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Effects of Vasoactive Intestinal Polypeptide on Neurones of the Rat Suprachiasmatic Nuclei *In Vitro*

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Abstract

The suprachiasmatic nuclei (SCN) of the hypothalamus house the main circadian pacemaker in mammals. Vasoactive intestinal polypeptide (VIP) is the most abundant neuropeptide in the SCN and has been shown to phase-shift the electrical activity rhythm of SCN cells *in vitro*. However, the effects of VIP on the cellular activity of rat SCN neurones are unknown. In this study, we examined the acute effects of VIP on the extracellularly recorded spontaneous firing rate of SCN neurones in an in-vitro hypothalamic slice preparation. Furthermore, with the use of receptor-selective agonists and antagonists, we determined which receptors might mediate the effects of VIP in the SCN. Approximately 50% of cells responded to VIP; the main type of response was suppression in firing rate, although a few cells were activated. Suppression responses to VIP were mimicked by the $VPAC_2$ receptor agonist Ro 25-1553 and blocked by the selective $VPAC_2$ receptor antagonist PG 99-465. The PAC_1 receptor agonist maxadilan evoked responses from 40% of SCN cells, and activations to this agonist were not altered by PG 99-465. Responses to VIP were not blocked by antagonists to ionotropic glutamate receptors, but the duration of suppression was modulated by the $GABA_A$ receptor antagonist bicuculline. Our data indicate that VIP alters the electrical activity of rat SCN neurones *in vitro*, via both $VPAC_2$ and PAC_1 receptors.

The main mammalian circadian pacemaker (or clock) is housed in the suprachiasmatic nuclei (SCN) of the hypothalamus (1, 2). This pacemaker is entrained by photic information relayed from the retina to the SCN directly via the retinohypothalamic tract and indirectly through cells of the retinally innervated intergeniculate leaflet that project to the SCN to form the geniculo-hypothalamic tract. Glutamate, and possibly pituitary adenylate cyclase activating peptide (PACAP), are the main neurotransmitters of the retinohypothalamic tract (3, 4), whereas the key transmitters of the geniculohypothalamic tract are GABA and neuropeptide Y (5, 6). A well-established characteristic of SCN neurones is that they exhibit a circadian rhythm in electrical activity with a peak firing rate at the middle of the subjective day (7–9). Exogenous application of a number of peptides and neurotransmitters contained in SCN cells and projections to the SCN can phase-shift the electrical firing rate rhythm of SCN cells in an in-vitro slice preparation [e.g. glutamate (7), neuropeptide Y (10) and PACAP (11)] indicating a possible involvement for these neurochemicals in the entrainment of the clock.

The 28-amino acid peptide vasoactive intestinal polypeptide (VIP) was originally isolated from porcine small intestine (12) and subsequently found to be distributed widely throughout the peripheral and central nervous systems, where it has been implicated in neuroendocrine functions (13). A large number of VIP-synthesizing neurones are found in the retinally innervated ventral region of the rodent SCN (14–16) and these cells give rise to extensive intra- and inter-SCN connections, and also project to regions outside the SCN (17–19). Recently, work from our laboratory has shown that application of VIP to rat SCN slices *in vitro* phase-shifts SCN cellular rhythms in a pattern similar to the phase-resetting effects of glutamate *in vitro* and light pulses *in vivo* (9). These data indicate a possible role for VIP in photic entrainment of the SCN clock.

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To date, three G-protein-coupled receptors for VIP and the related peptide PACAP have been identified: the high affinity PACAP receptor PAC_1, which binds PACAP with 1000-fold greater affinity than VIP, and the $VPAC_1$ and $VPAC_2$ receptors, which bind VIP and PACAP with equally high affinities (20). The $VPAC_1$ and $VPAC_2$ receptors are primarily coupled to the adenylate cyclase stimulating G-protein, Gs, whereas PAC_1 is coupled to Gs, and also to $G_{q/11}$, which stimulates the phospholipase C signalling cascade (20). It has been shown that $VPAC_2$ and PAC_1 receptors, but not the $VPAC_1$ receptor, are present in the SCN (21–24). Development of agonists selective for $VPAC_1$ and $VPAC_2$ receptors (25, 26) has enabled the determination of the receptors through which VIP may act. Using such agonists, we have shown that the phase-advancing effect of VIP on the rat SCN firing rate rhythm is likely mediated by the $VPAC_2$ receptor, rather than $VPAC_1$ or PAC_1 receptors (9). Further evidence to support the importance of the $VPAC_2$ receptor in photic entrainment of the clock was provided by a study using mice that overexpress the human $VPAC_2$ receptor (27). Compared with wild-types, these mice re-entrained much more rapidly to an 8-h advance in the light/dark cycle and exhibited a significantly shorter circadian period under constant darkness.

