Excitatory Actions of Vasoactive Intestinal Peptide on Mouse Thalamocortical Neurons Are Mediated by VPAC$_2$ Receptors

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Lee, Sang-Hun and Charles L. Cox. Excitatory actions of vasoactive intestinal peptide on mouse thalamocortical neurons are mediated by VPAC$_2$ receptors. J Neurophysiol 96: 858–871, 2006. Thalamic nuclei can generate intrathalamic rhythms similar to those observed at various arousal levels and pathophysiological conditions such as absence epilepsy. These rhythmic activities can be altered by a variety of neuromodulators that arise from brain stem regions as well as those that are intrinsic to the thalamic circuitry. Vasoactive intestinal peptide (VIP) is a neuropeptide localized within the thalamus and strongly attenuates intrathalamic rhythms via an unidentified receptor subtype. We have used transgenic mice lacking a specific VIP receptor, VPAC$_2$, to identify its role in VIP-mediated actions in the thalamus. VIP strongly attenuated both the slow, 2–4 Hz and spindle-like 5–8 Hz rhythmic activities in slices from wild-type mice (VPAC$_2^{+/+}$) but not in slices from VPAC$_2$ receptor knock-out mice (VPAC$_2^{-/-}$), which suggests a major role of VPAC$_2$ receptors in the antioscillatory actions of VIP. Intracellular recordings revealed that VIP depolarized all relay neurons tested from VPAC$_2^{+/+}$ mice. In VPAC$_2^{-/-}$ mice, however, VIP produced no membrane depolarization in 80% of neurons tested. In relay neurons from VPAC$_2^{+/+}$ mice, VIP enhanced the hyperpolarization-activated mixed cation current, I$_h$, via cyclic AMP activity, but VIP did not alter I$_h$ in VPAC$_2^{-/-}$ mice. In VPAC$_2^{-/-}$ mice, pituitary adenylyl cyclase activating-polypeptide (PACAP) depolarized the majority of relay neurons via I$_h$ enhancement presumably via PAC$_2$ receptor activation. Our findings suggest that VIP-mediated actions are predominantly mediated by VPAC$_2$ receptors, but PAC$_2$ receptors may play a minor role. The excitatory actions of VIP and PACAP suggest these peptides may not only regulate intrathalamic rhythmic activities, but also may influence information transfer through thalamocortical circuits.

INTRODUCTION

The thalamus serves as the gateway for relaying peripheral sensory-related information to the neocortex. In addition, the thalamus is capable of generating rhythmic activities similar to those observed during various arousal states and in certain pathophysiological conditions such as absence epilepsy (Steriade and Llinás 1988; von Krosigk et al. 1993; Steriade et al. 1993; Huguenard and Prince 1994; Warren and Jones 1994). The rhythmic activities arise from the reciprocal synaptic connectivity between thalamic relay nuclei and the adjacent thalamic reticular nucleus (TRN) in conjunction with the intrinsic properties of thalamic neurons (Deschênes et al. 1982; Jahnson and Llinás 1984a; von Krosigk et al. 1993). Although the cellular mechanisms required to maintain such rhythmic activities are well understood, those involved in the initiation and termination of these rhythmic activities are unclear. Certain neuromodulators, which arise from brain stem nuclei, such as acetylcholine, norepinephrine, and serotonin, have been found to attenuate the rhythmic activities (Lee and McCormick 1997; Lee and McCormick 1996; McCormick 1992). This termination of the rhythmic activity is closely related to changes in behavioral states.

There is increasing evidence that another group of neuromodulators namely, neuropeptides can also alter thalamic activity. A variety of neuropeptides (e.g., cholecystokinin, neuropeptide Y, nociceptin/orphanin FQ, somatostatin, substance P, and vasoactive intestinal peptide [VIP]), along with their respective receptors, have been localized within the thalamus and have been shown to alter the excitability of thalamic neurons by changing intrinsic properties of these cells such as resting membrane potential, input resistance, membrane conductance, and action potential firing mode or affecting synaptic transmission (Cox et al. 1995; Leresche et al. 2000; Sun et al. 2001; Sun et al. 2002; Lee and Cox 2003; Sun et al. 2003). Because neuropeptides have been found to be colocalized with classical neurotransmitters (i.e., γ-aminobutyric acid [GABA] and glutamate) in other brain regions, released in an activity-dependent manner, and produce long-lasting changes in neuronal excitability, they may play an important role in regulating thalamic activity (Lundberg and Hökfelt 1983).

VIP and pituitary adenylyl cyclase–activating polypeptide (PACAP) belong to the glucagons-secretin–VIP family of peptides. VIP was originally isolated from porcine intestine as a 28–amino acid peptide and is involved in many regulatory functions, including vasodilation, gastrointestinal secretion and motility, and glycogenolysis (Gozes and Brenneman 1989). VIP is also broadly distributed in the central and peripheral nervous system, where it has been found to produce a variety of actions (Gozes and Brenneman 1989). PACAP is a 38–amino acid peptide that shares 68% identity with VIP and was originally isolated from ovine hypothalamus and found to increase cyclic AMP (cAMP) accumulation in anterior pituitary cells (Miyata et al. 1989). PACAP is also localized within a variety of brain areas and peripheral organs and exerts a broad range of physiological actions, including the regulation of anterior pituitary cell function, adrenal gland activity, endocrine and exocrine pancreas secretions, and testicular spermatogenesis (Arimura and Shioda 1995).

