PACAP Potentiates L-Type Calcium Channel Conductance in Suprachiasmatic Nucleus Neurons by Activating the MAPK Pathway

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Dziema, Heather and Karl Obrietan. PACAP potentiates L-type calcium channel conductance in suprachiasmatic nucleus neurons by activating the MAPK pathway. J Neurophysiol 88: 1374–1386, 2002; 10.1152/jn.00896.2001. The endogenous pacemaker activity of the suprachiasmatic nuclei (SCN; the master clock in mammals) is regulated by photic information relayed from the retina to the SCN via the retinohypothalamic tract (RHT). Recent work has revealed that glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are stored in RHT nerve terminals and function in a coordinated manner to regulate clock timing. To address this interaction on a cellular level, Fura-2 Ca$^{2+}$ digital imaging was employed and the effects of PACAP on glutamate evoked Ca$^{2+}$ transients in SCN neurons were examined. Pretreatment of SCN neurons with PACAP markedly potentiated Ca$^{2+}$ transients elicited by both exogenous glutamate application and synthetically released glutamate. Many neurons became responsive to glutamate only after PACAP administration, suggesting that PACAP sets the lower concentration threshold required for glutamate to initiate a robust rise in postsynaptic cytosolic Ca$^{2+}$. Facilitation of glutamate-induced Ca$^{2+}$ transients was inhibited by nimodipine, indicating that PACAP potentiates L-type Ca$^{2+}$ channel activity. The modulatory actions of PACAP were inhibited by antagonizing signaling via the p42/44 mitogen-activated protein kinase (MAPK) signal transduction cascade. Immunocytochemistry and Western analysis confirmed that PACAP stimulates MAPK activity at doses and time points shown to potentiate Ca$^{2+}$ influx. Down-regulation of protein kinase C (PKC) with the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA) or PKC inhibition with bisindolylmaleimide attenuated the actions of PACAP, indicating that PKC also couples PACAP to potentiation of depolarization-induced Ca$^{2+}$ transients. The data presented here identify potentially important mechanisms by which PACAP regulates SCN physiology.

INTRODUCTION

As the master circadian clock in mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus drive a complex array of biochemical, physiological, and behavioral rhythms (reviewed by Miller et al. 1996; Moore 1997). The endogenous pacemaker activity of the SCN is regulated by a variety of stimuli, light being the most potent. Photic information is relayed from the eyes to the SCN via the retinohypothalamic tract (RHT). A number of studies have shown that light-induced secretion of glutamate from RHT nerve terminals is an essential event that links photic input to entrainment of the circadian clock. For example, infusion of ionotropic glutamate receptor antagonists into the region of the SCN blocks light-induced phase-shifts (Colwell and Menaker 1992; Colwell et al. 1991).

In addition to glutamate, the RHT also secretes modulatory peptides. One of these peptides, pituitary adenylate cyclase activating polypeptide (PACAP), has been shown to be a potent regulator of the circadian clock (reviewed by Hannibal et al. 1998). PACAP is a member of the glucagon/vasoactive intestinal peptide/secretin/growth hormone-releasing hormone family of structurally related peptides. Two forms of PACAP are found within the brain, a 38 amino acid residue form and a 27 residue peptide formed from internal cleavage of PACAP 38 (Miyata et al. 1989, 1990). PACAP 38 is the predominant form, comprising >90% of the PACAP isoform content in the brain (Arimura et al. 1991; Ghatié et al. 1993; Hannibal et al. 1995). Through two classes of G protein-coupled receptors, PACAP couples to the activation of signaling pathways that result in cAMP synthesis, and activation of phospholipase C, thus leading to inositol 1,4,5-tris-phosphate (IP3) production, protein kinase C (PKC) activation, and release of Ca$^{2+}$ from intracellular stores (Tanaka et al. 1996, 1997). Interestingly, PACAP has also been shown to stimulate activation of the p42/44 mitogen-activated protein kinase (MAPK) cascade in PC12 cells (Barrie et al. 1997), a neuron-like model cell line.

Recently, several studies have shown that PACAP alters the ability of glutamate to phase shift SCN firing patterns in slice (Chen et al. 1999; Harrington et al. 1999). It is of interest that immunohistochemical examination has colocalized expression of glutamate with PACAP in retinal ganglion nerve terminals that innervate the SCN (Hannibal et al. 2000), thus raising the possibility that PACAP and glutamate are co-released and function in a coordinated manner to regulate SCN timing.

To gain insight into the functional role of PACAP in the SCN, we examined the interactions between glutamate and PACAP at the level of Ca$^{2+}$ metabolism. Modulation of Ca$^{2+}$ was examined because of the proposed role glutamate-induced increases in cytosolic Ca$^{2+}$ play in initiating the series of transcriptional events that give rise to light-dependent entrainment of the circadian clock. Results obtained in this study reveal that PACAP significantly augments the ability of glutamate to elicit increases in cytosolic Ca$^{2+}$ levels via a mecha-

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nism requiring activation of the MAPK cascade. Portions of this work have been published in abstract form (Obrietan 2000).