Vasoactive intestinal polypeptide-induced changes in cell firing frequency have been described in several areas of the mammalian central nervous system, with the main effect of VIP being an activation in firing rate (28–30), although inhibitory actions have been observed (31). To date, however, there has been no comprehensive study describing the effects of VIP on the electrical activity of rodent SCN neurones. Since data from our laboratory and others (9, 27, 32) indicate that VIP and the $VPAC_2$ receptor have a role in the entrainment of the SCN circadian clock, the aim of the current study was to determine how exogenously applied VIP and selective VIP receptor agonists alter the extracellularly recorded spontaneous firing frequency of rat SCN neurones in an in-vitro brain slice preparation. To further define the receptor(s) that mediate the actions of VIP and other VIP receptor agonists in the SCN, we used a recently developed antagonist selective for the $VPAC_2$ receptor (33).

Methods

Slice preparation and maintenance

Male Wistar rats, aged 6–8 weeks (Charles River, Kent, UK), were housed in the animal unit at Manchester University, under a 12 : 12 h light/dark cycle for at least 10 days before to the start of the experiments. Lights-on was either at 07.00 h or 12.00 h, depending on the experimental protocol, and this was designated Zeitgeber time (ZT) 0.

Rats were killed by cervical dislocation and decapitation under halothane anaesthesia, in accordance with the UK Animals (Scientific Procedures) Act 1986. The brain was rapidly removed and dissected to form a tissue block containing the SCN. Coronal brain slices, 500 μm thick, were prepared using a Vibroslicer (Campden Instruments, Loughborough, UK) and transferred to a PDMI-2 submerged slice microincubator (Medical Systems Corp., NY, USA) with modifications for stabilization of the slice (34). Slices were maintained for 10–15 h by perfusion (approximately 1.5 ml/min) with oxygenated (95% O_2/5% CO_2) Earle's balanced salt solution (EBSS; Sigma, Poole, UK), supplemented with 24.6 mM glucose, 26.2 mM sodium hydrogen carbonate and 0.0005% gentamicin. The tissue bath and perfusion solutions were warmed to approximately 35 °C using a TC-202 temperature controller (Medical Systems Corp.). Slices were allowed to equilibrate for at least 1 h before electrophysiological recordings commenced.

Electrophysiological recording

Single unit activity of SCN neurones was recorded extracellularly with borosilicate glass electrodes (World Precision Instruments, Herts, UK) filled with 2 M NaCl (resistance approximately 5 MΩ). Action potential spikes were amplified (× 20 000), filtered (bandwidth 300 Hz to 3 kHz) and visualized using an oscilloscope. Data were averaged every 5 s and plotted as integrated histograms on a PC running Spike 3 software (Cambridge Electronic Design, Cambridge, UK). Only spikes at least three times the size of the background noise were recorded. Amplification, filtering and spike discrimination were performed using a NeuroLog modular system (Digitimer Ltd, Herts, UK). Cell firing frequency was recorded for at least 5 min, to establish a stable baseline firing rate, prior to drug treatment.

Drug treatment

VIP and the 38-amino acid peptide PACAP were obtained from BACHEM (Essex, UK). D-aminophosphopentanoic acid (D-AP5), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), bicuculline and *N*-methyl-D-aspartate (NMDA) were purchased from Sigma. The $VPAC_1$ and $VPAC_2$ receptor agonists [K15, R16, L27]VIP(1-7)/GRF(8-27) and Ro 25-1553, respectively (25, 26) and the $VPAC_2$ receptor antagonist PG 99-465 (33) were kindly supplied by Dr Patrick Robberecht (University of Brussels). Maxadilan, a PAC_1 selective agonist (35), was a generous gift from Professor Ethan Lerner (Harvard University). Drugs were dissolved in distilled water or DMSO (CNQX and PG 99-465) to stock concentration, aliquoted and stored at −20 °C. Immediately before use, an aliquot was thawed, diluted to the appropriate working concentration in EBSS and bath applied to the slice via the perfusion line for 5 min, unless stated otherwise. The final concentration of DMSO was never greater than 0.1% (v/v) and this alone was found to have no effect on SCN cell firing rate (data not shown).

Statistical analysis

Responses were considered significant if the neuronal firing frequency during the 5-min drug application was increased (activation) or decreased (suppression) by >20% of baseline firing activity recorded for 5 min immediately preceding the drug treatment. Day–night and neuroanatomical (ventral–dorsal) differences in the proportion of SCN cells responsive to VIP were assessed using chi-squared ($P < 0.05$). Where stated, values correspond to mean firing rate ± SEM.

Results

Electrophysiological responses of SCN neurones to VIP

In this study, 373 spontaneously firing SCN neurones were tested throughout the circadian cycle. Preliminary experiments, using a range of VIP concentrations (10 pM to 100 nM) (Figs 1 and 2), indicated that a threshold concentration to evoke responses occurred at approximately 1 nM. The concentration of 100 nM VIP was used throughout this study because it produced the maximal response in SCN neurones and has been shown to produce significant phase shifts in SCN electrical rhythms (9).