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VIP and PACAP can activate three different subtypes of receptors in the CNS: VPAC1, VPAC2, and PAC1 (Gozes and Brenneman 1989; Harmar et al. 1998). VIP and PACAP have similar affinities for VPAC1 and VPAC2 receptors; however, PACAP has a relatively higher affinity than VIP for PAC1 receptors. All three receptor subtypes are GTP-dependent protein-coupled receptors and are coupled to cAMP and/or inositol phosphate turnover (Ishihara et al. 1992; Deutsch and Sun 1992; Spengler et al. 1993; Van Rampilbergh et al. 1997; MacKenzie et al. 2001). These receptor subtypes are differentially distributed throughout the brain: VPAC1 receptors are most abundant in the cerebral cortex and hippocampus (Ishihara et al. 1992), VPAC2 receptors are primarily found in the thalamus and suprachiasmatic nucleus (Usdin et al. 1994; Sheward et al. 1995), and PAC1 receptors are more evenly distributed in various brain regions with relatively higher concentrations within the olfactory bulb, thalamus, hypothalamus, hippocampus, and cerebellum.

VIP is localized within TRN neurons, and these neurons in turn innervate almost all thalamic nuclei (Jones 1985). Both VPAC2 and PAC1 receptors are localized within primary sensory thalamic nuclei, including the ventrobasal nucleus (VB) and the dorsal lateral geniculate nucleus (US). We have shown elsewhere that VIP, via an unidentified receptor subtype, selectively depolarizes VB relay neurons and attenuates slow intrathalamic oscillations (Lee and Cox 2003; Sun et al. 2003). In a previous study, the VIP and PACAP depolarization of thalamic neurons was claimed to be via activation of PAC1 receptors based on agonist concentrations used (Sun et al. 2003). These pharmacological agents, however, are not particularly selective. In light of the availability of transgenic mice lacking the VPAC2 receptor, we can determine the role of VPAC2 receptor-mediated actions as well as PAC1 receptor-mediated actions on relay neurons. Our results indicate that VIP depolarizes relay neurons and attenuates intrathalamic oscillation via activation of VPAC2 receptors in mouse thalamus. PACAP, presumably through the activation of PAC1 receptors, also depolarizes relay neurons via a common mechanism, the enhancement of the hyperpolarization-activated mixed cation current, Ih. Finally, the VPAC2 receptor-mediated actions are required for the activation of the cAMP-dependent second messenger pathways. Some of these findings have been presented in abstract form (Lee et al. 2003).

**METHODS**

The general procedures used in these experiments were similar to those described elsewhere (Lee and Cox 2003). Mice deficient in VPAC2 receptor (VPAC2-/-) were originally generated by Dr. A. Harmar (Harmar et al. 2002). The University of Illinois colony of VPAC2-/- and VPAC2+/+ mice was derived from five breeding pairs of heterozygous mice (C57BL/6J) kindly provided by Dr. Harmar. The general procedures used for genotyping the mice in these experiments were similar to those previously described (Harmar et al. 2002). Young VPAC2-/- and VPAC2+/+ mice (postnatal age, 10–21 days) were deeply anesthetized with sodium pentobarbital (50 mg/kg) and decapitated. The brain was quickly removed and placed into cold, oxygenated physiological saline containing (in mM): 126.0 NaCl, 2.5 KCl, 1.25 MgCl2, 2.0 CaCl2, 0.5 Mg(H2PO4)2, 1.25 NaHCO3, 26.0 NaHCO3, and 10.0 glucose. This solution was gassed with 95% O2/5% CO2 to a final pH of 7.4.

**Intracellular recording procedures**

Intracellular recordings using the whole-cell configuration were obtained with the visual aid of an Axioskop 2FS equipped with differential interference contrast optics (Zeiss Instruments, Thornwood NY). Individual slices were transferred to a submersion-type recording chamber. A low-power objective (5x) was used to identify specific thalamic nuclei, and a high-power water immersion objective (63x) was used to visualize individual neurons. Recording pipettes were pulled from 1.5 mm outer diameter capillary tubing and had tip resistances of 3–6 MΩ when filled with the following intracellular solution (in mM): 117 K-glucuronate, 13 KCl, 1.0 MgCl2, 0.07 CaCl2, 0.1 EGTA, 10.0 HEPES, 2.0 Na2-ATP, and 0.4 Na-GTP. The pH was adjusted to 7.3 and osmolarity was adjusted to 290–300 mosm. Initial access resistances following break-in typically ranged from 10 to 25 MΩ and remained stable during most recordings included for analyses in this study. An Axoclamp2B amplifier (Axon Instrument, Foster City, CA) was used in bridge mode for voltage recordings (current clamp mode) or switching single-electrode voltage clamp mode for current recordings. Current and voltage protocols were generated by use of pClamp software (Axon Instruments), and data were stored on a computer. For current clamp recordings, an active bridge circuit was continuously adjusted to balance the drop in potential produced by passing current through the recording electrode. The apparent input resistances of individual neurons were calculated from the linear slope of the voltage-current relationship obtained by applying constant current pulses ranging from –100 to +40 pA (800 ms duration). During VIP and PACAP38 application, changes in input resistance were determined by membrane responses to single-intensity constant current hyperpolarizing pulses (5–40 pA, 500 ms, 0.2 Hz). For voltage clamp recordings, the amplifier was used in discontinuous mode. In these recordings, the switching frequency ranged from 2.5 to 3.5 kHz, with a gain of 150 to 800 pA/mV, and the headstage was continually monitored to ensure that the current transients had completely decayed before voltage measurements. Voltage-clamp recordings were limited to neurons that had a stable access resistance <30 MΩ.