METHODS

Tissue culture

SCN neurons were cultured from embryonic day 18 to postnatal day 1 Sprague-Dawley rat pups. Initially, pups were decapitated, and their brains were rapidly removed and placed in dissociation media [DM; (in mM) 90 NaSO\textsubscript{4}, 30 KSO\textsubscript{4}, 16 MgCl\textsubscript{2}, 0.25 CaCl\textsubscript{2}, 32 HEPES, and 0.01% phenol red (Sigma), pH 7.7]. Two techniques were used for isolation of the SCN. In our first technique, brains were cut into 500-μm coronal sections with a tissue chopper, the SCN-containing slice was identified using anatomical markers, and the SCN was excised from the slice with the use of micro-dissection scissors and a dissecting microscope. The tissue excised using this technique was used to analyze PACAP modulation of exogenously evoked Ca\textsuperscript{2+} transients. For the second technique, an ~1-mm cube of tissue containing the SCN and the surrounding hypothalamic tissue was excised from the ventral surface of the brain. The medial optic chiasm was used as a reference point for the excision. Tissue (SCN and non-SCN hypothalamic tissue) isolated using this co-culturing technique was used to analyze the modulatory effects of PACAP on Ca\textsuperscript{2+} transients initiated by synaptic glutamate release, and for Western and immunocytochemical analysis. Tissue was then washed three times in DM, finely minced with a razor blade, and digested with a mild protease solution (100 units/ml papain latex, Worthington; 4.5 mg cysteine, finely minced with a razor blade, and digested with a mild protease

Ca\textsuperscript{2+} imaging: analysis

Calibrated cytosolic Ca\textsuperscript{2+} data from single neurons were analyzed with Igor Pro software (WaveMetrics) as described previously (Obrietan and van den Pol 1995; Obrietan et al. 1995). For assays that measure the effects of PACAP on Ca\textsuperscript{2+} transients initiated by synaptic glutamate release, the mean Ca\textsuperscript{2+} level in the presence of glutamate receptor antagonists was subtracted from the mean Ca\textsuperscript{2+} rise initiated by antagonist withdrawal. Data were analyzed over a 30-s period, 120 s after withdrawal of glutamate receptor antagonists. Data are presented as mean Ca\textsuperscript{2+} rise ± SE. To assess the effects of PACAP on exogenously-induced Ca\textsuperscript{2+} transients, the mean baseline (unstimulated) Ca\textsuperscript{2+} level over a 20-s period prior to stimulation was subtracted from the maximal evoked Ca\textsuperscript{2+}. Data are presented as the peak Ca\textsuperscript{2+} rise ± SE.

Immunocytochemistry

Cells were stimulated for 5 min and rapidly fixed in ice-cold 90% methanol and 10% acetic acid for 5 min. Cells were then washed three times in phosphate-buffered saline (PBS), and then blocked with 1% normal goat serum and 10% bovine serum albumin (BSA) in PBS with 0.1% Triton X-100 (PBST) and 0.02% azide for 2 h at room temperature. Next, the tissue was incubated overnight at 4°C with an affinity-purified rabbit polyclonal antibody against the activated forms of erk-1 and erk-2 (1:5,000 dilution; Sigma). Membranes were treated with a normal goat serum and 10% bovine serum albumin (BSA) in PBS with 0.25% Triton X-100 (PBST) and 0.02% azide for 2 h at room temperature. After washing, the tissue was mounted using gel mount (Biomedia Inc.) and visualized using an MRC-600 scanning laser confocal microscope.

Western blotting

Cultured cells were stimulated for 5 min and then lysed in 100 μl of hot 6× sample buffer. Lysates were heated to 90°C for 10 min, vortexed for 15 s, and followed by centrifugation for 7 min at 15,000 g. Extract (30 μl/lane) was loaded onto a 10% SDS-PAGE gel and electrophoresed using standard procedures. Protein was transblotted onto polyvinylidene fluoride (PVDF) (Immobilon P; Millipore). Following blocking with 10% (wt/vol) powdered milk dissolved in PBST, membranes were incubated (4°C overnight) in PBST with a mouse monoclonal antibody against the activated forms of erk-1 and erk-2 (1:5,000 final dilution; Sigma). Membranes were treated with a rabbit anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:2,000; NEN) and immunoreactivity was developed using the Western-CDP star alkaline phosphatase chemiluminescent detection system (NEN). Membranes were then probed overnight with a goat polyclonal antibody against total erk-1 and erk-2 (1 μg/μl final
Calbiochem. Rp-cAMPs was acquired from LC laboratories.

B1–B4 IBMX. After 5 min, media was aspirated, and the reaction was terminated by 5 mM; Sigma). Control cells were mock stimulated and treated with media containing PACAP and 3-isobutyl-1-methylxanthine (IBMX, 1 mM; H9262) and 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 2-amino-5-phosphonopentanoic acid (AP5) were acquired from Sigma-RBI. Bisindyl-maleimide, Gö 6976, U0126, and PD 98059 were acquired from Calbiochem. Rp-cAMPs was acquired from LC laboratories.

cAMP assay
cAMP levels were measured by determining the ratio of cAMP to a total ATP, ADP, and AMP pool as described by Wong et al. (1991). Briefly, cells were incubated in standard tissue culture media containing 4 µCi/ml [3H]adenine (ICN) for 8 h. Cells were stimulated with media containing PACAP and 3-isobutyl-1-methylxanthine (IBMX, 1 mM; Sigma). Control cells were mock stimulated and treated with IBMX. After 5 min, media was aspirated, and the reaction was terminated by administration of 5% trichloroacetic acid supplemented with 1 µM cAMP. An analogous procedure was used to examine forskolin (Sigma)-mediated increases in cAMP accumulation. Dowex AG 50WX4 and neutral alumina chromatography were used to isolate soluble nucleotides. A scintillation counter was used to measure the ratio of [3H]ATP, [3H]ADP, and [3H]AMP to [3H]cAMP. Experiments were performed in triplicate.

