L-type calcium channels and MAP kinase contribute to thyrotropin-releasing hormone-induced depolarization in thalamic paraventricular nucleus neurons

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Kolaj M, Zhang L, Renaud LP. L-type calcium channels and MAP kinase contribute to thyrotropin-releasing hormone-induced depolarization in thalamic paraventricular nucleus neurons. Am J Physiol Regul Integr Comp Physiol 310: R1120–R1127, 2016. First published March 23, 2016; doi:10.1152/ajpregu.00082.2016.—In rat paraventricular thalamic nucleus (PVT) neurons, activation of thyrotropin-releasing hormone (TRH) receptors enhances neuronal excitability via concurrent decrease in a G protein-coupled inwardly rectifying K (GIRK)-like conductance and opening of a cannabinoid receptor-sensitive transient receptor potential canonical (TRPC)-like conductance. Here, we investigated the calcium (Ca$^{2+}$) contribution to the components of this TRH-induced response. TRH-induced membrane depolarization was reduced in the presence of intracellular BAPTA, also in media containing nominally zero [Ca$^{2+}$]o, suggesting a critical role for both intracellular Ca$^{2+}$ release and Ca$^{2+}$ influx. TRH-induced inward current was unchanged by T-type Ca$^{2+}$ channel blockade, but was decreased by blockade of high-voltage-activated Ca$^{2+}$ channels (HVACCs). Both the pharmacologically isolated GIRK-like and the TRPC-like components of the TRH-induced response were decreased by nifedipine and increased by BayK8644, implying Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels. Only the TRPC-like conductance was reduced by either thapsigargin or dantrolene, suggesting a role forryanodine receptors and Ca$^{2+}$-induced Ca$^{2+}$ release in this component of the TRH-induced response. In pituitary and other cell lines, TRH stimulates MAPK. In PVT neurons, only the GIRK-like component of the TRH-induced current was selectively decreased in the presence of PD98059, a MAPK inhibitor. Collectively, the data imply that TRH-induced depolarization and inward current in PVT neurons involve both a dependency on extracellular Ca$^{2+}$ influx via opening of L-type Ca$^{2+}$ channels, a sensitivity of a TRPC-like component to intracellular Ca$^{2+}$ release via ryanodine channels, and a modulation by MAPK of a GIRK-like conductance component.

thyrotropin-releasing hormone; thalamic paraventricular neurons; L-type Ca$^{2+}$ channels; TRPC-like and GIRK-like currents; mitogen-activated protein kinase

THYROTROPIN-RELEASING HORMONE (TRH), initially isolated from hypothalamic tissue, is the hormone responsible for regulating the release of thyroid-stimulating hormone and prolactin from adenohypophyseal thyrotrophs and lactotrophs, respectively (reviewed in Ref. 26). The subsequent discovery of TRH-like immunoreactivity in neurons and axons in discrete extrahypothalamic regions of the brain together with evidence of TRH binding and mRNA for TRH receptors has triggered numerous investigations, which collectively suggest that TRH and its receptors contribute to a broad range of physiological functions that include arousal, thermogenesis, locomotion, analgesia, mood, and cognition (for reviews, see Refs. 26, 31, 50). In addition, potential therapeutic roles for TRH and TRH analogs are being sought in areas of neuroprotection, neuropsychiatric and mood disorders, narcolepsy, and certain forms of epilepsy (13, 50). Concurrent with these developments, investigations have sought to define the cellular mechanisms of TRH actions at its receptors in neurons, where TRH has long been considered to have a neurotransmitter role. Consistent with this notion, exogenous application of TRH has been shown to influence (usually enhance) neuronal excitability through a postsynaptic action in many central nervous system (CNS) regions. One area of current interest is the thalamic paraventricular nucleus (PVT), where our recent observations revealed that activation of TRH receptors was found to enhance neuronal excitability via concurrent decrease in a G protein-coupled inwardly rectifying K (GIRK)-like conductance and opening of a cannabinoid receptor-sensitive transient receptor potential canonical (TRPC)-like conductance (52, 53). The present study is an extension of this investigation, with a focus on the role of Ca$^{2+}$ influx, Ca$^{2+}$ release, and modulation by MAPK.