One hundred and fifty-two cells were tested with VIP (100 nM) alone, of which 79 (52%) responded. The type of response most frequently seen following a 5-min application of VIP was suppression in firing rate (59/79 cells, 75%) (Fig. 2A). On average, the firing rate decreased by 53% compared to baseline and returned to prestimulus levels within 5–20 min. In 20/79 (25%) VIP-responsive cells, activation in SCN neuronal firing rate was observed (Fig. 2B). The mean amplitude of the activations was 130% of baseline firing rate and, as with suppressions, the firing rate generally

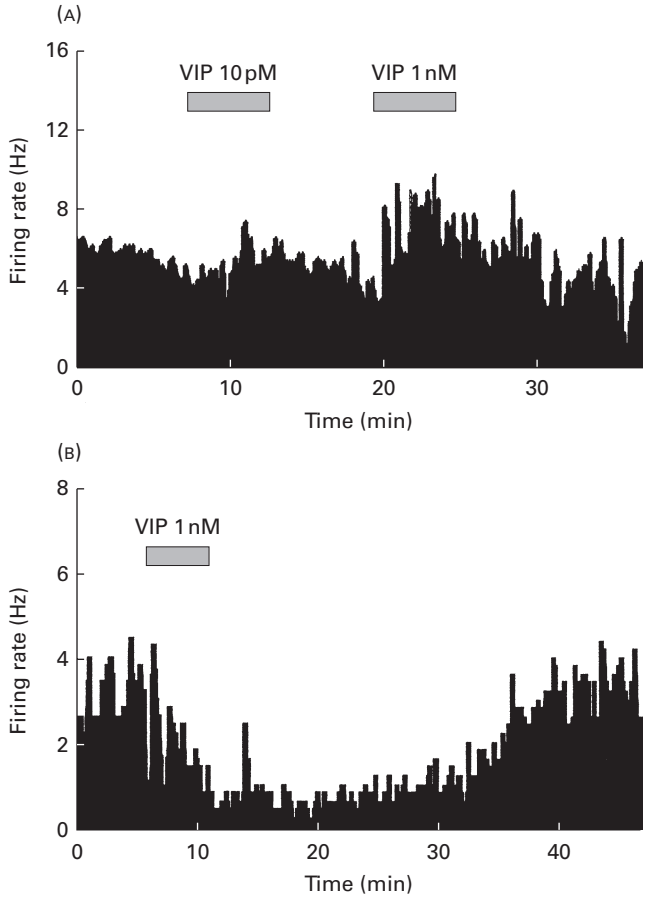

FIG. 1. Integrated firing rate histograms showing effects of low concentrations of vasoactive intestinal polypeptide (VIP) on suprachiasmatic nuclei (SCN) cell firing frequency. (A) VIP (10 pM) did not evoke changes in neuronal firing rate. In contrast, VIP (1 nM) elicited both activations and (B) suppressions in SCN neuronal activity. Grey bars show timing and duration of VIP application.

returned to baseline within 5–20 min (Table 1). However, the firing rate of some cells (10/79) did not return to baseline levels after more than 30 min following VIP-induced activation or suppression. In 10 cells that did not respond to VIP or other agonists, 20 μM NMDA was applied to test whether these cells responded to a suitable stimulus. Nine of these cells responded with a large transient activation in firing rate (Fig. 2C).

To determine whether there was temporal variation in responsiveness to VIP, cells were grouped according to the time of day they were recorded, either in the projected day (ZT0-12) or projected night-time (ZT12-24) of the slice. Of 110 cells tested between ZT0-12, 47 (43%) responded to VIP, 35 of which were suppressed and 12 were activated. The 42 cells treated with VIP between ZT12-24 showed a significantly higher proportion of responses (76%) of which 24 were suppressions and eight were activations (Fig. 2D) (chi-squared = 13.64, d.f. = 2, $P < 0.001$). Dividing the cells into those recorded from the dorsal or the ventral region of the SCN indicated that there were no significant regional differences in the proportion of SCN cells responding to VIP (chi-squared, $P > 0.05$). Of the 80 cells recorded in the dorsal SCN region, 41 (53%) responded to VIP (29 suppressed and 12 activated). In the ventral SCN region, 38 of 72 cells (53%) responded to VIP (30 suppressed and eight activated).

Effects of VIP receptor agonists and antagonists on SCN cell firing rate

To ascertain which receptor(s) mediate the responses to VIP, cells were treated for 5 min with agonists selective for the three known VIP/PACAP receptors. The $VPAC_1$ receptor agonist [K15, R16, L27]VIP(1-7)/GRF(8-27) (100 nM) evoked a response (activation) from only one of 11 cells tested. In a separate sample of 11 VIP-responsive cells, this $VPAC_1$ receptor agonist failed to significantly alter electrical activity (Fig. 3A), indicating that these actions of VIP on rat SCN neurones were not mediated by the $VPAC_1$ receptor.