**Extracellular recording procedures**

Extracellular multiple unit recordings were obtained using sharpened tungsten microelectrodes (Frederick Haer, Inc., Bowdoinham, ME). All data were digitized (1–2 kHz) and stored using pClamp software (Axon Instruments). Monopolar electrical stimulation was applied to either TRN or internal capsule by use of sharpened tungsten electrodes (200–600 kΩ). Analyses of intrathalamic rhythmic activities were similar to those described previously (Lee and Cox 2003). In brief, autocorrelograms were constructed from the extracellular multiple-unit data during a 3–9 s period with a bin size of 30 ms to quantify degree of synchrony and duration of intrathalamic oscillations (Minianalysis, Synaptosoft Inc., Fort Lee, NJ). Three measures were used to quantify oscillatory activity in autocorrelograms: number of peaks, oscillation amplitude (Amposc), and frequency of oscillation. Alterations in Amposc indicate a change in the number of unit discharges in the rhythmic activity. The number of peaks indicates the number of cycles in the rhythm, and the oscillation frequency reflects the principle frequency of the rhythmic activity.
Pharmacological agents

Concentrated stock solutions of VIP and PACAP38 were prepared in distilled water and diluted in physiological saline to a concentration of 0.4–4.0 μM. VIP and PACAP38 were applied by injecting a bolus into the input line of the chamber using a motorized syringe pump. On the basis of the rate of agonist injection and the rate of chamber perfusion, the final bath concentration of drugs was estimated to be one eighth of the concentration introduced in the flow line and this dilution is reflected within the results (Cox et al. 1995; Lee and Cox 2003). Control injections of physiological saline did not alter the intrathalamic activity during extracellular recording or intrinsic properties (e.g., membrane potential, input resistance) of neurons during current-clamp recordings, which suggests that the temporary increase in flow rate during the bolus injections had no effect on the recordings. VIP and PACAP38 were purchased from Calbiochem (San Diego, CA) and ZD7288 from Tocris (Ellisville, MO). All remaining compounds were purchased from Sigma (St. Louis, MO).

Statistics

Data are presented as means ± SD. Most statistical analyses consist of Mann-Whitney U test and, when appropriate, the Wilcoxon test for paired samples. In some noted instances, a students’ t-test or paired t-test was used for testing statistical significance. Results were considered statistically significant when P < 0.05.

RESULTS

VIP attenuates intrathalamic rhythms via activation of VPAC2 receptors

Extracellular recording electrodes were placed in the VB to monitor intrathalamic rhythmic activity that arises from the reciprocal synaptic connectivity between VB and the adjacent TRN. Single electrical stimuli in or near TRN typically evoked a spindle-like rhythmic activity (5–8 Hz) in slices from VPAC2+/+ and VPAC2−/− mice that could last for many seconds (Fig. 1Ai). These spindle-like rhythms were produced in 76% of slices from VPAC2+/+ mice (13/17 slices) and 78% of slices from VPAC2−/− mice (14/18 slices). This incidence rate of obtaining the rhythmic activity was not significantly different between the two groups (P > 0.5, Fisher’s Exact test). The autocorrelogram clearly indicates a highly synchronized response with duration of approximately 2 s (approximately 10 cycles) in a slice from a VPAC2+/+ mouse (Fig. 1B, left panel, black line) and a similar duration (2 s, approximately 12 cycles) in a slice from a VPAC2−/− mouse (Fig. 1B, right panel, black line). Under these conditions, the frequency and duration of the rhythmic activity were stable from trial to trial. From the autocorrelograms, we quantified the number of peaks and interburst frequency. The interburst periods of the spindle-like activity produced in slices from VPAC2+/+ and VPAC2−/− mice were very stable from trial to trial (Figs. 1Aiii and 2Aii). The autocorrelograms clearly indicate a highly synchronized response that lasts four cycles (approximately 1.5 s) in a slice from a VPAC2+/+ mouse (Fig. 1B, left panel, gray line) and three cycles (approximately 1.5 s) in a slice from a VPAC2−/− mouse (Fig. 1B, right panel, gray line). The interburst periods and the number of peaks of the rhythmic activity did not significantly differ in slices from VPAC2+/+ and VPAC2−/− mice (Fig. 1, C and D; P > 0.05; Mann-Whitney U test).

We next tested the effects of VIP on intrathalamic rhythmic activity in slices from VPAC2+/+ mice (n = 6) and from VPAC2−/− mice (n = 5). VIP (0.5 μM, 60 s duration) dramatically suppressed the spindle-like rhythmic activity in all slices from VPAC2+/+ mice (Fig. 2 Ai, left column). This VIP concentration was used because it produced a maximal depolarization in rat thalamic relay neurons (Lee and Cox 2003). The contour plot illustrates that the maximum effect occurred 120 s after VIP treatment and recovered near predrug levels within 5 min following VIP application (Fig. 2Aii, left column). VIP significantly reduced the number of peaks and Amppeaks in all VPAC2+/+ slices (Fig. 2B, P < 0.05; Mann-Whitney U test). In addition to the suppression of the rhythmic activity, the interburst period was shortened from 220 ms to 150 ms after VIP application in two slices in which VIP severely attenuated but did not eliminate the rhythmic activity. Following the addition of BMI (10 μM) to transform the spindle-like rhythm to a slow-wave rhythm, VIP (0.5 μM, 60 s) also dramatically suppressed the slow rhythmic activity in a reversible manner in all slices tested from VPAC2+/+ mice (Fig. 2Ai, ii, left panel; n = 7). In slices from VPAC2−/− mice, this relatively high concentration of VIP (0.5 μM) did not alter the spindle-like or slow-wave rhythmic activity (Fig. 2Ai, ii, iii). Our population data indicate that VIP did not significantly alter the number of peaks, Amppeaks, or interburst period in slices from VPAC2−/− mice (Fig. 2B, P > 0.1, Mann-Whitney U test). Our data indicate that the antissorciatory action of VIP is mediated through the activation of VPAC2 receptors.

VIP depolarizes relay neurons in VPAC2+/+ slices, not in VPAC2−/− slices

We next examined the effects of VIP on thalamic relay neurons in slices from VPAC2+/+ and VPAC2−/− mice. Intracellular recordings were obtained from 68 relay neurons from VPAC2+/+ mice and 61 relay neurons from VPAC2−/− mice. The average resting membrane potential (VPAC2+/+: −67.7 ± 3.1 mV; VPAC2−/−: −68.7 ± 2.5 mV) and apparent input resistance (VPAC2+/+: 210.0 ± 82.5 MΩ; VPAC2−/−: 218.2 ± 87.0 MΩ) of relay neurons from VPAC2+/+ and VPAC2−/− mice did not differ significantly (P > 0.1, Mann-Whitney U test).

