKB 2400 Gel Scan XL version 1) and normalized with ethidium bromide staining of 28S ribosomal RNA (Bonini and Hofmann, 1991), as described previously (Takimoto et al., 1993).

Nuclear transcription assay. Nuclei were prepared using 0.05% Triton X-100, and nuclear run-on assay was performed with $\sim 5 \times 10^6$ nuclei and 50 μ Ci UTP (~3000 Ci/mmol), as described previously (Takimoto et al., 1993). Incorporation of radioactivity into RNAs was determined by measuring trichloroacetic acid-precipitable activity. Approximately 3×10^6 cpm ³²P-labeled RNAs were hybridized with a nylon membrane that was previously linked to linearized plasmid DNAs. The membranes were washed and exposed to an x-ray film at -80°C for 2–5 d. The hybridization signals were measured by enhanced laser densitometry (Takimoto et al., 1993).

Immunoblot analysis. Cells grown on plastic dishes were washed with Tris-buffered saline and collected with the same solution containing 1 mM EDTA. The collected cells were lysed in a solution [20 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM iodoacetamide, and 1% Triton X-100]. After removing nuclear debris by centrifugation, the Triton extract was used for immunoblot analysis. For some experiments, post-nuclear membrane fraction was prepared by homogenization and differential centrifugation of cells. Briefly, the collected cells were lysed in a hypotonic solution. After centrifugation of the cell lysate, the pelleted membranes were homogenized in a solution [0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide]. The homogenate was centrifuged at 1000 $\times$ g for 10 min to remove nuclei. The post-nuclear membrane fraction was then obtained by centrifugation of the low-speed supernatant at 100,000 $\times$ g for 1 hr. Protein concentration was determined using Bio-Rad protein assay solution (Bio-Rad, Hercules, CA) with human immunoglobulin as a standard.

Protein (50 or 100 μ g) for the Triton extract, or 10 μ g protein from the post-nuclear membrane fraction were separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was coated with 5% nonfat dry milk in phosphate-buffered saline. The membrane was then probed with one of three anti-channel antibodies: affinity-purified anti-Kv1.5 antibody at 1:200 dilution (Takimoto et al., 1993), anti-Kv1.4 antibody at 1:2000 dilution (Sheng et al., 1992), or affinity-purified anti-Kv2.1 antibody at 1:400 dilution (anti-GST-*drk1*, Trimmer, 1991; or anti-KC, Sharma et al., 1993). Bound antibodies were detected with peroxidase-conjugated anti-rabbit IgG,

followed by enhanced chemiluminescence method (ECL, Amersham, Arlington Heights, IL). Immunoreactive proteins were determined by measuring intensity of the signals on the films by enhanced laser densitometry, as described previously (Takimoto et al., 1993).

Electrophysiology. Cells grown on 35 mm dishes were untreated, or pretreated with 1 μ M TRH for either 1.5 hr or 12 hr. Prior to recording, TRH was removed by replacing the culture medium with Ca²⁺-free bath saline [155 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 20 mM glucose, 10 mM Na-HEPES (pH 7.4)]. Recording was performed within 1 hr after replacing the medium. Patch pipettes were filled with a solution [140 mM KCl, 2 mM MgCl₂, 10 mM K-EGTA, 10 mM Na-HEPES (pH 7.2)]. After formation of gigaohm seal, the bath solution was replaced by perfusing with Ca²⁺-free bath saline supplemented with 1 mM Na-EGTA. K⁺ currents were measured by the standard whole-cell patch-clamp method (Hamill et al., 1981), as described previously (Takimoto et al., 1993).