By contrast, the $VPAC_2$ receptor agonist Ro 25-1553 (100 nM) evoked responses from nine of 20 (45%) spontaneously discharging SCN cells (seven suppressed, two activated) (Fig. 3B). In a different sample of six VIP-responsive SCN neurones (five suppressed, one activated), Ro 25-1553 evoked similar changes with respect to direction (five suppressed, one activated), time course and magnitude (Table 1). Furthermore, in five cells that did not respond to VIP, Ro 25-1553 similarly failed to evoke significant changes in cellular activity. These data show that VIP-evoked changes in firing rate are mimicked by the $VPAC_2$ receptor agonist Ro 25-1553.

To further examine the possibility that the actions of VIP are mediated by the $VPAC_2$ receptor, we assessed the responsiveness of SCN cells to VIP in the presence of the $VPAC_2$ receptor antagonist PG 99-465 (100 nM). Application of the antagonist alone did not evoke a response in any cell tested. Of 10 cells tested with VIP, following pretreatment with PG 99-465, only two responded: one activation and one suppression (Fig. 3C), demonstrating that the $VPAC_2$ receptor antagonist reduced the response rate of cells to VIP.

In a different sample of cells, application of the PAC_1-selective agonist maxadilan (100 nM) evoked responses in four of 10 (40%) SCN neurones (one suppressed, three activated) (Fig. 3D). These activations were characterized by a 'bursty' pattern of discharge rate. To confirm that maxadilan evoked these actions via the PAC_1 receptor, maxadilan responses were examined in the presence of the $VPAC_2$ receptor antagonist PG 99-465 (100 nM). Of seven cells tested, four responded to the PAC_1 agonist (one suppressed, three activated) following pretreatment with PG 99-465 (Figs 3E and Table 1). The similarity of the proportion of cells responding to maxadilan in the presence (57%) or absence of PG 99-465 (40%) supports the contention that maxadilan is selective for the PAC_1 receptor.

Since PACAP is thought to act as a mixed agonist at both $VPAC_2$ and PAC_1 receptors, responses to PACAP were examined in the presence and absence of the $VPAC_2$ receptor antagonist, PG 99-465. In the absence of this antagonist, PACAP (100 nM) evoked three activations and nine suppressions from 36 cells tested. These responses to PACAP were similar in duration and amplitude to those evoked by