In endocrine tissues, TRH acts through a G protein-coupled receptor (GPCR) to promote an increase in phospholipase C (PLC) activity, an initial release of Ca$^{2+}$ from intracellular stores and a later influx of Ca$^{2+}$ through L-type voltage-gated Ca$^{2+}$ channels (reviewed in Ref. 19). TRH also stimulates MAPK (33). However, in neurons, various investigations have not provided consistency in the role of Ca$^{2+}$ entry vs. intracellular Ca$^{2+}$ release in their response to TRH, or a relationship with MAPK. For example, in locus coeruleus, TRH-induced membrane depolarization was resistant to bath-applied nifedipine and TRH-induced inward current remained unaffected after intracellular application of BAPTA, a Ca$^{2+}$ chelator (21). Also, in a majority of dissociated septal neurons, response to TRH was abolished in media containing nominally zero Ca$^{2+}$, while other neurons remained responsive (43). In lateral hypothalamic orexin neurons, the TRH-induced inward current was reduced after substitution of strontium for Ca$^{2+}$ in the bathing media (16). By contrast, the TRH-induced current in dissociated hippocampal CA1 neurons was similar to control in nominally zero Ca$^{2+}$ media but was blocked after application of either thapsigargin in the external media or by intracellular BAPTA (10). Here, in PVT neurons in which the majority of neurons respond to exogenous TRH (52), we sought to address the role of Ca$^{2+}$ influx vs. intracellular Ca$^{2+}$ release, as well as the link to MAPK. We investigated the Ca$^{2+}$ dependency of both the TRPC-like and GIRK-like components of the TRH-induced current. The results indicate a dependency on extracellular Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels, a sensitivity of the TRPC-like component to Ca$^{2+}$-induced Ca$^{2+}$ release

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(CICR) via ryanodine channels, and a modulation by MAPK of the GIRK-like component.

**MATERIALS AND METHODS**

Experimental protocols were approved by the Ottawa Hospital Research Institute Animal Care and Use Committee. We used 3–8-wk-old Wistar rats of either sex that were housed in pairs in a temperature-controlled (22–24°C) environment with a 12:12-h light-dark (LD) cycle, with food and water available ad libitum. Our earlier studies of anterior PVT neurons revealed a diurnal variation in some of their intrinsic properties, notably resting membrane potential, low threshold-activated Ca2+ currents (Ih), hyperpolarization-activated cation currents (Ih), and undefined potassium conductances (25). To minimize any diurnal variations, all animals were killed by guillotine at zeitgeber time (ZT) 2–6, during their subjective quiet-day period. After quick removal, the brain was immersed in oxygenated (95% O2-5% CO2) and cooled (<4°C) in artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 127 NaCl, 3.1 KCl, 1.3 MgCl2, 2.4 CaCl2, 26 NaHCO3, and 10 glucose (osmolality 300–310 mosmol/kgH2O; pH 7.3). Brain slices 350–400 μm in thickness were cut in the coronal plane with a Vibratome 3000 Plus Sectioning System (Vibratome, St. Louis, MO), incubated in gased ACSF for >1 h at room temperature, and then transferred to a recording chamber and superfused (2–4 ml/min) with oxygenated ACSF at 32–34°C. In all experiments, the blind-patch technique was used for whole cell current-clamp and voltage-clamp recordings using either an Axopatch 1D or Axopatch 200A, respectively (Molecular Devices, Sunnyvale, CA). Data were filtered at 2 kHz, continuously monitored and stored on disk. A Digidata 1200B interface with Clampex software (pClamp 9; Molecular Devices, Sunnyvale, CA) was used to eliminate the contribution of GIRK channels to Voltage-clamp recordings in voltage-clamp mode. Recordings in voltage-clamp mode in the presence of 100 nM TRH (100 nM for 60 s) was significantly reduced when compared with controls (Fig. 1, A, B, D; n = 5; one-way ANOVA, F = 17.024, P < 0.001). There was no diurnal variation in resting membrane potential between these two groups (one-way ANOVA, F = 2.254, P = 0.143). To evaluate a dependency on Ca2+ influx, we used ACSF with nominally zero Ca2+. Here, the TRH-induced depolarization was also significantly reduced (Fig. 1, C and D; n = 6; one-way ANOVA, F = 23.705, P < 0.001). The data suggest major roles both for Ca2+ influx and intracellular Ca2+ in the TRH-induced response.