Results

TRH rapidly downregulates Kv1.5 gene expression

Previously, we have shown that Kv1.5 mRNA and immunoreactive protein rapidly turn over with half-lives of ~35 min, and ~4 hr, respectively, in GH₃ cells (Takimoto et al., 1993). Therefore, changes in Kv1.5 gene transcription can lead to rapid and substantial changes in the channel message and protein. Thus, we measured Kv1.5 mRNA expression following 1 μ M TRH application to GH₃ cells. As can be seen in Figure 1, steady state Kv1.5 mRNA level rapidly dropped to ~50% of control level in response to the neuropeptide. The decrease in Kv1.5 mRNA was dose dependent, with a half-maximal decrease being obtained with ~4 nM TRH (Fig. 2). This sensitivity to TRH is similar to the affinity of the TRH receptor in GH₃ cells (Hinkle, 1989), and *Xenopus* oocytes or COS cells expressing cloned rat TRH receptors (Straub et al., 1990; de la Pena et al., 1992). Thus, TRH rapidly downregulates Kv1.5 mRNA via a high affinity TRH receptor.

To test whether the TRH-induced downregulation of Kv1.5 mRNA might be due to a reduction in mRNA synthesis, we measured Kv1.5 gene transcription with nuclear run-on assays (Fig. 3A,B). Transcription of Kv1.5 gene markedly decreased with 1 hr of TRH treatment, but nearly recovered to the control level at 3 hr. Thus, TRH rapidly and transiently inhibits transcription of the Kv1.5 gene.

TRH-induced downregulation of Kv1.5 mRNA expression does not require protein synthesis or the presence of serum factors

Although Kv1.5 gene transcription rapidly decreased upon TRH application, the inhibition of the channel gene transcription might be indirectly mediated by another protein that is induced by TRH. TRH has been shown to induce expression of the immediate early genes *c-fos* and *c-jun* in GH₃ cells (Carr et al., 1993). Expression of these immediate early gene products reaches significant levels within an hour. Therefore, TRH might inhibit Kv1.5 gene expression indirectly by inducing the expression of these or other immediate early genes. To examine this possibility, GH₃ cells were pretreated with 0.25 mM cycloheximide to inhibit protein synthesis. A half hour after cycloheximide application, the cells were incubated with 1 μ M TRH for 3 hr in the continued presence of the inhibitor (Fig. 4). As previously reported, inhibition of protein synthesis increases steady state level of Kv1.5 mRNA, probably due to stabilization of the message (Takimoto et al., 1993). However, TRH still decreased Kv1.5 mRNA in the cycloheximide-treated cells (Fig. 4). Therefore, downregulation of Kv1.5 mRNA does not require synthesis of regulatory proteins.

Previously, we have demonstrated that the glucocorticoid ag-

Figure 1. TRH rapidly decreases Kv1.5 mRNA expression. *A*, Northern blot analysis of 5 μ g total RNA isolated from GH₃ cells treated with 1 μ M TRH for various times. Arrows on the left represent positions of 18S and 28S ribosomal RNA on the blot. The difference in loading of RNAs in each lane was determined by measuring the intensity of ethidium bromide staining of 28S ribosomal RNA (Bonini and Hofmann, 1991). The relative intensities of 28S ribosomal RNA staining in each lane of the presented blot were 1, 1.28, 1.24, 1.74, 1.67, 1.87, from time 0 to 24 hr, respectively. *B*, Kv1.5 mRNA levels were determined by measuring the 3.5 kb bands on Northern blots and normalized for loading. Points and error bars represent the means and SE, respectively ($N = 4$ for each time point). Asterisk (*) and double asterisk (**) indicate $p < 0.05$ and $p < 0.01$ to control level (time 0), respectively, by two-tailed Bonferroni test.

onist dexamethasone directly activates transcription of the Kv1.5 gene. Therefore, it was possible that endogenous glucocorticoids in the culture medium might upregulate the basal Kv1.5 mRNA level to some extent. If so, TRH might inhibit glucocorticoid receptor activity and thereby decrease Kv1.5 mRNA expression. However, we found that removing the serum from the medium did not affect steady state level or the TRH-induced downregulation of Kv1.5 mRNA (Fig. 4). Moreover, downre-