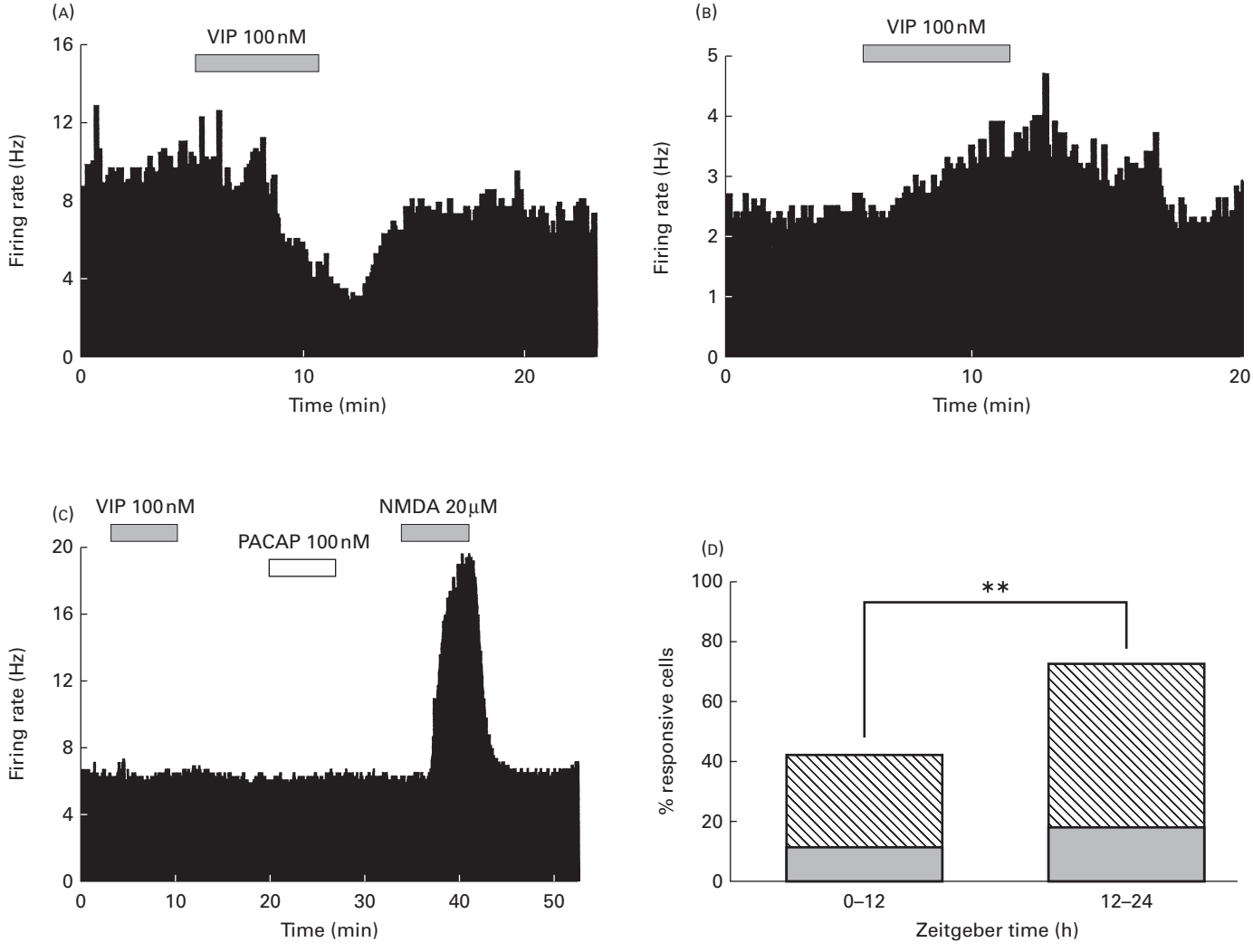

FIG. 2. Integrated firing rate histograms showing typical (A) suppression and (B) activation responses in suprachiasmatic nuclei (SCN) cells and (C) a nonresponsive SCN cell following application of vasoactive intestinal polypeptide (VIP) (100 nM). Application of *N*-methyl-D-aspartate (NMDA) (20 μM) to cells that did not respond to VIP or pituitary adenylate cyclase activating peptide (PACAP) evoked large transient increases in neuronal firing rate (C). Dark grey bars show time and duration of VIP application. White bar shows timing and duration of PACAP application. Light grey bar shows time and duration of NMDA application. (D) The overall proportion of cells responding to VIP during the subjective night was significantly greater than during the subjective day (chi-squared = 13.64, d.f. = 2, $P < 0.001$). Filled areas of bars denote activation responses and hatched areas denote suppressions.

TABLE 1. Summary of Effects of Vasoactive Intestinal Polypeptide (VIP), VIP Receptor Selective Agonists and VIP in the Presence of $VPAC_2$, GABA and Glutamate Receptor Antagonists.

Drug treatment	Cells tested	No. cells activated	No. cells suppressed	Average activation (% of baseline)	Average suppression (% of baseline)	Duration of response (min)
VIP (100 nM)	152	20	59	130 ± 32	−53 ± 3	10.0 ± 0.9
VIP (100 nM) and PG 99-465 (100 nM)	10	1	1	100	−64	22.0
VIP (100 nM) and bicuculline (20 μM)	23	2	11	42	−56 ± 9	12.0 ± 3.8
VIP (100 nM) and D-AP5 (50 μM) and CNQX (20 μM)	15	2	6	51	−87 ± 6	9.1 ± 1.3
Ro 25-1553 (100 nM)	20	2	7	50	−80 ± 9	24.0 ± 4.0
Maxadilan (100 nM)	10	3	1	56 ± 18	−34	6.3 ± 1.8
Maxadilan (100 nM) and PG 99-465 (100 nM)	7	3	1	59 ± 24	−94	11.8 ± 5.2
PACAP (100 nM)	36	3	9	72 ± 8	−38 ± 9	8.4 ± 2.2
[K15, R16, L27]VIP(1-7)/GRF(8-27) (100 nM)	11	1	0	63	NA	13