Similar to what we had previously observed in rat thalamic relay neurons, VIP (0.5 μM) consistently depolarizes thalamic relay neurons from wild-type mice (Fig. 3A). This VIP concentration (0.5 μM) depolarized all relay neurons tested from VPAC2+/+ mice with an average amplitude of 3.9 ± 1.5 mV (n = 23). The latency to peak of the VIP-mediated depolarization occurred 115 ± 26 s (n = 23) after VIP application, and the depolarization lasted an average of 333 ± 71 s in 12 of 17 neurons that completely recovered to the pre-VIP membrane potential. A smaller depolarization was produced by lower VIP concentration (0.1 μM, 3.4 ± 0.8 mV, n = 3, data not shown).

J Neurophysiol • VOL 96 • AUGUST 2006 • www.jn.org
A reduction in the apparent input resistance was commonly associated with the VIP-mediated membrane depolarization (Fig. 3A). In a subset of neurons in which the membrane potential was manually repolarized to predrug levels during the peak of the VIP-mediated depolarization, the input resistance was reduced by 36\% (n = 4).

In slices from knock-out (VPAC2−/−) animals, VIP (0.5 μM) did not alter the membrane potential in the majority of neurons (8/10 cells, Fig. 3Bi); however, a much smaller depolarization was observed in two neurons (Fig. 3Bii, 1.7 ± 0.8 mV). The scatter plot in Fig. 3C illustrates the range of responses to VIP (0.5 μM) in neurons from VPAC2+/+ (●) and VPAC2−/− (▲) animals. It is clear that the excitatory action of VIP on thalamic relay neurons is predominantly mediated via activation of VPAC2 receptors.

In our previous study in rat thalamus, we found that VIP enhances I_h in relay neurons (Lee and Cox 2003). We sought to extend this finding to the mouse thalamus but, more important, determine whether the alteration of I_h is abolished in the VPAC2−/− animals. Voltage-clamp recordings were used to characterize the VIP-mediated conductance changes in relay neurons from VPAC2+/+ and VPAC2−/− animals. Long-duration (2.4 s) voltage step commands (−50 to −120 mV; 10 mV increments; 0.125 Hz) were used to activate I_h in relay neurons. The current responses to the step commands consisted of an initial instantaneous response followed by a slow inward current that typically reached a steady state after a couple of seconds (Fig. 4A). We calculated I_h as the difference between the initial instantaneous response (I_{in}) and the steady-state level (I_{ss}). Little I_h was evoked in response to the initial small
FIG. 2. VIP strongly attenuates intrathalamic oscillations in VPAC2+/+ slices, but not in VPAC2−/− slices. A: in control conditions, single stimulus (●) in internal capsule evokes 5–8 Hz rhythmic activities in slices from VPAC2+/+ and VPAC2−/− mice. VIP (0.5 μM) strongly attenuates this oscillation in VPAC2+/+ slices, but does not alter activity in VPAC2−/− slices. The addition of BMI (10 μM) transforms the fast rhythmic activity to a slower 2–4 Hz rhythmic activity in slices from both animal types. VIP strongly attenuates this slow oscillation in VPAC2+/+ slices, but not in VPAC2−/− slices. Aii: contour plots of experiments in Ai illustrating the time course of VIP effect on intrathalamic rhythmic activity. Aiii: the degree of synchrony and duration of the rhythmic activity were quantified from autocorrelograms constructed from the raw data. The autocorrelograms illustrate the synchronized rhythmic activity in prior to (black trace) and following (red trace) VIP application. B: summary of VIP effects on number of peaks, Amp, and oscillation period as calculated from the autocorrelograms. The number in each bar indicates the number of slices tested. *, P < 0.05.
The fact that VIP produced a small depolarization in 2 of 10 neurons in slices from VPAC2−/− mice suggests VIP may also act on other receptor subtypes (i.e., VPAC1, PAC1). Both VPAC2 and PAC1 receptors are localized in the thalamus (Masuo et al. 1992; Hashimoto et al. 1996; Shioda et al. 1997). Because VIP has high affinity for both VPAC1 and VPAC2 receptors, but a much lower affinity (approximately 1,000-fold less) for the PAC1 receptor, it is possible that the VIP concentrations used in our current study (0.5 μM) could also activate PAC1 receptors. It is important to note, however, that this VIP-mediated action was only in a small minority of neurons in the VPAC2−/− slices. To probe for PAC1 receptor-mediated actions, we next used the agonist, PACAP38, which has a relatively higher affinity for PAC1 receptors.

In neurons from VPAC2−/− mice, PACAP38 (0.5 μM) depolarized 10 of 11 neurons tested (Fig. 5, A and C). The average response to PACAP38 (0.5 μM) was 2.6 ± 0.9 mV (n = 10). Of the 10 cells tested, 5 were in control physiological solution, and the remaining 5 in TTX (1.0 μM). The amplitudes of the PACAP38-mediated responses did not significantly differ between these two conditions (P > 0.1; Mann-Whitney U test), and thus these data have been combined. The latency to peak of the PACAP38-mediated depolarization occurred 176 ± 48 s (n = 10) after application, and the membrane potential partially recovered to predrug levels. Similar to VIP, the PACAP38-mediated membrane depolarization was associated with a decrease in apparent input resistance (17 ± 16%, n = 5).

Because PACAP38 has a similar affinity for VPAC2 and PAC1 receptors, PACAP38 application may equally activate both of these two receptor subtypes (Gottschall et al. 1990; Lam and Srivastava 1990). In slices from VPAC2−/− mice, PACAP38 (0.5 μM) produced a depolarization in 9 of 11 neurons, which averaged 3.4 ± 0.9 mV (n = 9; Fig. 5, B and C). Of the 9 cells tested, 4 were in control ACSF, and the remaining 5 in 1.0 μM TTX. The amplitude of the PACAP38-mediated depolarization did not differ between these two conditions, and thus these data have been combined (P > 0.1, t-test). The latency to peak of the PACAP38-mediated depolarization occurred 169 ± 42 s (n = 9) after application and the membrane potential partially recovered to predrug levels. The PACAP38-mediated membrane depolarization was associated with a reduction in apparent input resistance (32 ± 18%, n = 5; P < 0.05, paired t-test).