Materials
PACAP1–38 (human, ovine, rat) was acquired from Bachem. NMDA, glutamate, nimodipine, bicuculline, TTX, PMA, forskolin, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 2-amino-5-phosphonopentanoic acid (AP5) were acquired from Sigma-RBI. Bisindyl-maleimide, Gö 6976, U0126, and PD 98059 were acquired from Calbiochem. Rp-cAMPs was acquired from LC laboratories.

RESULTS

Given that PACAP is a known neuropeptide, we were interested in examining the physiological ramifications of coordinated activation of peptidergic and glutamatergic signaling. To assess potential interactions, we examined the ability of PACAP to modulate Ca2+ transients initiated by exogenous glutamate application.

In an initial set of experiments, the capacity of PACAP to elicit a rise in cytosolic Ca2+ levels in SCN neurons was examined. Perfusion of 200 nM PACAP triggered Ca2+ transients in approximately 45% of all neurons examined (n = 157; Fig. 1A). Often, the Ca2+ rise initiated by PACAP consisted of rapid and sharp transients that persisted for several minutes after withdrawal of PACAP from the perfusion solution. These data are suggestive of Ca2+ release from internal stores, as has been previously reported by Kopp et al. (1999) for SCN neurons. Lower doses of PACAP (0.1–1.0 nM) triggered Ca2+ transients in <1% of neurons (n = 157). Next, we assessed the modulatory effects of PACAP on glutamate-evoked increases in cytosolic Ca2+. Toward this end, SCN neurons were repeatedly challenged with glutamate (3 µM) both before and after PACAP (200 nM) administration. Before PACAP administration, glutamate triggered relatively low-amplitude Ca2+ transients (Fig. 1, B1–B4 and C). However, after 2 min of PACAP administration, there was a significant (P < 0.0001, 2-tailed Student’s t-test) increase in the amplitude of glutamate-mediated Ca2+ transients. Before PACAP administration, the mean Ca2+ rise elicited by glutamate was 40 ± 6 nM; after PACAP administration the mean Ca2+ rise was 104 ± 11 nM (n = 34). Interestingly, some neurons were responsive to glutamate only after PACAP administration (Fig. 1, B2 and B3).

Potentiation of glutamate-evoked Ca2+ transients was independent of whether PACAP elicited a Ca2+ response. Along these lines, potentiation was observed both in neurons that exhibited a Ca2+ response to PACAP (Fig. 1, B3 and B4) and in neurons where no change in basal Ca2+ was observed (Fig. 1, B1 and B2). NMDA was added at the end of many experiments to verify that the recorded cells were neurons. In these experiments and other experiments where exogenous agents were applied to evoke Ca2+ responses, tetrodotoxin (TTX; 500 nM) was added to all perfusion buffers to inhibit the potentially complicating effects of action potential–mediated transmitter release.

Glutamate-induced Ca2+ transients are mediated in part by membrane depolarization-dependent opening of voltage-activated Ca2+ channels (VACC). To assess whether PACAP-induced potentiation of glutamate responses is due to modulation of VACC activity, SCN neurons were repeatedly challenged with bath applications of K+ (15 mM). K+-induced membrane depolarization triggered rapid and reproducible Ca2+ transients (Fig. 2A). As was observed with glutamate, PACAP (200 nM) administration significantly (P < 0.0001, 2-tailed Student’s t-test) potentiated K+-evoked Ca2+ transients (Fig. 2, A and D). The potentiating actions of PACAP were dose-dependent; at 200 nM, PACAP elicited a ≥20% potentiation of K+-evoked Ca2+ transients in 55% of neurons, whereas at 2 nM, 7% of neurons exhibited reproducible potentiation of ≥20% (Fig. 2C). Next, we assessed whether potentiation of VACC activity is the mechanism by which PACAP facilitates glutamate-induced Ca2+ transients. Toward this end, cells were treated with the L-type VACC inhibitor nimodipine (5 µM), and the capacity of PACAP to potentiate glutamate-evoked Ca2+ transients was assessed. Previous work on SCN neurons showed that the majority of VACC-induced Ca2+ influx in cell bodies is mediated by L-type channels (Obrietan and van den Pol 1997). Under this condition, K+ (15 mM) evoked a relatively modest 23 ± 3 nM Ca2+ rise (Fig. 2, B and D). In the absence of functional L-type VACCs, PACAP did not significantly (P > 0.05, 2-tailed Student’s t-test) potentiate glutamate (3 µM)-evoked Ca2+ transients (Fig. 2, B and D). Likewise, pretreatment of SCN cells with nimodipine blocked PACAP-mediated potentiation of depolarization-evoked Ca2+ transients (Fig. 3). Collectively, these results reveal that PACAP-dependent potentiation of L-type VACC is an underlying mechanism that mediates facilitation of glutamate-evoked Ca2+ transients.