Figure 2. TRH downregulates Kv1.5 mRNA expression via a high affinity receptor. GH₃ cells were treated with various concentrations of TRH for 3 hr. Total RNAs were isolated, and the 3.5 kb Kv1.5 mRNA levels were measured by Northern blot analysis. Points and error bars represent the means and SE, respectively ($N = 4$ for each dose). The concentration required for the half-maximal reduction of the message was calculated to be ~ 4 nM by fitting the data to the Hill equation with Hill coefficient fixed at 1 using GRAPHPAD INPLOT version 4.0 (San Diego, CA).

gulation of Kv1.5 mRNA was similar in the presence or absence of 100 nM dexamethasone (Fig. 4). Hence, TRH-induced downregulation of Kv1.5 mRNA expression does not depend on glucocorticoid receptor activity or any other serum factors.

TRH downregulates Kv2.1 mRNA, but not Kv1.4 or α_{1D} calcium channel mRNAs

GH₃ cells express at least four distinct voltage-gated K⁺ channel genes (Meyerhof et al., 1992); Kv1.5 and Kv2.1, which encode noninactivating delayed rectifier K⁺ currents (Frech et al., 1989; Swanson et al., 1990), and Kv1.4 and a homolog of a mouse *shal* gene, which encode inactivating transient K⁺ currents (Stuhmer et al., 1989; Tseng-Crank et al., 1990; Baldwin et al., 1991; Pak et al., 1991; Meyerhof et al., 1992). Using rat cDNA probes, we have detected significant amounts of mRNAs encoding the first three K⁺ channels in GH₃ cells (Levitan et al., 1991; Attardi et al., 1993; Takimoto et al., 1993). To test the specificity of the TRH effect, we measured steady state levels of Kv1.4 and Kv2.1 mRNAs in GH₃ cells following neuropeptide application (Fig. 5*A,B*). Kv1.4 cDNA probe gave multiple bands on Northern blot (Fig. 5*A*): molecular sizes of mRNAs detected with Kv1.4 probe were approximately 12, 11, 6, and 4.5 kilobase (kb) (Fig. 5*A*). Kv2.1 cDNA probe detected 11 and 6 kb mRNAs with the 11 kb message being more abundant. Based on the probe lengths and exposure times, we estimated that the 11 kb Kv2.1 mRNA was about 10 times less abundant than the 6 kb Kv1.4 and 3.5 kb Kv1.5 transcripts in GH₃ cells. Figure 5*B* shows TRH-induced changes in the 6 kb Kv1.4 mRNA and the 11 kb Kv2.1 mRNA. We found that TRH was capable

Figure 3. TRH rapidly reduces transcription of Kv1.5 gene. *A*, GH₃ cells were untreated (Control), or treated with 1 μ M TRH for either 1 hr (TRH 1 h) or 3 hr (TRH 3 h). After transcription reaction, approximately 3×10^6 cpm of ³²P-labeled RNAs were hybridized with the nylon membrane that was previously linked to the linearized Kv1.5 cDNA clone (Kv1.5), β -actin cDNA clone (Actin), and the vector alone (pGEM). After washing, the membranes were exposed to an x-ray film for 3 d at -80°C . *B*, Hybridization signals were measured by densitometry. Columns represent transcription activity of Kv1.5 gene relative to that of β -actin in the same blot with error bars indicating SE ($N \geq 3$ for each treatment). Note that Kv1.5 gene transcription in TRH-treated cells for 1 hr is significantly lower than that in control untreated cells ($p < 0.05$, two-tailed t test).

of lowering Kv2.1 mRNA to $\sim 50\%$ of control level. In contrast, the 6 kb (Fig. 5*B*) or the two larger (measured in combination, data not shown) Kv1.4 mRNAs were not significantly affected within 24 hr. Likewise, voltage-gated calcium channel α_{1D} mRNA (Fig. 5*A,B*), as well as cyclophilin and glyceraldehyde phosphate dehydrogenase mRNA levels (data not shown), did not decrease. Treatment of cells with vehicle alone did not significantly affect any mRNA levels within 24 hr (data not shown). Therefore, TRH specifically downregulates expression of Kv1.5 and Kv2.1 K⁺ channel mRNAs.