Because relay neurons from VPAC2−/− mice may have more receptors (i.e., VPAC2, PAC1), it is interesting that the PACAP38-mediated depolarization in relay neurons from VPAC2−/− mice did not significantly differ from that in relay neurons from VPAC2−/− mice (P > 0.1; Mann-Whitney U test). Thus, we next tested whether VIP and PACAP38 share identical pathways involved in membrane depolarization. We attempted whether VIP could occlude the membrane depolarization produced by PACAP38. As illustrated in Fig. 5D, VIP (0.5 μM) depolarized all relay neurons tested from VPAC2−/− slices (n = 6, 4.2 ± 1.1 mV). At the peak of VIP effect, the membrane potential was manually adjusted to resting levels and PACAP38 (0.5 μM) was applied. Under these conditions, PACAP38 produced no obvious depolarization in all cells.
PACAP38 enhances \( I_h \) in relay neurons from VPAC2\(^{-/-} \) slices

Our initial focus in this study was to identify the specific receptor subtype underlying the VIP-mediated depolarization in thalamic relay cells; however, in light of the small population of neurons in which VIP still depolarizes neurons in the VPAC2\(^{-/-} \) animal, this animal model also provides a unique tool to study the role of PAC1 receptors in the thalamus. Voltage-clamp recordings were used to characterize the conductance changes produced by PACAP\(_{38} \) in relay neurons from VPAC2\(^{-/-} \) mice. Analogous to our current clamp recordings above, PACAP\(_{38} \) (0.05–0.5 \( \mu \)M) produced an inward current that partially recovered to predrug holding current levels (Fig. 6A). In TTX (1.0 \( \mu \)M), PACAP\(_{38} \) (0.05–0.5 \( \mu \)M) produced an inward current that averaged 18 ± 15 pA (n = 8), and the time to peak of the inward current averaged 198 ± 56 s, which is similar to our current clamp recordings. Slow voltage command ramps (−60 mV to −110 mV, 2 s duration, 0.1 Hz) were used to determine the voltage dependence of the PACAP\(_{38} \)-mediated alteration in conductance (Fig. 6, A and B). In the predrug condition, the current response to the ramped voltage command is nonlinear; during the ramped voltage command there is an increase in conductance at more hyperpolarized potentials, which is likely a result of \( I_h \) activation. To quantify changes in the “resting” conductance of the neuron, we analyzed the initial portion of the current response near resting membrane potential (−60 to −80 mV). The resting conductance of the neurons prior to PACAP\(_{38} \) averaged 4.7 ± 0.8 \( n \)S. Following PACAP\(_{38} \) application, the conductance was significantly increased to 5.6 ± 2.6 \( n \)S (n = 9; P < 0.02, Wilcoxon test). Of the nine cells tested, 0.1 \( \mu \)M PACAP\(_{38} \) was applied to four cells, 0.5 \( \mu \)M to four cells, and 0.05 \( \mu \)M to the remaining cell, and these data have been combined for this analysis.

To determine the voltage dependence of the conductance altered by PACAP\(_{38} \), we calculated the difference in current responses before and after PACAP\(_{38} \) application (Fig. 6C). The PACAP\(_{38} \)-mediated conductance (\( I_{\text{diff}} \)) was usually linear over the voltage range of −60 mV to −90 mV. Extrapolating the linear fit of \( I_{\text{diff}} \) indicated that the conductance sensitive to PACAP\(_{38} \) had a reversal potential of −47 ± 4 mV (n = 6, Fig. 6C). These results are consistent with our previous study testing VIP on rat thalamic relay neurons (Lee and Cox 2003). In light of our previous work demonstrating that the VIP-mediated response was completely attenuated by the \( I_h \) blocker, ZD7288, we next tested whether the PACAP\(_{38} \)-mediated response was also sensitive to this agent. In ZD7288 (100 \( \mu \)M), PACAP\(_{38} \) (0.5 \( \mu \)M) did not produce inward current, nor
Role of cAMP in VIP-mediated actions

Previous studies have demonstrated that increases in cAMP activity can enhance Ih in thalamic relay neurons (Budde et al. 1997; Lüthi and McCormick 1999; Sun et al. 2003). In light of the absence of the VIP-mediated enhancement of Ih in neurons from VPAC2−/− mice, we tested whether this pathway may have been disrupted by the genetic manipulation. Voltage steps commands were used to activate Ih in neurons of slices from VPAC2+/+ and VPAC2−/− animals (Fig. 7). Similar to VIP, the membrane permeable, cAMP analogue, 8-cpt-cAMP (1 mM; 60 s application) produced a statistically significant depolarizing shift in the Ih activation function (4.8 ± 0.4 mV; n = 5) in neurons from VPAC2+/+ mice (Fig. 8A, P < 0.01; paired t-test). In relay neurons from VPAC2−/− mice, 8-cpt-cAMP (1 mM) also produced a significant depolarizing shift in the Ih activation function (4.2 ± 1.1 mV; n = 5; P < 0.02; paired t-test, Fig. 7B). These results indicate that the deletion of VPAC2 receptor gene did not disrupt the modulation of Ih by cAMP, which thereby suggests that the lack of VIP mediated responses in the VPAC2−/− mice is not due to a disruption of the second messenger pathway.