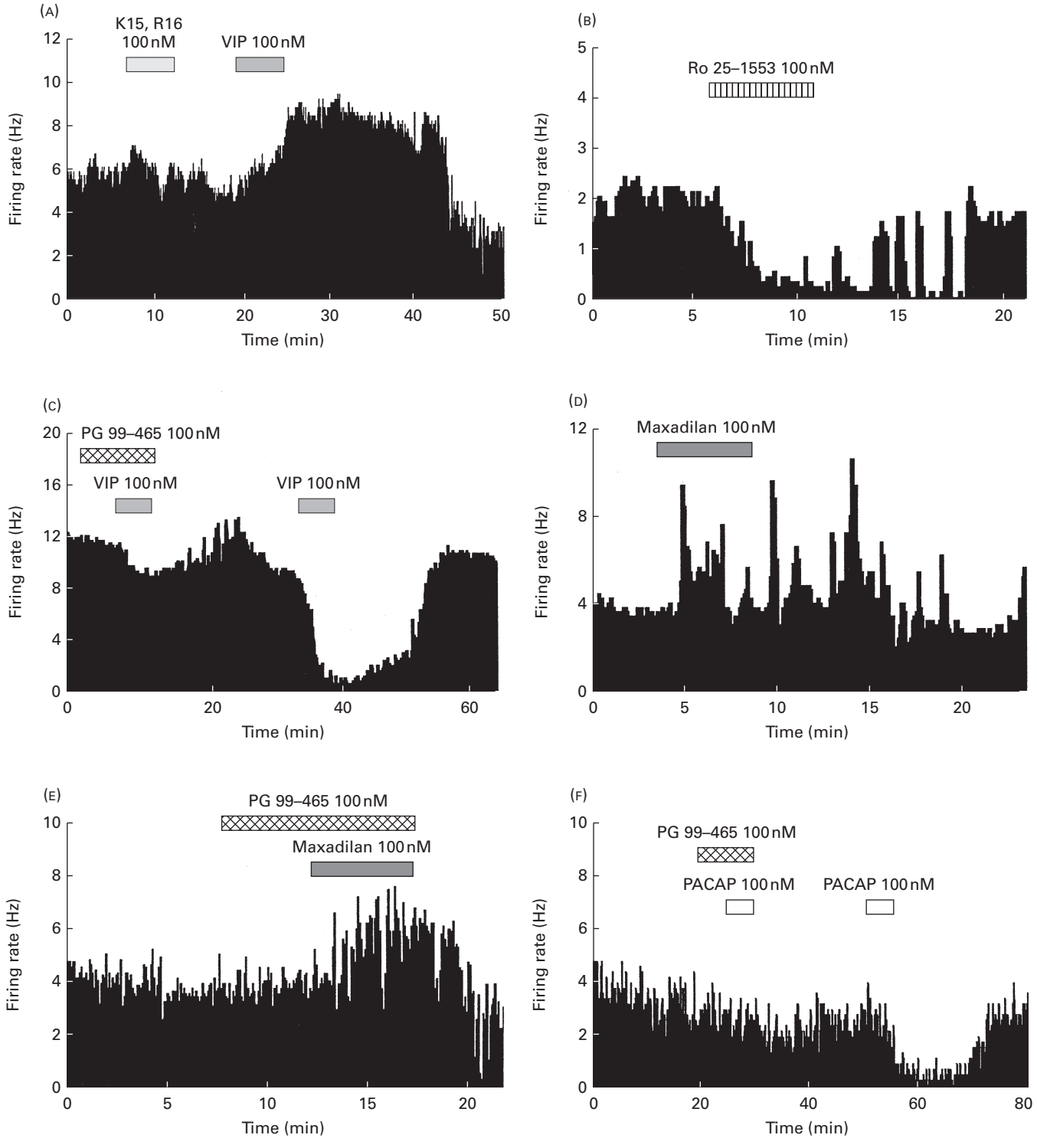

FIG. 3. Integrated firing rate histograms showing (A) a typical nonresponse to the $VPAC_1$ receptor agonist [K15, R16, L27]VIP(1-7)/GRF(8-27) (100 nM); (B) a typical suppression response to the $VPAC_2$ receptor agonist Ro 25-1553 (100 nM); (C) attenuation of a vasoactive intestinal polypeptide (VIP)-induced response by the $VPAC_2$ receptor antagonist PG 99-465 (100 nM); (D) a typical activation response to the PAC_1 agonist maxadilan (100 nM); (E) no attenuation of the maxadilan-induced activation by PG 99-465 (100 nM); and (F) partial antagonism by PG 99-465 of a suppression evoked by pituitary adenylate cyclase activating peptide (PACAP) (100 nM). Bars show timing and duration of drug application; dark grey bars for VIP, light grey bar for [K15, R16, L27]VIP(1-7)/GRF(8-27), white bar for PACAP, striped bars for Ro 25-1553 and maxadilan and hatched bars for PG 99-465.