Potentiation of Ca2+ transients mediated by spontaneous glutamate release

Next, the modulatory actions of PACAP on Ca2+ transients mediated by synaptically released glutamate were assessed. To perform these experiments, SCN neurons were co-cultured with neurons from the surrounding hypothalamic areas, thereby providing glutamatergic synaptic inputs. As previously described (Obrietan and van den Pol 1995), complex Ca2+ transients are initiated by the removal of the ionotropic glutamate receptor antagonists AP5 (100 µM) and DNQX (10 µM)
from the perfusion solution (Fig. 4). The ability of ionotropic glutamate receptor antagonists to suppress spontaneous Ca\(^{2+}\) transients indicates that glutamate is secreted from the cultured cells. Similar results are observed with the addition and removal of TTX from the perfusion solution, indicating that action potentials stimulate glutamate release from synaptically-coupled neurons (Obrietan and van den Pol 1995). Under conditions of spontaneous glutamate-mediated Ca\(^{2+}\) transients, the administration of PACAP (200 nM) triggered a significant (P < 0.001, 2-tailed Student’s t-test) increase in Ca\(^{2+}\) levels (Fig. 4). Initially, the withdrawal of AP5 and DNQX triggered a 67 ± 5 nM Ca\(^{2+}\) rise; PACAP increased the Ca\(^{2+}\) rise to 95 ± 5 nM, n = 57. On reintroduction of glutamate receptor antagonists at the end of the experiment, cytosolic Ca\(^{2+}\) returned to basal levels, indicating that PACAP was eliciting a modulatory action on the secretion and/or response to synaptically released glutamate. Interestingly, some neurons only exhibited robust Ca\(^{2+}\) transients on PACAP administration (Fig. 4, bottom), indicating that PACAP receptor activation is required for robust glutamate-mediated Ca\(^{2+}\) transients.

To characterize further its modulatory actions, PACAP was applied in the presence of AP5 and DNQX and the residual effect on Ca\(^{2+}\) transients mediated by synaptic glutamate release was assessed. Under this condition, a 90-s application of PACAP (200 nM) potentiated Ca\(^{2+}\) transients triggered by subsequent withdrawals of AP5 and DNQX (Fig. 5A). In ~35% of the cells examined, 200 nM PACAP initiated at least a twofold increase in the mean Ca\(^{2+}\) rise (n = 90). The modulatory actions of PACAP persisted for an extended period (>8 min) after it was washed from the perfusion chamber. Consistent with its effects on K\(^+\) channels, PACAP (200 nM) potentiation of Ca\(^{2+}\) transients was independent of whether PACAP elicited a Ca\(^{2+}\) rise. Horizontal bars below Ca\(^{2+}\) traces in A and B denote time in minutes (min); vertical bars to the left of each trace denote the calibrated cytosolic Ca\(^{2+}\) values. To gain an appreciation for the heterogeneity of response characteristics, traces from several neurons are shown for each experiment. C: graphical representations of mean glutamate-evoked Ca\(^{2+}\) transients before and after PACAP administration. The filled bars represent the responses to the two consecutive glutamate-evoked Ca\(^{2+}\) transients immediately before PACAP administration; the two open bars represent the two evoked Ca\(^{2+}\) transients immediately after PACAP administration. Error bars denote SE. *: P < 0.0001, two-tailed Student’s t-test.

FIG. 1. PACAP potentiates glutamate-mediated Ca\(^{2+}\) transients in SCN neurons. A). Administration of 1 nM PACAP (bar = 120 s) did not alter basal Ca\(^{2+}\) levels in the two representative traces. However, administration of 200 nM PACAP (bar = 120 s) elicited a robust Ca\(^{2+}\) rise and complex Ca\(^{2+}\) transients in the upper trace. No effect of 200 nM PACAP was observed in the lower trace. Similar heterogeneous response characteristics have been reported for SCN neurons (Kopp et al. 1999). NMDA (30 μM; arrow) was added at the end of the experiment to verify that the cells were neurons. B: neurons were repeatedly exposed to glutamate (arrowheads: 3 μM, 10 s exposure duration). Before administration of PACAP (200 nM, 120 s exposure duration), glutamate-evoked Ca\(^{2+}\) transients were relatively weak or absent. However, after bath application of PACAP, peak Ca\(^{2+}\) transients stimulated by glutamate were markedly enhanced. To inhibit the complicating effects of action potential-mediated transmitter release, all bath solutions contained tetrodotoxin (TTX: 500 nM). Note that potentiation of Ca\(^{2+}\) transients was independent of whether PACAP elicited a Ca\(^{2+}\) rise. Horizontal bars below Ca\(^{2+}\) traces in A and B denote time in minutes (min); vertical bars to the left of each trace denote the calibrated cytosolic Ca\(^{2+}\) values. To gain an appreciation for the heterogeneity of response characteristics, traces from several neurons are shown for each experiment. C: graphical representations of mean glutamate-evoked Ca\(^{2+}\) transients before and after PACAP administration. The filled bars represent the responses to the two consecutive glutamate-evoked Ca\(^{2+}\) transients immediately before PACAP administration; the two open bars represent the two evoked Ca\(^{2+}\) transients immediately after PACAP administration. Error bars denote SE. *: P < 0.0001, two-tailed Student’s t-test.
tiated glutamate-mediated Ca\(^{2+}\) transients. This finding indicates that PACAP-mediated potentiation of glutamatergic neurotransmission does not result from modulation of GABA\(_A\) receptor activity. Interestingly, some neurons (Fig. 5B, bottom) were responsive to withdrawal of glutamate receptor antagonists only after PACAP application.