**RESULTS**

We first evaluated the TRH response under current-clamp conditions. During intracellular dialysis with 10 mM BAPTA in the pipette media, the membrane depolarization induced by a brief bath application of TRH (100 nM for 60 s) was significantly reduced compared with controls (Fig. 1, A, B, D; n = 5; one-way ANOVA, F = 17.024, P < 0.001). There was no diurnal variation in resting membrane potential between these two groups (one-way ANOVA, F = 2.254, P = 0.143). To evaluate a dependency on Ca2+ influx, we used ACSF with nominally zero Ca2+. Here, the TRH-induced depolarization was also significantly reduced (Fig. 1, C and D; n = 6; one-way ANOVA, F = 23.705, P < 0.001). The data suggest major roles both for Ca2+ influx and intracellular Ca2+ in the TRH-induced response.

**TRH-induced inward current depends on Ca2+ entry through L-type Ca2+ channels.** Recordings in voltage-clamp mode in the presence of 1 μM TTX confirmed a dependency of the TRH-induced inward current on external Ca2+. In ACSF containing nominally zero Ca2+, the response to TRH (100 nM applied for 60 s) was significantly reduced (Fig. 2, A and E; one-way ANOVA, F = 10.984, P = 0.003). Because TRH

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**Fig. 1. External Ca2+ plays a major role in thyrotropin-releasing hormone (TRH) receptor signaling.** A: whole cell current-clamp recording illustrates the typical response of paraventricular thalamic nucleus (PVT) neurons to a 60-s bath application of 100 nM TRH (bar). B and C: whole cell current-clamp recordings from two other PVT neurons show smaller TRH responses when TRH was applied either during the internal perfusion with 10 mM BAPTA (B) or in artificial cerebrospinal fluid (ACSF) with nominally 0 mM Ca2+ (for 15 min; C). D: summary histogram illustrates TRH-induced membrane depolarization in the presence of 100 nM TRH alone (black bar; for 60 s) or when TRH was applied either during internal perfusion with 10 mM BAPTA or in ACSF with nominally 0 mM Ca2+ (gray bars). Data are expressed as means ± SE, **p < 0.001.**
Ca$^{2+}$ illustrate a reduced TRH-induced inward current in the presence of L-type
0.001. A voltage range at which low-voltage activated T-type Ca$^{2+}$
evaluated the TRH-induced current in the presence of a Z941
responses occurred from a holding potential around
200 nM), nifedipine (3
traces done in the presence of 1
receptor to both G protein-coupled inwardly rectifying K (GIRK)- and tran-
coadministration of L-type Ca$^{2+}$
enhanced amplitude and duration of TRH-induced inward current during
Fig. 2. Activation of L-type Ca$^{2+}$
things was identical with control data (Fig. 3
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of K⁺ conductances (Ref. 52), the amplitude of the TRH-induced inward current was significantly reduced by thapsigargin (Fig. 3B; one-way ANOVA, F = 10.476, P = 0.005), suggesting an involvement of CICR in TRH-induced opening of TRPC-like channels.