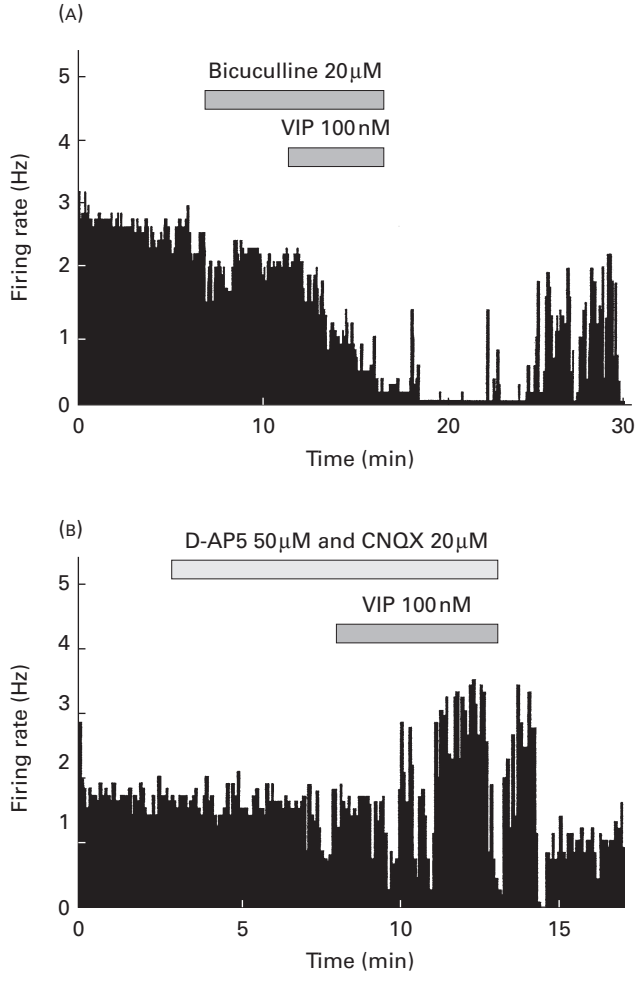

FIG. 4. Integrated firing rate histograms showing that VIP-evoked responses were not blocked by (A) the $GABA_A$ receptor antagonist bicuculline (20 μM) or (B) a cocktail of the ionotropic glutamate antagonists D-AP5 (50 μM) and CNQX (20 μM). Dark grey bars depict timing and duration of vasoactive intestinal polypeptide (VIP) application. Light grey bars indicate timing and duration of antagonist application.

equimolar concentration of VIP or maxadilan (Table 1). In a different sample of four PACAP-responsive cells, PG 99-465 (100 nM) partially blocked the magnitude and duration of PACAP-evoked suppressions (n = 3) (Fig. 3F), but had no effect on the activation (n = 1). These data confirm that PACAP acts via both $VPAC_2$ and PAC_1 receptors to alter SCN neuronal activity.

Effects of GABA and glutamate receptor antagonists on VIP-evoked responses

Since GABA and glutamate are thought to be the main inhibitory and excitatory neurotransmitters, respectively, in the SCN (3, 36), the responsiveness of SCN cells to VIP was examined in the presence of the $GABA_A$ receptor antagonist bicuculline, or a combination of the ionotropic glutamate receptor antagonists D-AP5 and CNQX. Bicuculline (20 μM) was applied to 23 cells for a 10 min period and 5 min into the treatment, VIP (100 nM) was introduced into the test solution. Responses to bicuculline alone were seen in five of these cells (two suppressed, three activated). Thirteen cells responded to VIP in the presence of bicuculline: 11 were suppressed, two were activated (Fig. 4A, Table 1). Responsiveness to bicuculline did not appear to influence the probability of a cell responding to VIP (three of five bicuculline-sensitive cells responded to VIP; 10/18 bicuculline-insensitive cells responded to VIP). In a separate group of VIP-suppressed neurones (n = 4), bicuculline pretreatment failed to block the direction or amplitude of VIP-evoked suppression in all cells, but did reduce the duration of the suppression in three of these cells (with bicuculline, duration = 4.7 ± 1.0 min; without bicuculline, duration = 12.5 ± 3.2 min).

A further 15 cells were pretreated for 5 min with a combination of D-AP5 (20 μM) and CNQX (50 μM) which were then coapplied with VIP for 5 min. Eight cells (53%) responded to VIP in the presence of D-AP5 and CNQX: six were suppressed and two were activated (Fig. 4B). Responses to VIP in the presence of CNQX/D-AP5 showed a similar magnitude and duration to that observed with VIP alone (Table 1).

Controls

To ensure that changing the perfusion tube between EBSS and drug-containing solutions did not evoke changes in cell firing frequency, control experiments were performed in which the tube was moved from the flask containing standard EBSS bathing solution to another flask, also containing EBSS. Of 36 cells where this procedure was performed, only four (11%) showed a significant response, confirming that changes in firing rate observed following drug application were likely due to the presence of pharmacological compounds.

Discussion

Vasoactive intestinal polypeptide tested at a concentration (100 nM) known to reset the rat SCN clock (9) was found to evoke predominantly suppressions in SCN cellular activity, some which were modulated by bicuculline, implicating the involvement of GABAergic mechanisms in these suppressions. Day–night differences in responses to VIP were found with an increased percentage of SCN cells responding during the night-time. In addition to suppressions, increases in electrical activity to VIP were seen at all day–night phases, suggesting that different receptors may mediate these suppressive and activational effects. To identify these receptors, $VPAC_1$, $VPAC_2$ and PAC_1 receptor-selective agonists were applied to SCN neurones. As with VIP, these peptides were used at a concentration of 100 nM, as both functional and binding studies indicate that maximal responses are produced by this concentration (25, 33, 35). The selective $VPAC_1$ receptor agonist [K15, R16, L27]VIP(1-7)/GRF(8-27) evoked responses in only approximately 5% of SCN neurones. Furthermore, in VIP-responsive SCN neurones, this agonist failed to evoke significant alterations in electrical activity. When considered together with *in situ* hybridization and radioreceptor data showing the absence of $VPAC_1$ mRNA

expression and $VPAC_1$ binding sites in the rat SCN (21, 37), our data strongly imply that the $VPAC_1$ receptor is either not present or expressed at levels too low to impact on the electrophysiological actions of VIP in the SCN.