To verify that SCN neurons exhibit PACAP-evoked potentiation of glutamatergic neurotransmission, we isolated neurons from the SCN and the surrounding hypothalamic region separately and irreversibly labeled the cells from surrounding hypothalamic region with a fluorescent chloromethyl derivative tag (CellTracker). The tissue was then co-cultured and the fluorescent tag was used to distinguish the two populations of neurons. Relative to fura-2 that labels all cells (Fig. 5C2), CellTracker was found in a subset of the cells (i.e., the non-SCN cells: Fig. 5C1). Using this criterion, SCN cells were specifically monitored for PACAP-induced potentiation of synapticly evoked Ca\(^{2+}\) transients. Figure 5C3 shows the response characteristics of two SCN cells. In both cells, the administration of PACAP triggers a dramatic potentiation of Ca\(^{2+}\) influx mediated by the synaptic release of glutamate. These data confirm that the potentiating actions of PACAP are observed in SCN cells.

### Second messenger signaling events that couple PACAP to modulation of Ca\(^{2+}\) transients

To begin to understand how PACAP potentiates Ca\(^{2+}\) transients, we examined second messenger signaling pathways activated by PACAP. Because the modulatory actions of PACAP are independent of whether PACAP elicits a Ca\(^{2+}\) rise, it is unlikely that signaling pathways activated by increased cytosolic Ca\(^{2+}\) are involved [i.e., Ca\(^{2+}\)/calmodulin-dependent protein kinases: (CaMKs)]. Initially, the contribution of cAMP-dependent signaling was examined. Toward this end, SCN neurons were incubated with \(^{3}\)H-ATP for 6 h and stimulated with increasing concentrations of PACAP or the adenyl cyclase activator forskolin to generate dose-response curves. After 5 min, cells were lysed and cAMP accumulation was measured. PACAP (Fig. 6A) as well as forskolin (data not shown) significantly increased cAMP production. Figure 6B shows dose-response curves for the effects of PACAP and forskolin on cAMP accumulation. The EC\(_{50}\) for PACAP was 0.7 nM, and the EC\(_{50}\) for forskolin was 26 nM. These results indicate that PACAP is a more potent activator of adenyl cyclase than forskolin.

**FIG. 2.** PACAP potentiates glutamate-evoked Ca\(^{2+}\) transients via an L-type Ca\(^{2+}\) channel-dependent mechanism. A: SCN neurons were repeatedly stimulated with potassium (K\(^+\), arrowheads = 10 s exposure, 15 mM) both before and after PACAP (200 nM) administration (bar = 120 s). B: cells were continuously perfused with the L-type Ca\(^{2+}\) channel blocker nimodipine (5 µM). Blocking L-type Ca\(^{2+}\) channels blocked PACAP (200 nM, bar = 90 s)-induced potentiation of glutamate (3 µM: arrows = 10 s application)-evoked Ca\(^{2+}\) transients. All bath solutions contained TTX. C: PACAP-induced potentiation of K\(^+\)-evoked Ca\(^{2+}\) transients is dose-dependent. The percentage of neurons exhibiting PACAP-induced potentiation of ≥20% are shown for each PACAP concentration. D: graphical representations of mean evoked Ca\(^{2+}\) responses for experiments represented in A and B. The two bars per condition represent the responses to two consecutive evoked Ca\(^{2+}\) transients either immediately before or immediately after PACAP administration. Error bars denote SE. **: P < 0.0001, 2-tailed Student’s t-test.
shown) stimulated robust increases in cAMP. To determine whether PACAP potentiates via a cAMP-dependent mechanism, data from the forskolin dose-response curve was used to determine the forskolin concentration that raised cAMP to a level equivalent to a concentration of PACAP (200 nM) that triggered robust potentiation. This forskolin concentration (150 nM; Fig. 6A) was then assayed to determine whether elevated cAMP levels potentiate depolarization-induced Ca\(^{2+}\) rises.

Contrary to the effects of PACAP, forskolin did not potentiate the evoked Ca\(^{2+}\) rise (Fig. 6, B and D). Rather, increased cAMP tended to dampen the K\(^{+}\)-evoked Ca\(^{2+}\) rise. In a second experiment to test the role of the cAMP/PKA signaling pathway, SCN neurons were incubated with the PKA inhibitor Rp-cAMPS (200 mM), and the effects of PACAP on evoked Ca\(^{2+}\) responses were tested. Disruption of PKA-dependent signaling did not block PACAP-induced potentiation of depolarization-induced Ca\(^{2+}\) rises (Fig. 6, C and D). Together, these results indicate that PACAP does not potentiate depolarization-induced Ca\(^{2+}\) rises via a cAMP/PKA-dependent mechanism.

Next, we examined whether PKC-dependent signaling couples PACAP to facilitation of evoked Ca\(^{2+}\) transients. For this study, PKC levels in cultured cells were down-regulated by pretreatment (24 h) with phorbol 12-O-tetradecanoylphorbol-13-acetate (PMA; 1 μM). PKC down-regulation attenuated PACAP (200 nM)-induced potentiation of Ca\(^{2+}\) transients stimulated by K\(^{+}\) (15 mM; Fig. 7, A and D). Although attenuation was observed, PACAP still elicited a statistically significant potentiation of K\(^{+}\)-evoked Ca\(^{2+}\) transients. The effects of two PKC inhibitors, bisindolylmaleimide (a broad range inhibitor) and Gö 6976 (inhibitor of Ca\(^{2+}\)-dependent PKC isoforms), were also examined. Similar to the effect of 12-O-tetradecanoylphorbol 13-acetate (TPA) down-regulation, bisindolylmaleimide reduced, but did not eliminate, PACAP-mediated potentiation of depolarization-induced Ca\(^{2+}\) rises (Fig. 7, B and D). In the presence of bisindolylmaleimide, PACAP elicited a ≥20% potentiation of K\(^{+}\)-evoked Ca\(^{2+}\) transients in 32% of neurons (n = 88), whereas 55% of neurons exhibited PACAP potentiation of ≥20% under normal physiological