CICR can be mediated through activation of either inositol 1, 4, 5-trisphosphate receptors (IP3Rs) or ryanodine receptors (RyRs). Data from our previous investigation indicated that IP3Rs are unlikely to be involved in the TRH-induced inward current (52, 53). However, L-type Ca²⁺ channels may be functionally coupled with RyRs (6). To address a potential L-type channel coupling with RyRs, we next bath-applied dantrolene (20 μM), a specific RyR inhibitor and obtained results that were similar to thapsigargin, i.e., the net TRH-induced inward current was significantly reduced (Fig. 3B; one-way ANOVA, F = 10.389, P = 0.003). In addition, the TRH response was unchanged in low Na⁺ ACSF (Fig. 3B; one-way ANOVA, F = 0.864, P = 0.370) but significantly smaller in ACSF containing 1.3 mM Ba²⁺ (Fig. 3B: one-way ANOVA, F = 20.356, P < 0.001). These data suggest that CICR through functional coupling between L-type Ca²⁺ channels and RyRs contributes to the TRH-induced activation of TRPC-like channels.

MAPK participates in TRH-induced suppression of GIRK-like channels. Investigations in various endocrine-like cell lines reveal that activation of TRH receptors can lead to stimulation of MAPK (33, 41). To assess a possible participation of MAPK in the TRH-induced response in PVT neurons, we evaluated the impact of PD98059, a specific inhibitor of MAPK (1) on the TRH-induced current. In the presence of PD98059 (50 μM), the net TRH-induced inward current displayed no reversal potential, and the amplitude was reduced when compared with control (Fig. 4, A and B; one-way ANOVA, F = 8.817, P = 0.006), suggesting that activation of MAPK could be involved in the regulation of TRH-induced decrease of a GIRK-like conductance. In support of this, we noted that the TRH-induced current was significantly reduced when PD98059 was applied under conditions in which the activity of TRPC-like channels was inhibited (low Na⁺ data in Fig. 4B; one-way ANOVA, F = 34.764, P < 0.001). By contrast, when tested in ACSF containing 1.3 mM Ba²⁺, PD98059 did not alter the TRH-induced increase in TRPC-like channel conductance (Ba²⁺ data in Fig. 4B; one-way ANOVA, F = 0.370, P = 0.526).
By contrast, the data from the TRH-induced responses in PVT neurons suggest that influx via L-type Ca\(^{2+}\) channels is a critical early step in TRH receptor signaling, a judgment based on the following observations. First, TRH-induced depolarization is decreased in the presence of ACSF containing nominally zero Ca\(^{2+}\), attesting to the importance of extracellular Ca\(^{2+}\). Second, although TRH-induced inward current is not affected after blockade of T-type Ca\(^{2+}\) channels with Zn\(^{2+}\), sensitivity to Cd\(^{2+}\) supports an involvement of high-voltage activated Ca\(^{2+}\) channels (HVACCs). While Cd\(^{2+}\) at the concentration used here has been reported to impair T-type channel current in cultured thalamic neurons (34), we have observed no significant influence on T-type Ca\(^{2+}\) channel current in PVT neurons in our slice preparations (see Fig. 2C in Ref. 55). Since the TRH-induced inward current is insensitive to coapplication of ω-Agatoxin IVA/ω-conotoxin GVIA, yet modulated with nifedipine and BayK8644, the pharmacological profile seems consistent with an engagement of L-type HVACCs (5). It has been shown that nifedipine may interfere with T-type Ca\(^{2+}\) channel function (37). However, its main target appears to be the Ca\(_{v}3.2\) subtype, which has the lowest expression in PVT neurons (see Fig. 3B in Ref. 25). The TRH-induced response component that is mediated by the closing of GIRK-like channels is not blocked by agents acting on PC-PLC and cannabinoid receptors (53) or on CICR (Fig. 3, current study) but is almost equally as reduced as the TRPC-mediated response component in the presence of nifedipine or BayK8644 (Fig. 2E). Therefore, these data suggest that activation of the L-type HVACC is a foremost step in the TRH receptor signaling pathways for both closure of the GIRK-like channels and opening of the TRPC-like channels. To the best of our knowledge, an involvement of L-type HVACCs in neuronal responses to TRH has not been verified previously. Of interest, and for similarity, direct activation of L-type HVACCs by G protein-coupled receptors in CNS neurons has been reported for cholinergic receptors (54, 56), orexin receptors (23, 48), and glutamate metabotropic receptors (56, 58).