Because VIP receptors are linked to G-proteins that activate adenylate cyclase (AC), we next tested whether exogenous application of membrane-permeable cAMP analogs would reproduce and/or occlude VIP-mediated membrane depolarizations. In TTX (1 μM), 8-cpt-cAMP (10−1, 000 μM) produced a membrane depolarization in 20 of 25 relay neurons from cells tested from VPAC2+/+ mice (Fig. 8, A and B). 8-cpt-cAMP (100 μM) produced a reversible depolarization that averaged 1.1 ± 0.6 mV (n = 9). The latency to peak of the 8-cpt-cAMP-mediated depolarization occurred 124 ± 28 s after application with an average duration of 324 ± 79 s for six neurons that completely recovered to predrug levels. The 8-cpt-cAMP depolarization was associated with a decrease in apparent input resistance (Fig. 8, A and B). We next determined whether the 8-cpt-cAMP-mediated depolarization was concentration dependent. Low 8-cpt-cAMP concentration (10 μM) depolarized one of four relay neurons. Increasing the 8-cpt-cAMP concentration (500 and 1000 μM) produced larger membrane depolarizations (2.2 ± 1.4 mV; n = 12; Fig. 8, B and C). The average duration of the depolarization was 527 ± 203 s for six neurons that completely recovered to the pre-8-cpt-cAMP membrane potential. At these higher concentrations, 8-cpt-cAMP reduced the input resistance by 18 ± 12% (n = 5; P > 0.03). Our population data indicate a dose dependent increase in membrane depolarization by 8-cpt-cAMP (Fig. 8C). In eight neurons, repeated applications of 8-cpt-cAMP, low-concentration (10 or 100 μM) followed 12 min later by a higher 8-cpt-cAMP concentration (100 or 1000 μM) resulted in a larger depolarization by the higher concentration in all cells (Fig. 8D).

We next attempted to test whether activation of the cAMP pathway by 8-cpt-cAMP could occlude the membrane depolarization produced by VIP. As illustrated in Fig. 8E, VIP (0.1 μM) depolarized a relay neuron from a VPAC2+/+ slice. Following VIP washout, 8-cpt-cAMP (500 μM) was bath-applied and subsequently depolarized the neuron. The membrane potential was then manually adjusted to resting levels and VIP was reapplied in the presence of 8-cpt-cAMP. In this case, VIP produced no obvious depolarization (Fig. 8E). Following washout of 8-cpt-cAMP (20 min), VIP once again depolarized the neuron. For this experiment, higher concentration of 8-cpt-cAMP (1000 μM) was used in four neurons in which 0.5 μM VIP was applied, and a lower concentration of
8-cpt-cAMP (500 μM) was used in the remaining four cells. In all cells tested, the response to VIP-mediated depolarization was significantly attenuated in the presence of 8-cpt-cAMP (Fig. 8F, n = 8; P < 0.01, Wilcoxon test).

DISCUSSION

Our study provides several novel findings regarding peptide actions on thalamic neurons and intrathalamic circuit activity. First, we provide evidence that both spindle-like (5–8 Hz) and slow-wave (2–4 Hz) rhythmic activity can be produced in the mouse in vitro slice preparation. VIP attenuates both types of intrathalamic rhythmic activities, and this action is mediated through the activation of VPAC2 receptors. The VIP-mediated depolarization of relay neurons requires the activation of VPAC2 receptors and engages cAMP-dependent mechanisms leading to the enhancement of Ih. Finally, we provide evidence that activation of PAC1 receptors produces excitatory responses in thalamic relay neurons via a similar mechanism as that with VPAC2 receptor activation. Key aspects of our findings are illustrated in Fig. 9. VIP primarily binds to VPAC2 receptors, which in turn activate AC and increase cytosolic cAMP concentrations, leading to an enhancement of Ih and ultimately depolarization of the relay neurons. In addition, PACAP38 depolarizes thalamic relay neurons in mice lacking the VPAC2 receptor, presumably via activation of PAC1 receptors. Activation of PAC1 receptors also enhances Ih in a similar manner as for VPAC2 receptors. Our current working hypothesis regarding VIP-mediated actions in the thalamus is that burst activity of VIP-containing TRN neurons, which is common during rhythmic activity, causes synaptic release of VIP. The VIP, in turn, activates VPAC2 receptors on relay neurons and enhances Ih via a cAMP-dependent process, thereby producing a membrane depolarization in relay neurons. This depolarization biases the thalamic relay neurons toward tonic firing mode, thereby attenuating the rhythmic activity. Thus, VIP may act as an endogenous modulator of intrathalamic circuit activity.

Antioscillatory actions of VIP

Intrathalamic rhythmic activities result from the intrinsic properties of thalamic neurons and the reciprocal synaptic connections between thalamic relay nuclei and the TRN (Steriade and Llinás 1988; von Krosigk et al. 1993; Steriade et al. 1993; Huguenard and Prince 1994; Warren and Jones 1994). Spindle-like and slow-wave rhythms have been observed in vivo in many animal species, but in vitro such rhythms have clearly been observed in ferrets, guinea pigs, and, to a lesser extent, in rats. Despite the presence of slow-wave rhythms in mice, spindle-like rhythms have not been obvious (Warren et al. 1994; Huntsman et al. 1999). Our study provides clear evidence that the mouse thalamus is clearly capable of both spindle-like rhythmic activities, and attenuating GABA_A receptor-mediated activity transformed the spindle-like rhythmic...
activity to a slow-wave rhythmic activity similar to that observed in other animal models (Huguenard and Prince 1994; Bal et al. 1995; McCormick et al. 1995). The presence of these intrathalamic rhythms in wild-type mice allows the power of transgenic approaches to be used for the studies of these activities, in a manner similar to our use with the VPAC2−/− mouse.

In our earlier study, we found that VIP attenuates slow-wave rhythmic activity via an unidentified receptor subtype in rat thalamic slices (Lee and Cox 2003). In the present study, we found that VIP attenuates both spindle-like and slow 2–4 Hz intrathalamic rhythms in slices from wild-type (VPAC2+/+) mice. In slices from VPAC2 receptor knock-out animals (VPAC2−/−), VIP did not alter the rhythmic activity. These data clearly indicate that the antioscillatory actions of VIP are mediated via activation of VPAC2 receptors.