![FIG. 3. PACAP potentiates Ca\(^{2+}\) transients via an L-type VACC-dependent mechanism. Cells were pretreated (10 min) and continuously perfused with the L-type Ca\(^{2+}\) channel blocker nimodipine (5 μM). Under this condition, potassium (K\(^{+}\), arrowheads =10 s exposure, 15 mM)-evoked Ca\(^{2+}\) transients were largely inhibited. In the absence of functional L-type Ca\(^{2+}\) channels PACAP (200 nM, bar =120 s) did not significantly affect the magnitude of the depolarization-evoked Ca\(^{2+}\) transient. A: two representative SCN neuronal traces are shown. All bath solutions contained TTX. B: graphical representations of mean evoked Ca\(^{2+}\) responses for the experiment represented in A. The two bars per condition represent the responses to the two consecutive evoked Ca\(^{2+}\) responses immediately before and immediately after PACAP administration. Error bars denote SE.](image)

![FIG. 4. Ca\(^{2+}\) transients evoked by synaptic glutamate release are potentiated by PACAP. Withdrawal of ionotropic glutamate receptor antagonists D, L-2-amino-5-phosphonovalerate (AP5: 100 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX: 10 μM) from the perfusion media triggered spontaneous Ca\(^{2+}\) transients. Brief (60 s) PACAP (200 nM) administration triggered a dramatic increase in the magnitude of the spontaneous Ca\(^{2+}\) transients. Robust induction was also observed in neurons that did not exhibit a marked Ca\(^{2+}\) rise on withdrawal of glutamate receptor antagonists from the perfusion solution (bottom trace). Administration of AP5 and DNQX at the end of the experiment returned Ca\(^{2+}\) to basal levels, indicating that PACAP modulates cytosolic Ca\(^{2+}\) levels by augmenting the excitatory actions of synaptically-released glutamate.](image)
conditions (Fig. 2C). The inhibitor of Ca\(^{2+}\)-dependent PKC isoforms, Gö 6976, did not affect the potentiating actions of PACAP (Fig. 7, C and D). The substantial reduction in PACAP-mediated potentiation resulting from TPA and bisindolylmaleimide indicates that PKC activity contributes to PACAP-induced potentiation of depolarization-evoked Ca\(^{2+}\) transients.

Finally, the potential contribution of the MAPK pathway was examined. To assess whether the MAPK pathway was activated by PACAP, cultured SCN neurons were stimulated...
with PACAP (1 or 200 nM; 5 min) and then lysed, and cell extracts were immunobotted for the activated (i.e., dually phosphorylated; Thr-202 and Tyr-204) forms of the extracellular signal-regulated kinases erk-1 and erk-2 (here, collectively referred to as ERK). Relative to control, mock-treated cultures, 200 nM PACAP stimulated robust ERK activation, whereas 1 nM PACAP triggered a more modest increase in ERK activity (Fig. 8A). Pretreatment (20 min) of SCN neurons with the MEK inhibitors U0126 (10 μM) or PD 98059 (50 μM) blocked PACAP (200 nM)-induced activation of ERK (Fig. 8B). Membranes were also probed with an antibody that detects total ERK. Immunocytochemistry was used to show that PACAP (200 nM) stimulates ERK activation (Fig. 8C). The PACAP-induced ERK activity profile is similar to the profile for PACAP-induced potentiation of Ca\(^{2+}\) transients, and thereby raises the possibility that the MAPK pathway couples PACAP to potentiation of Ca\(^{2+}\) transients.

To assess the contribution of the MAPK pathway to PACAP-dependent facilitation of Ca\(^{2+}\) transients, SCN cells were pretreated (20 min) and continuously perfused with MEK inhibitors. Using the same protocol as above (Fig. 2A), cells were repeatedly challenged with K\(^+\) (15 mM) before and after administration (2 min) of PACAP (200 nM; Fig. 9, A and B). In the presence of U0126 (10 μM), K\(^+\) triggered reproducible
Ca^2+ transients; however, the ability of PACAP to potentiate these transients was blocked (Fig. 9, A and C). As with U0126, PD 98059 (50 μM) blocked PACAP-mediated potentiation of K^+-evoked Ca^2+ transients (Fig. 9, B and C). Together these findings reveal that PACAP stimulation of the MAPK pathway is required for Ca^2+ transient potentiation.

**DISCUSSION**

Within the SCN, release of glutamate from RHT nerve terminals plays a central role in light-induced entrainment of the circadian clock. The phase regulating effects of light are the likely result of glutamate-induced increases in cytosolic Ca^2+ within SCN neurons. Thus modulators of glutamate signaling could play an important role in regulating the phase-shifting actions of glutamate. For this study, we examined the modulatory actions of PACAP 1–38. The data presented here reveal PACAP as a potent regulator of glutamate-induced Ca^2+ transients. Many neurons became responsive to glutamate only after PACAP administration, suggesting that PACAP may set the lower concentration threshold required for glutamate to initiate a robust rise in postsynaptic cytosolic Ca^2+. This effect of PACAP is mediated by MAPK pathway-dependent potentiation of L-type VACC activity.