Figure 4. TRH downregulates Kv1.5 mRNA expression regardless of the presence or absence of cycloheximide, serum, or dexamethasone. GH₃ cells were preincubated for 30 min in the presence of 0.25 mM cycloheximide and then treated or untreated with 1 μ M TRH for 3 hr in the continued presence of the inhibitor (Chx, Chx+TRH). Dexamethasone was added simultaneously with TRH into the medium, and the cells were cultured for 3 hr (Dex, Dex+TRH). For serum deprivation, cells grown on dishes were washed and cultured with serum-free medium for 15–24 hr before application of TRH (–Serum, –Serum+TRH). Total RNAs were isolated, and Kv1.5 mRNA levels were determined by measuring the 3.5 kb channel mRNA by Northern blot analysis. Columns represent the 3.5 kb Kv1.5 mRNA level relative to control level (untreated cells cultured in the presence of serum) with error bars indicating SE ($N > 3$ for each treatment). Note that TRH significantly decreases Kv1.5 mRNA level in the presence of cycloheximide, in the absence of serum, or in the presence of dexamethasone ($p < 0.05$, two-tailed t test).

TRH-induced downregulation of K⁺ channel mRNAs is associated with decreases in the K⁺ channel immunoreactive proteins and K⁺ currents

Immunoreactive channel proteins were then measured using antibodies specific for each K⁺ channel polypeptide. As previously shown (Takimoto et al., 1993), anti-Kv1.4 antibody detects two distinct sizes (96 kDa and 82 kDa) of proteins in GH₃ cell extract, while anti-Kv1.5 antibody exhibits monospecific binding to the 76 kDa protein (Fig. 6*A*). In addition, we attempted to measure Kv2.1 immunoreactive protein in GH₃ cell extract using antibodies specific for the Kv2.1 polypeptide. We used two antibodies: one was raised against a fusion protein containing amino acids 506–533 of rat Kv2.1 polypeptide (GST-drk1, Trimmer, 1991), and the other was against a synthetic peptide corresponding to the carboxyl terminal amino acids 837–853 (KC, Sharma et al., 1993). Both antibodies detected two proteins; a faint and diffuse band of ~ 130 kDa, and a strong tight band of ~ 120 kDa (Fig. 6*A* with anti-GST-drk1, data with anti-KC not shown).

Using these antibodies, we measured changes in immunoreactive channel proteins following 1 μ M TRH application (Fig. 6*a,b*). Immunoblots showed that Kv1.5 immunoreactive protein decreased about twofold within 12 hr of the treatment.

Figure 5. TRH decreases Kv2.1 mRNA, but not Kv1.4 or α_{1D} calcium channel mRNAs. *Top*, Northern blot analyses of 5 μ g total RNA isolated from GH₃ cells treated with 1 μ M TRH for various times. Kv1.4 probe detects four major bands ($\sim$ 12, $\sim$ 11, $\sim$ 6, and $\sim$ 4.5 kb). Kv2.1 probe gives one major band of $\sim$ 11 kb and a minor band of $\sim$ 4.5 kb. α_{1D} calcium channel probe provides a single band of $\sim$ 8 kb. Arrows on the left indicate positions of 18S and 28S ribosomal RNA on the blots. *Bottom*: time course changes following 1 μ M TRH application in the three channel mRNAs; Kv1.4 mRNA (6 kb), Kv2.1 mRNA (11 kb), and α_{1D} calcium channel mRNA (16 kb). For control, channel mRNA levels were determined in the same preparations of cells except that solvent alone was applied to the medium. No significant changes in any channel mRNA levels were obtained within 24 hr. Points and error bars represent the means and SE, respectively ($N \geq 4$). Asterisk (*) and double asterisks (**) indicate $p < 0.05$ and $p < 0.01$ to control level (time 0), respectively, by two-tailed Bonferroni test.