L-type Ca\(^{2+}\) channels are formed by the Ca\(_{v}1.1\) family, which consists of four isoforms of the α\(_{1}\) pore-forming subunit (Ca\(_{v}1.1\) to Ca\(_{v}1.4\); reviewed in Refs. 51 and 59). While L-type HVACCs are commonly activated at suprathreshold membrane potentials, a functional contribution to resting membrane potential has also been reported (29). Although there is the possibility that poor spatial clamping of dendrites may allow the voltage to be more positive and, thus, contribute to Ca\(^{2+}\) influx at remote sites, we noted minimal inward current under basal conditions at −63 mV. Recently, it has been reported that
the Ca_{1.3} gene product, in particular, can be activated at relatively hyperpolarized membrane potentials that may overlap with low-threshold T-type channels (49). Indeed, L-type Ca_{1.3} isofoms are present in PVT (17, 42). However, our data indicate that blockade of T-type channels had no influence on the TRH-induced inward current.

Chelation of intracellular Ca^{2+} ions with BAPTA decreased the TRH-induced response, suggesting that Ca^{2+}-dependent intracellular processes have a role. Consistent with this hypothesis, the data show that both thapsigargin and dantrolene decrease the TRPC-mediated component of the TRH-induced response, implying that CICR through ryanodine receptors is part of the signaling pathway leading to opening of TRPC-like channels in PVT neurons. A functional coupling between ryanodine receptors and L-type HVACCs has been established in neurons (6). Interestingly, it has also recently been suggested that there is a cross-signaling between L-type HVACCs and RyRs that control Ca^{2+} influx during neuronal activity in lateral geniculate thalamic cortical relay neurons (35).

We recently reported observations suggesting that activation of TRH receptors can lead to synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG; 53). 2-AG is perhaps the most abundant endocannabinoid in the rat brain (2). Since 2-AG synthesis can depend on an increase in postsynaptic intracellular Ca^{2+} concentration (32, 38, 40), data from the current study would be in agreement with an involvement of intracellular Ca^{2+} as part of one or more pathways that lead to activation of cannabinoid receptors and consequent opening of TRPC-like channels. TRH-induced currents have a profile suggestive of TRPC4/5-like channels (52). In addition, it has been proposed that enhanced Ca^{2+} entry through either CICR or voltage-dependent Ca^{2+} channels is sufficient for TRPC5 channel activation (14). Therefore, we might speculate that Ca^{2+} ions have a dual function, one as a possible cofactor for 2-AG synthesis, another as a TRPC-like channel gating molecule.

Activation of many GPCRs leads to an increase in MAPK activity. In pituitary cells expressing endogenous TRH receptors and in a number of cell lines expressing transfected TRH receptors, exposure to TRH has been shown to stimulate MAPK (22, 33, 46). MAPK cascades can couple GPCRs to the nucleus (15), and MAPK is required for TRH stimulation of transcription of certain genes (46). Comparatively little is known with respect to a role of MAPK in TRH receptor signaling in neurons. In a study focusing on a TRH-induced increase in spontaneous inhibitory postsynaptic currents, exposure to the MAPK kinase inhibitor PD98059 reduced ongoing events to ~60% of control but did not block TRH-induced events, suggesting that the MAPK pathway was not involved (9). Conversely, our observations in PVT neurons do suggest an involvement of MAPK in the TRH-induced current, specifically in the closure of GIRK-like channels. This was perhaps surprising given the fact that TRH responses mediated by the opening of TRPC-like channels involves activation of endogenous cannabinoid receptors (53), and MAPK is known to be part of their signaling pathways (3). On the other hand, a coupling between GIRK channels and MAPK has been suggested before (7, 27). Although we have not pursued the role of MAPK further here, it interesting to note that participation of both L-type HVACCs and MAPK are essential in prokineticin 2 receptor-mediated depolarization of neurons in the subfor-