**Evoked Ca^2+ rises and PACAP**

One of the well-characterized physiological effects of PACAP is mobilization of intracellular Ca^2+ stores. Indeed, our data reveal that PACAP elicits marked Ca^2+ transients in a large percentage of cells. PACAP-mediated increases in cytosolic Ca^2+ are triggered by activation of PLC, which in turn triggers IP3 production, thereby leading to release of Ca^2+ from intracellular stores (Tanaka et al. 1996, 1997). This effect has been observed in a variety of cell types including neurons cultured from the SCN (Kopp et al. 1999).

Although Ca^2+ mobilization is of significant interest, our focus was on the ability of PACAP to modulate depolarizing Ca^2+ responses. Toward this end, neurons were repeatedly stimulated with glutamate or depolarizing levels of potassium, thus creating a baseline response profile to which we could compare glutamate-evoked responses after PACAP adminis-

![Image](http://jn.physiology.org/)
With this paradigm, we found that PACAP triggered a marked potentiation of evoked Ca\(^{2+}\)/H\(^{+}\) transients. In fact, in some neurons, responsiveness was established only after pretreatment with PACAP. Maintaining TTX in the perfusion buffers throughout the experimental period obviated the complicating influence of action potential mediated transmitter release. Thus the observed actions of PACAP were the result of postsynaptic modulation of evoked Ca\(^{2+}\)/H\(^{+}\) transients.

Rapid, glutamate-evoked, cytosolic Ca\(^{2+}\)/H\(^{+}\) transients are initiated by the opening of ionotropic glutamate receptor channels, and secondarily, by depolarization-mediated activation of VACCs. To determine whether PACAP was functioning as a modulator of glutamate- or VACC-mediated responses, the modulatory actions of PACAP on K\(^+-\)evoked Ca\(^{2+}\) transients were tested. Depolarization-mediated activation of VACC was potentiated by PACAP. Nimodipine, an L-type Ca\(^{2+}\) channel inhibitor, blocked PACAP-mediated potentiation of glutamate-evoked as well as depolarization-evoked Ca\(^{2+}\) transients, indicating that PACAP was facilitating Ca\(^{2+}\)/H\(^{+}\) influx by potentiating the actions of L-type VACCs. PACAP-mediated potentiation of Ca\(^{2+}\)/H\(^{+}\) influx may result from the modulation of L-type VACC conductance properties. Conversely, PACAP may regulate cellular excitability by closing K\(^+-\) channels, thereby triggering membrane depolarization. Data from a number of studies support both mechanisms of action (Bruch et al. 1997; Chatterjee et al. 1996; Chik et al. 1996; Darvish and Russell 1998; Ichinose et al. 1998; Tanaka et al. 1996, 1997). Thus, in addition to potentiating responses to glutamate, PACAP may function as a broad-spectrum modulator of transmitters that trigger membrane depolarization.

PACAP potentiated the evoked responses of a subpopulation (55%) of SCN neurons. It is unclear why potentiation was not observed in all cells tested. One possible explanation is that the effects of PACAP are phase-dependent. In dispersed culture, SCN neurons are rhythmic, but asynchronous (Welsh et al. 1995), and thus it is conceivable that only a subset of cells are ever in the correct phase to elicit a modulatory response by PACAP. Another possible explanation is that only certain subsets of SCN cells contain the intracellular machinery required for PACAP to potentiate evoked Ca\(^{2+}\)/H\(^{+}\) responses.
Potentiation of synaptic transmission

PACAP-induced potentiation of Ca\(^{2+}\) transients was also observed under conditions of synaptic glutamate release. In this approach SCN neurons were cultured with glutamatergic neurons from the surrounding hypothalamic area. A rise in neuronal Ca\(^{2+}\) levels was initiated by removing ionotropic glutamate receptor antagonists from the perfusion media. This Ca\(^{2+}\) rise was blocked by TTX, indicating its action potential–dependent origin. Whole cell patch-clamp recording has also been used to examine membrane depolarization and an increase in spontaneous excitatory postsynaptic potentials mediated by the withdrawal of glutamate receptor antagonist (Obrietan et al. 1995). With this assay, we observed that bath application of PACAP triggered a robust potentiation of glutamate-mediated Ca\(^{2+}\) transients. Additionally, some cells became responsive to tonic glutamate release only on PACAP receptor stimulation. These effects were purely modulatory in nature since Ca\(^{2+}\) returned to basal levels after reintroduction of glutamate receptor antagonists. Given that the modulatory actions elicited by PACAP were similar regardless of whether glutamate was exogenously applied or synaptically released, it is likely that the effects of PACAP were via a postsynaptic site of action, although a presynaptic modulatory action of PACAP cannot be ruled out.

To validate that SCN neurons exhibit PACAP-evoked potentiation of glutamatergic neurotransmission, co-cultured cells from surrounding hypothalamic region were irreversibly labeled with a fluorescent tag. The fluorescent tag was then used to identify the two populations of neurons. This approach allowed us to specifically record from SCN neurons, and thereby confirm that PACAP potentiates the effects of synaptically released glutamate in cells derived from the SCN. However, since the majority of endogenous activity assays did not use this technique to discriminate between the two populations, we cannot exclude the possibility that we also recorded from some non-SCN cells.

Several studies have shown that PACAP exerts modulatory actions via both presynaptic and postsynaptic mechanisms. Along these lines, PACAP increases the frequency of postsynaptic potentials in the hippocampus (a presynaptic effect; Roberto and Brunelli 2000), whereas in supraoptic neurons, PACAP has little effect on the frequency of spontaneous postsynaptic currents, but rather, triggers membrane depolarization via a postsynaptic mechanism and thereby increases the neuronal firing rate (Shibuya et al. 2000). It is important to note that the blockade of GABA\(_\text{A}\) receptor activity with bicuculline did not alter the ability of PACAP to modulate Ca\(^{2+}\) transients elicited by synaptic glutamate release, indicating that PACAP does not facilitate glutamate signaling by modulating the inhibitory actions of GABA at the GABA\(_\text{A}\) receptor.