Kv2.1 immunoreactive protein also significantly decreased at 12 hr. In contrast, Kv1.4 immunoreactive protein decreased slightly only after 24 hr. Treatment of cells with vehicle alone did not significantly change the levels of any of the immunoreactive proteins within 24 hr (data not shown). Thus, the neuropeptide TRH acts within hours to specifically reduce two delayed rectifier K⁺ channel proteins.

To test if this decrease in the immunoreactive channel proteins might be associated with changes in density and properties of voltage-gated K⁺ currents, whole-cell patch-clamp recordings were performed following preincubation of cells with 1 μ M TRH for either 1.5 hr or 12 hr (Fig. 7). Prior to recording, TRH was removed by replacing the medium with Ca²⁺-free saline to eliminate acute effects caused by the presence of the neuropeptide. Pretreatment with TRH for 1.5 hr did not significantly alter voltage-gated K⁺ current ($N = 6$, data not shown). In contrast, pretreatment with TRH for 12 hr decreased total voltage-gated outward K⁺ current density approximately twofold (Fig. 7A). This was largely due to a $\sim$ 60% decrease in the noninactivating delayed rectifier component of the K⁺ current (Fig. 7A,B). A less dramatic and significant decrease in the inactivating component was also seen (Fig. 7A,B). No obvious changes in the kinetics of either component were obtained. Hence, the TRH-induced inhibition of expression of Kv1.5 and Kv2.1 gene prod-

ucts is associated with a decrease in voltage-gated K⁺ current density.

Discussion

Many neurotransmitters produce acute effects on excitability by modulating the gating of K⁺ channels already present in plasma membrane. For example, the neuropeptide TRH acts within seconds to modulate the gating of Ca²⁺-activated K⁺ current via a high affinity receptor in GH₃ cells (Ritchie, 1987). Our results demonstrate that TRH acts within hours to inhibit expression of the Kv1.5 and Kv2.1 genes and that this downregulation of K⁺ channel gene expression is associated with a decrease in K⁺ current density. Thus, neurotransmitters can produce long-term changes in electrical activity by regulating K⁺ channel gene expression.

TRH rapidly and reversibly decreased expression of two K⁺ channel mRNAs expression as well as Kv1.5 gene transcription. The reversibility of TRH action might be caused by a decrease in the effective concentration of applied TRH in the medium or the downregulation of TRH receptors. However, an earlier study demonstrated that the prolactin-secreting activity of TRH in the medium of GH₃ cultures is not changed until 24 hr (Hinkle and Tashjian, 1973). Furthermore, we found that different doses of TRH (0.1 and 1 μ M) caused similar time course

Figure 6. TRH influences expression of voltage-gated K⁺ channel immunoreactive proteins. *Top*: immunoblot analyses of three voltage-gated K⁺ channel proteins. Each lane represents 50 μ g protein of the Triton extract for Kv1.4 and Kv1.5, or 100 μ g protein of the Triton extract for Kv2.1 from GH₃ cells treated with 1 μ M TRH for various times. Major immunoreactive proteins detected by Kv1.4 antibody are ~95 kDa and ~82 kDa, while Kv1.5 antibody reacts with ~76 kDa protein (Takimoto et al., 1993). Kv2.1 antibody (anti-GST-*drk1*) detects two proteins of ~130 and ~120 kDa, the larger of which could be seen only after extended exposure of the film. *Arrows* on the left indicate positions of size marker proteins; from top, 97.4 kDa, 66.2 kDa, 55 kDa, and 42.7 kDa. *Bottom*: Time course changes in three K⁺ channel immunoreactivities following 1 μ M TRH application. The panel for Kv1.4 immunoreactivity shows the change in ~95 kDa immunoreactive protein. The ~82 kDa Kv1.4 immunoreactive protein was also changed similarly to the larger immunoreactive protein. For measuring Kv2.1 immunoreactivity, both the post-nuclear membrane fraction and the Triton extract were used for immunoblot analysis. The results with the two preparations were consistent in several parallel experiments. For control, channel immunoreactivities were determined in the same preparations of cells except that vehicle alone was applied to the medium. No significant changes in any channel immunoreactive proteins were seen within 24 hr of vehicle treatment. *Points and error bars* represent the means and SE, respectively. *N* $\geq$ 4 for each time point. *Asterisk* (*) indicate *p* < 0.05 compared to control (time 0) by two-tailed Bonferroni test.