In situ hybridization studies indicate a high level of expression of $VPAC_2$ receptor mRNA in the rat SCN (21–23), while the electrophysiological data of this study strongly implicate this receptor in the actions of VIP in the SCN. Ro 25-1553, a highly selective agonist of the $VPAC_2$ receptor (26), typically evoked suppression responses in SCN neurones with a similar duration and magnitude to those elicited by VIP in these cells. Furthermore, decreases in firing rate in response to VIP were almost completely blocked by pretreatment with the $VPAC_2$ receptor-selective antagonist, PG 99-465. These results raise the possibility that the $VPAC_2$ receptor mediates the VIP-induced suppressions in firing rate in rat SCN neurones. The predominance of suppressions to Ro 25-1553 indicates that stimulation of this receptor most likely reduces SCN neuronal activity. In other areas of the brain where the $VPAC_2$ receptor is expressed, such as the midbrain and preoptic area neurones, VIP also suppresses electrical activity (31).

Previous investigations have revealed the presence of ^{125}I-PACAP binding sites and PAC_1 receptor mRNA expression in the rat SCN (11, 24, 38). Consistent with these studies, we found that the selective PAC_1 receptor agonist, maxadilan, evoked changes in SCN cellular activity at nanomolar concentrations. The most frequent response to maxadilan was activation, although some suppressions were seen. These responses were not altered by the $VPAC_2$ receptor antagonist, PG 99-465, confirming the selectivity of maxadilan for the PAC_1 receptor. Although the possibility that some actions of maxadilan may involve transsynpatic mechanisms cannot be excluded, the predominance of activation responses to this PAC_1 receptor agonist indicates that PAC_1 receptor stimulation most likely leads to increased cellular activity in the rat SCN.

Pituitary adenylate cyclase activating polypeptide, which binds to both $VPAC_2$ and PAC_1 receptors, evoked suppressions (75% of responses) and activations from rat SCN cells. The suppressions were attenuated by PG 99-465, indicating that these suppressive actions of PACAP were mediated via the $VPAC_2$ receptor, while some of the activational effects of PACAP were mediated via the PAC_1 receptor. The idea that PAC_1 receptor activation evokes increases in neuronal firing rate is further supported by the finding that in areas where PAC_1 is the predominant receptor type, such as the paraventricular and supraoptic nuclei (39, 40), the main type of electrophysiological response to PACAP is excitation (41, 42).

The increased numbers of SCN cells responding to VIP during the night-time coincides with the phase of the circadian cycle at which VIP resets the rodent SCN circadian clock, both *in vivo* (32) and *in vitro* (9). The levels of VIP mRNA in the adult rodent SCN do not appear to show prominent changes across the circadian cycle (43), indicating that gating of the resetting actions of VIP presumably involves either altered expression of VIP receptors (i.e. $VPAC_2$ and PAC_1) and/or intracellular sensitivity to the second messenger pathways activated through stimulation of these receptors. Significant variation in the expression of mRNAs for $VPAC_2$ and PAC_1 in the rat SCN has been shown with high levels reported during the late night phases (23, 24). The absence of phase-advancing effects of PACAP compared to VIP or Ro 25-1553 during the late night (9) suggests that it is stimulation of the $VPAC_2$ receptor and its signal transduction pathway, rather than the PAC_1 receptor, that mediates the actions of VIP on SCN clock cells. Although the receptor *in situ* hybridization studies do not directly demonstrate when the levels of $VPAC_2$ and PAC_1 receptor proteins peak, it is possible that the phase-dependent responses of SCN cells to VIP are due to phase-specific availability of the two receptors in the SCN.

In this study, we found that VIP and PACAP evoked mainly suppressions, while maxadilan evoked mostly activations. Since the $VPAC_2$ receptor agonist Ro 25-1553 also evokes primarily suppressions, and because the $VPAC_2$ receptor antagonist PG 99-465 attenuated PACAP elicited suppressions, it is tempting to speculate that the $VPAC_2$ receptor mediates suppressions and the PAC_1 receptor mediates activations. However, maxadilan also elicited suppressions indicating that the receptor-based mechanisms underpinning these effects are complex. Consistent with this notion, a number of PAC_1 isoforms are expressed in the rat SCN (44) and it is unclear what role(s) these might play in rhythm processes. Furthermore, it is apparent that some of the effects of VIP are transsynpatic, and involve recruitment of GABAergic mechanisms since the $GABA_A$ receptor antagonist bicuculline modulated the duration of VIP suppressions. Clearly, further studies using intracellular and whole-cell patch recording techniques are required to fully characterize the cellular actions of VIP and PACAP and to differentiate the roles of the $VPAC_2$ and the PAC_1 receptors in the rodent SCN.

In conclusion, our data show that approximately 50% of rat SCN neurones respond to VIP, while 33% respond to PACAP. These actions are mediated by both $VPAC_2$ and PAC_1 receptors, which are known to be present in the SCN. Furthermore, it appears that some of the actions of VIP in the SCN are dependent on $GABA_A$ receptors but not ionotropic glutamate receptors. Additional studies using PG 99-465 blockade of $VPAC_2$ receptors on the phase-resetting actions of VIP and PACAP *in vivo* and *in vitro* will provide further evidence for the role of these peptides and their receptors in the entrainment of the SCN clock.

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