Second messenger pathways

PACAP triggers a wide array of molecular physiological responses (reviewed by Vaudry et al. 2000). This diversity of actions may be in part the result of PACAP’s ability to activate a variety of signaling pathways. One goal of this investigation was to identify the second messenger signaling pathways that couple PACAP to potentiation of glutamate-induced Ca\(^{2+}\) transients. Although PACAP triggered Ca\(^{2+}\) transients in a subpopulation of neurons, this physiological response was not required for PACAP to potentiate evoked Ca\(^{2+}\) transients, suggesting that Ca\(^{2+}\)-inducible second messenger signaling pathways, such as CaMKs, are not involved in PACAP-mediated potentiation of transients. Increased cAMP is also not likely to be directly involved, given that forskolin-mediated elevation in cAMP did not potentiate evoked Ca\(^{2+}\) transients. Furthermore, inhibition of PKA with Rp-cAMPS did not affect the potentiating actions of PACAP. Previous work using a similar model system revealed that alterations in cAMP (either increased or decreased) tended to reduce rather than potentiate the amplitude of evoked Ca\(^{2+}\) responses (Obrietan and van den Pol 1997). Considering these results, we focused on the potential role of the MAPK pathway.

Pretreatment with the MEK inhibitors U0126 or PD 98059 blocked PACAP-induced potentiation of Ca\(^{2+}\) transients initiated by exogenous application of K\(^+\). Inhibitor results were complemented by Western and immunocytochemical data, showing that PACAP triggers ERK phospho-activation. Similar results for PACAP-induced ERK activation have been observed in cerebellar granule cells (Villalba et al. 1997).

Although the mechanism by which MAPK signaling potentiates L-type Ca\(^{2+}\) channels is not known, there are several potential routes. The most direct model would involve ERK or one of the downstream ERK activated kinases phosphorylating the L-type channel. The phosphorylation could potentiate L-type Ca\(^{2+}\) currents by triggering a shift in the voltage-dependence of channel activation. Indeed, several studies have shown that the L-type channel conductance properties can be regulated in a phosphorylation-dependent manner (reviewed by Catterall 2000). Interestingly, work in cardiomyocytes has shown that leukemia inhibitory factor (LIF) enhances L-type Ca\(^{2+}\) currents through a mechanism that requires the MAPK pathway: PD 98059 completely inhibited the LIF-induced increase in L-type Ca\(^{2+}\) currents (Murata et al. 1999). Additionally, signaling via the MAPK pathway has been shown to trigger activation of the L-type Ca\(^{2+}\) channel in neuroblast cells (Ekinci et al. 1999). These observations, coupled with our data, raise the possibility that the MAPK pathway plays a general role as a modulator of cellular excitability.

The potential role of PKC as a signaling intermediate coupling PACAP to potentiation of depolarization-induced Ca\(^{2+}\) influx was also examined. Toward this end, a combination of PKC inhibitors and long-term TPA treatment (to down-regulate PKC expression) was employed. Both PKC down-regulation and pretreatment with bisindolylmaleimide attenuated the potentiating actions of PACAP. Importantly, significant residual modulatory activity persisted after disruption of PKC signaling. These data suggest that PKC activity contributes to, but is not essential for, PACAP-mediated potentiation of depolarization-induced Ca\(^{2+}\) transient. It is interesting to note that in some model systems, PKC can trigger MAPK pathway activation (Corbit et al. 2000; Troller et al. 2001). Additional studies will be required to identify the exact mechanism by which PKC contributes to this process.

MAPK, PACAP, and the circadian clock

The results presented here reveal a new role for the MAPK pathway in the SCN as a modulator of glutamatergic neurotransmission. Within the past several years, a number of reports
PACAP POTENTIATES L-TYPE $\mathrm{Ca}^{2+}$ CHANNELS IN SCN NEURONS

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have begun to examine the role of signaling via the MAPK pathway in the circadian clock. These studies have revealed that photic input during the subjective night, but not during the subjective day, triggers a marked increase in MAPK activation in the SCN (Obrietan et al. 1998), and that the MAPK pathway couples glutamate-receptor activation to gene expression in SCN neurons (Obrietan et al. 1999). A role for the MAPK pathway in endogenous circadian timing has been identified in immortalized cells, where TPA-induced circadian oscillations in gene expression are inhibited by blocking the MAPK pathway (Akashi and Nishida 2000). In addition, MAPK pathway activation is rhythmically regulated in the avian pineal gland (Sanada et al. 2000). Likewise, the activation state of the MAPK pathway is regulated in a circadian-dependent manner in the SCN (Obrietan et al. 1998), and that the MAPK pathway is regulated in a circadian-dependent manner in the SCN. These studies have revealed that the MAPK pathway is regulated in a circadian-dependent manner in the SCN (Obrietan et al. 1998), and that the MAPK pathway is regulated in a circadian-dependent manner in the SCN (Obrietan et al. 1998), and that the MAPK pathway is regulated in a circadian-dependent manner in the SCN (Obrietan et al. 1998). In conclusion, the results presented here identify new modulatory roles for PACAP and the MAPK pathway in the SCN.

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