changes in Kv1.5 mRNA expression (data not shown). Likewise, removal of the serum from the medium did not affect the transience of the TRH effect on Kv1.5 mRNA. Thus, the reversibility of TRH action is not due to degradation of the neuropeptide. Moreover, it has been shown that the downregulation of TRH receptor in GH₃ cells is a relatively slow process: the number of available receptors decreases only to ~90% and ~70% of control level 1 hr and 12 hr after the neuropeptide application, respectively (Hinkle and Tashjian, 1975). Our results indicate that Kv1.5 gene transcription and mRNA expression nearly recover to control levels at 3 hr and 6 hr, respectively. Therefore, the temporality of TRH effects is not simply caused by a reduction in the number of available receptors. Rather, it is likely that one application of TRH produces sequential events that include inhibition and subsequent recovery of Kv1.5 gene transcription on a time scale of several hours. Because of the short half-lives of Kv1.5 mRNA and protein (~35 min and ~4 hr, respectively, Takimoto et al., 1993), this rapid change in channel gene transcription might account for the subsequent rapid and substantial changes in channel mRNA and protein.

TRH up- and downregulates prolactin and TRH receptor

mRNAs, respectively, in GH₃ cells. The increase in the hormone mRNA is largely due to an activation of gene transcription (Gershengorn, 1986; Hinkle, 1989), whereas the decrease in the receptor mRNA, at least in part, results from destabilization of the transcript (Narayanan et al., 1992). In this study, TRH appeared to decrease expression of the two K⁺ channel mRNAs. Although Kv2.1 gene transcription was not examined because of its lower expression, we found that TRH reduced Kv1.5 gene transcription. Our results also indicate that the TRH-induced downregulation of Kv1.5 mRNA expression is not mediated by induction of the immediate early genes or antagonism of glucocorticoid action. Therefore, it is likely that TRH directly inhibits transcription of at least one K⁺ channel gene.

The two K⁺ channels that are downregulated by TRH encode delayed rectifier K⁺ currents when individually expressed in *Xenopus* oocytes (Frech et al., 1989; Swanson et al., 1990). However, we found that long-term TRH treatment decreased both delayed rectifier and transient components of K⁺ currents. The less dramatic decrease in the transient K⁺ current might arise for a number of reasons. First, TRH might also decrease expression of another K⁺ channel gene that encodes a transient K⁺ current. PCR analysis indicates that GH₃ cells express an-

other voltage-gated K^+ channel gene homologous to a mouse *shal* gene (Meyerhof et al., 1992). Heterologous expression of the mouse *shal* gene in *Xenopus* oocytes produces a transient K^+ current (Baldwin et al., 1991; Pak et al., 1991). Therefore, it is certainly possible that TRH might also reduce the expression of this K^+ channel gene. Second, it was recently reported that brain dendrotoxin-binding voltage-gated K^+ channels are present in a tight association with another small protein, named β subunit (Scott et al., 1994), and that this association influences current kinetics (Heinemann et al., 1994). Thus, $Kv1.5$ and/or $Kv2.1$ homomeric channels may encode a transient K^+ current in GH_3 cells by associating with a β subunit. Finally, a pore-forming functional voltage-gated K^+ channel consists of four protein subunits, which can be identical gene products or distinct gene products in the same subfamily (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990; Sheng et al., 1993; Wang et al., 1993). Therefore, $Kv1.5$ and/or $Kv2.1$ gene products might be associated with other channel gene products in the corresponding subfamily and thereby contribute to the transient K^+ current in GH_3 cells. For instance, we found significant $Kv1.4$ immunoreactive protein in GH_3 cell extract. Thus, by inhibiting $Kv1.5$ channel expression, TRH might reduce the number of $Kv1.4$ – $Kv1.5$ heteromeric channels, which are known to inactivate (Po et al., 1993; Lee et al., 1994). Indeed, our finding that TRH decreased both the delayed rectifier and transient K^+ currents raises the possibility that altering expression of one channel gene might affect the expression of multiple heteromeric and homomeric channels.