The antioscillatory actions of VIP are likely a consequence of the depolarizing actions of VIP on thalamic relay neurons. Within mouse thalamic relay neurons, we found that VIP produced a long-lasting robust membrane depolarization. Despite numerous reports of VIP-mediated membrane depolarizations in different regions of the CNS, the identification of the specific receptors underlying these actions has remained elusive because of poorly selective pharmacological agents for VIP/PACAP receptors (Phillis et al. 1978; Jeflinja et al. 1982; Kohlmeier and Reiner 1999; Liu and Morris 1999; Lee and Cox 2003; Sun et al. 2003). The development of this VPAC2−/− mouse may serve as a valuable tool to investigate the functional role of VPAC2 receptors in various brain regions (Harmar et al. 2002; Aton et al. 2005). Immunocytochemical studies indicate that VPAC2 and PAC1 receptors are present within thalamic nuclei, though distinguishing the functional consequences of these specific receptor subtypes has been difficult with current pharmacological agents (Usdin et al. 1994; Sheward et al. 1995; Vertongen et al. 1997; Burgunder et al. 1999).

The integral role of VPAC2 receptors

The VPAC2−/− mice have served as a very important tool in further understanding of VIP-mediated actions in the thalamus. In slices from VPAC2+/+ mice, we have found that in relay neurons VIP produces a membrane depolarization that is associated with a decreased input resistance. Voltage clamp recordings clearly indicate that VIP enhances Ih, which is similar to what has been observed in rat thalamic neurons (Lee and Cox 2003; Sun et al. 2003). In slices from VPAC2−/− mice, however, the VIP-mediated depolarization in response to a relatively high VIP concentration (0.5 μM) is absent in 80% of the neurons and is severely reduced in amplitude in the remaining cells. In addition, VIP does not alter Ih in relay neurons in slices from VPAC2−/− mice. Thus, our data strongly support the notion that the excitatory actions of VIP are predominantly via VPAC2 receptors. Our results differ from the conclusions of Sun et al. (2003), who concluded that the predominant action of VIP/PACAP was due to PAC1 receptors based on (1) the concentration dependence of their agonists responses (PACAP more potent than VIP) and (2) the VPAC1 receptor agonist, (Ala) VIP, did not depolarize thalamic relay neurons, which suggests the lack of VPAC1 receptor-mediated actions on thalamic relay neurons (Nicole et al. 2000; Sun et al. 2003). Our results strongly indicate the role of the VPAC2 receptor, unless there is a complete remodeling of the VIP/PACAP receptor distribution as a result of the VPAC2 knock-out, but such a remodeling has not been observed in other brain regions of these VPAC2 mice and thus would be specific only to the thalamic nuclei (Aton et al. 2005).

As mentioned above, although the majority of relay neurons from VPAC2−/− mice did not respond to VIP, in the remaining 20% of neurons, VIP produced a smaller depolarization. Because VIP has a lower affinity for PAC1 receptors relative to VPAC2 receptors, the VIP concentration (0.5 μM) used in this study could possibly activate PAC1 receptors. Our finding...
supports such a conclusion because the agonist, PACAP38, which has a higher affinity for PAC1 receptors than VIP, depolarized the majority of neurons from VPAC2−/− mice. In light of the anatomical data indicating the presence of VPAC2 and PAC1 receptors in thalamic relay nuclei, the VPAC2−/− mice serve as a potentially useful model to understand PAC1-mediated actions within thalamic neurons. We found that PACAP38 not only depolarized thalamic neurons from VPAC2−/− mice, but our voltage clamp recordings also reveal that PACAP38 also increases the conductance of relay neurons and appears to also enhance Ih, similar to that observed by VIP via VPAC2 receptors. Thus, our data clearly indicate that activation of PAC1 receptors can enhance Ih, but, perhaps more important, also suggest that these two different receptors may converge onto a common effector mechanism to alter the excitability of thalamic neurons. Consistent with this idea, our data strongly suggest that VIP occludes PACAP38-mediated membrane depolarization in thalamic neurons from VPAC2−/− mice.

The dependence of VIP-mediated response on cAMP

VIP-sensitive receptors are G protein–dependent receptors that have been found to engage cAMP synthesis as well as inositol phosphate turnover pathways (Ishihara et al. 1992; Deutsch and Sun 1992; Spengler et al. 1993; Van Rampelbergh et al. 1997; MacKenzie et al. 2001). In addition, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels that underlie Ih, are modulated by alteration in cAMP concentrations (Beaumont and Zucker 2000; Wainer et al. 2001). Several neuromodulators within the thalamus (e.g., norepinephrine, serotonin, VIP, PACAP), which activate receptors coupled to cAMP-dependent pathways, enhance Ih and thereby depolarize thalamic neurons (McCormick and Pape 1990; Pape 1996; Sun et al. 2003; Frere and Luthi 2004; present study). Our data demonstrate that cAMP analogues enhance Ih, and produce membrane depolarizations similar to VIP. Furthermore, we have found that activation of cAMP can occlude the VIP-mediated membrane depolarization, which indicates the dependence of the depolarizing action on cAMP dependent

**FIG. 8.** cAMP analogue depolarizes relay neurons in VPAC2−/− slices. A: in TTX, 8-cpt-cAMP (0.01, 0.1 mM) produces small reversible membrane depolarizations in relay neurons from VPAC2−/− slices. B: in a different neuron, 8-cpt-cAMP (0.1, 1 mM) produces small depolarizations. C: summary of 8-cpt-cAMP-mediated membrane depolarizations in VB neurons. Cell counts for each concentration are listed in parentheses. D: scatter plot indicating the effect of multiple 8-cpt-cAMP doses to individual neurons. In eight neurons, low 8-cpt-cAMP concentration (0.01, 0.1 mM) application was followed 10 min later by a higher 8-cpt-cAMP concentration (0.1, 1 mM) application. Note the dose-dependent increases in depolarizations produced by 8-cpt-cAMP. E: cAMP analogue occludes VIP-mediated membrane depolarization. VIP produces a reversible depolarization in a relay neuron from VPAC2−/− slice. Following washout of VIP, 8-cpt-cAMP (0.5 mM) is applied and depolarizes the neuron. The membrane potential is manually adjusted to resting levels and VIP is applied again. This time VIP produces no obvious depolarization. Following washout of 8-cpt-cAMP, VIP once again depolarizes the neuron. F: summary of VIP-mediated membrane depolarization in thalamic neurons. Cell counts for each condition are listed in parenthesis.
Processes. Our findings are consistent with previous reports that VPAC2 receptors are primarily coupled to cAMP-dependent pathways (Lutz et al. 1993; Lutz et al. 1999; Itri and Colwell 2003).