Neurotransmitter inhibition of K^+ channel expression might be important under a variety of physiological, pharmacological, and pathological conditions. For example, studies with *Aplysia* sensory neurons have established that gene expression is required for transmitter-induced long-term enhancement of excitability and synaptic transmission (Goelet et al., 1986; Montarolo et al., 1986; Dale et al., 1987; Sholz and Byrne, 1987), and that long-term memory acquisition is associated with a persistent decrease in voltage-gated K^+ current (Sholz and Byrne, 1987). These effects might be due to neurotransmitter inhibition of voltage-gated K^+ channel gene expression. The reduction in K^+ current, due to a decrease in the number of channels, might slow action potential repolarization and, hence, promote Ca^{2+} entry and synaptic transmission. Similarly, the known long-term enhancement of prolactin secretion induced by TRH may involve inhibition of K^+ channel gene expression (Dannies and Tashjian, 1973). Likewise, TRH-induced inhibition of K^+ channel expression may also occur in CNS where the neuropeptide and transcripts for its receptor are found (Jackson and Reichlin, 1974; Winokur and Utiger, 1974; Calza et al., 1992; Satoh et al., 1993). We also propose that the inappropriate release of neurotransmitters might be responsible for seizure-induced downregulation of K^+ channel mRNAs. Drug-induced seizure activity temporarily decreased two voltage-gated K^+ channel mRNAs in rat hippocampal neurons (Tsaur et al., 1992). Our results suggest that decreases in channel mRNAs would reduce K^+ channel protein expression. This could, in turn, produce hyperexcitability that promotes further seizure activity and epilepsy. Finally, K^+ channels are the targets of acute modulation by hormones, neurotransmitters, and several clinically useful drugs. Indeed, $Kv1.5$ is thought to encode channels that are sensitive to quinidine (Snyders et al., 1992), verapamil (Rampe et al., 1993a), and an antihistamine (Rampe et al., 1993b).

Figure 7. TRH treatment for 12 hr decreases voltage-gated K^+ current density. *A*, GH_3 cells were untreated (control) or pretreated with $1 \mu M$ TRH for 12 hr (TRH). Prior to recording, TRH was removed by replacing the culture medium with Ca^{2+} -free bath saline, and recording was performed between 10 and 60 min after replacing the medium. Total K^+ currents (I_k) was evoked by depolarization from -70 mV to various test potentials ranging from -50 to $+50$ mV. A noninactivating component of the current [$I_{k(DR)}$] was isolated using a 200 msec duration prepulse to 0 mV with a 50 msec duration repolarization to -50 mV preceding each test pulse potential. An inactivating component of the currents was obtained by subtracting the noninactivating component from the total current [$I_k - I_{k(DR)}$]. Currents were normalized to membrane capacitance to account for variation of cell size. *B*, Representative current traces evoked by pulses to $+50$ mV. *C*, control cell; *T*, TRH-treated cell. *C*, Peak current densities were determined from current traces of $I_{k(DR)}$ and $I_k - I_{k(DR)}$. Each point and error bar represent the mean and SE, respectively. $N = 12$ for control, and $N = 13$ for TRH treatment. Asterisk (*) indicates $p < 0.05$ compared to control (two tailed t test).

Therefore, neurotransmitter regulation of K^+ channel gene expression might produce long-term changes in electrical activity and also alter the efficacy of physiological and pharmacological modulators.

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