**Functional role of VIP/PACAP in the thalamus**

Thalamic neurons discharge action potentials in two basic firing modes: tonic and burst. The firing mode of thalamic neurons is dependent on the low threshold, transient calcium current, IT (Jahnsen and Llinás 1984a; Steriade and Llinás 1988; Cranelli et al. 1989). At relatively hyperpolarized membrane potentials, channels underlying IT are deactivated, and subsequent depolarization can occur (Jahnsen and Llinás 1984a; Steriade and Llinás 1988; Cranelli et al. 1989). At relatively depolarized membrane potentials IT is inactivated, and subsequent depolarization gives rise to a transient calcium-dependent depolarization, a low-threshold calcium spike, on which a short-duration (100–200 ms), high-frequency burst (>200 Hz) of sodium-dependent action potentials can occur (Jahnsen and Llinás 1984a; Steriade and Llinás 1988; Cranelli et al. 1989). At relatively depolarized membrane potentials IT is inactivated, and subsequent depolarization of the membrane potential will result in tonic action potential discharge at a frequency that is relatively linear with the degree of membrane depolarization (Jahnsen and Llinás 1984a; Jahnsen and Llinás 1984b; Huguenard and McCormick 1992; Zhan et al. 1999).

Several neuromodulators depolarize thalamic neurons, predisposing the neurons to a tonic mode and thereby terminating the intrathalamic rhythmic activity. It is interesting that many of these modulators alter the excitability of thalamic neurons via common effector mechanisms. Two obvious candidates are the resting K current, K\(_{\text{leak}}\), and I\(_{\text{h}}\). Within thalamic neurons, acetylcholine, glutamate (via metabotropic receptors), norepinephrine, serotonin, cholecystokinin, as well as histamine depolarize thalamic neurons by decreasing a resting leak potassium current and thereby alter the action potential discharge mode of these neurons and ultimately terminating the rhythmic activity (McCormick and Prince 1986; McCormick and Prince 1987; McCormick and Prince 1988; McCormick and Wang 1991; McCormick and Williamson 1991; McCormick and von Krosigk 1992; McCormick 1992; Cox et al. 1995; Cox et al. 1997; Cox and Sherman 1999). Furthermore, norepinephrine, serotonin, and histamine have been found to enhance I\(_{\text{h}}\), thus leading to membrane depolarization and ultimately terminating the intrathalamic oscillation (Pape and McCormick 1989; McCormick and Pape 1990; McCormick and Williamson 1991). Our data strongly indicate VIP and PACAP38 depolarize relay neurons via an enhancement of I\(_{\text{h}}\) (Lee and Cox 2003; Sun et al. 2003). A common aspect of these modulators is their ability to engage particular intracellular second-messenger pathways namely, cAMP which ultimately alters I\(_{\text{h}}\).

Despite sharing a common effector mechanism, a key to understanding the functional aspects of VIP and PACAP likely depends on the source of these peptides. VIP-containing neurons are in the TRN and therefore VIP is endogenous to the intrathalamic circuit, and its release is likely correlated to intrathalamic circuit activity (Burgunder et al. 1999). Other neuropeptides (e.g., cholecystokinin, somatostatin, neuropeptide Y, and VIP) can also alter the firing mode of thalamic neurons and attenuate intrathalamic rhythmic activity (Cox et al. 1997; Leresche et al. 2000; Sun et al. 2002; Lee and Cox 2003; Sun et al. 2003). Similar to VIP, these other neuropeptides are localized within the thalamic neurons and may play a potentially important role in regulating thalamic neuron excitability, but further studies are needed. In contrast to the above-mentioned neuropeptides, PACAP-containing neurons that innervate the thalamus originate from brain stem regions in a somewhat similar manner as other known modulators such as acetylcholine, norepinephrine, serotonin, and histamine (Hashimoto et al. 1996; Shioda et al. 1997; Hannibal 2002). The activity of neurons in these areas is closely associated with levels of arousal and sleep-wake states (Steriade et al. 1993; McCormick and Bal 1997). Thus, the functional distinction between peptides such as VIP and PACAP, which appear to share common cellular mechanisms, likely involves their sites of origin. In the case of these two peptides, VIP appears to be endogenous to the thalamic circuitry, whereas PACAP would presumably be released by increase activity of brain stem neurons.

Our working hypothesis is that high-frequency burst activity of TRN required for intrathalamic rhythmic activity may be optimal for peptide release and therefore produce the release of VIP. This synaptically released VIP activates VPAC2 receptors on relay neurons, increases cAMP activity, enhancing I\(_{\text{h}}\), and thereby depolarizes the relay neurons. This depolarization and decrease of membrane resistance biases the cells to tonic-discharge mode and may contribute to the termination of rhythmic activity. We speculate that VIP acts as an intrinsic modulator of intrathalamic oscillation that could regulate the duration of rhythmic activity. We predict that overall decreases in VIP release could lead to prolonged durations of intrathalamic rhythmic activities similar to that during absence seizure activity. The role of neuropeptides in the thalamus may not
only influence rhythmic activities, but considering their long-lasting actions, these compounds may also play an important role in regulating the overall information transfer through thalamocortical circuits.

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