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Abstract The suprachiasmatic nucleus (SCN) in the hypothalamus serves as the pacemaker for mammalian circadian rhythms. In a hamster brain slice preparation, the authors were able to record spontaneous activity from SCN cells for up to 4 days in vitro and verify a self-sustained rhythm in firing. The phase of this rhythm was altered by the concentration of glucose in the bathing medium, with time of peak firing advanced for a 20 mM glucose condition and slightly delayed for a 5 mM glucose condition, relative to 10 mM. The advancing effect of 20 mM glucose and the delaying effect of 5 mM glucose were not maintained during a 2nd day in vitro after changing the bathing medium back to 10 mM glucose, thus indicating the effect was not a permanent phase shift of the underlying oscillation. In experiments recording from cell-attached membrane patches on acutely dissociated hamster SCN neurons, exchanging the bathing medium from high (20 mM) to zero glucose increased potassium (K^+)-selective channel activity. With inside-out membrane patches, the authors revealed the presence of a glybenclamide-sensitive K^+ channel (190 pS) and a larger conductance (260 pS) Ca^{2+}- dependent K^+ channel that were both reversibly inhibited by ATP at the cytoplasmic surface. Furthermore, 1 mM tetraethylammonium chloride was demonstrated to advance peak firing time in the brain slice in a similar manner to a high concentration of glucose (20 mM). The authors interpret the results to imply that SCNs are sensitive to glucose, most probably via ATP modulation of K^+ channel activity in these neurons. Tonic modulation of K^+ channel activity appears to alter output of the pacemaker but does not reset the phase.

Key words ATP, brain slice, ATP-sensitive K^+ channel, calcium-dependent K^+ channel, circadian, glucose, suprachiasmatic

INTRODUCTION

Mammalian biological rhythms with circadian periodicity are controlled by a pacemaker in the brain's hypothalamus. This pacemaker, the suprachiasmatic nucleus (SCN), is able to generate a self-sustained circadian oscillation and imposes circadian rhythmicity on other systems in the brain and body (for a review, see Harrington et al., 1994). Circadian rhythms are reset according to environmental cues to allow synchronization with an entraining cycle such as the light:dark cycle (Mistlberger and Rusak, 1994).

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The SCN expresses circadian or daily rhythmicity even when isolated in vitro (Gillette, 1991). This can be measured by several techniques including a daily variation in spontaneous firing rate of SCN neurons in a brain slice preparation with spontaneous firing usually maximal in the middle of the subjective day. Time of day is generally standardized to the animals' previous housing conditions by arbitrarily labeling zeitgeber time (ZT) 12 the time of lights off in the light:dark cycle. Average peak time of firing in rat SCN brain slices is reported to occur between ZT 5.0 (Rangarajan et al., 1994) and ZT 7.9 (Gillette, 1986), with most studies reporting average peak times between ZTs 6.0 and 7.0 (Prosser and Gillette, 1989; Shibata et al., 1992; Shirakawa and Moore, 1994; Starkey et al., 1995). In one study using golden hamsters, the peak time of firing was much earlier, occurring at ZT 0.15 (Cote and Harrington, 1993), whereas another study using golden hamsters reported peak times between ZTs 6.0 and 8.0, similar to those reported using rats (Mason, 1991). One difference between these experiments using hamsters was the concentration of glucose in the bathing media: 20 mM (Cote and Harrington, 1993) and 10 mM (Mason, 1991). Here, we present data that indicate that glucose concentration is a critical factor in determining time of peak firing in SCN slices.

Effects of glucose on neurons are well documented (Oomura et al., 1969, 1974). Glucose can induce excitation (Ashford et al., 1988), and its depletion can hyperpolarize neurons (Spuler et al., 1988). The discovery in cardiac muscle of ATP-sensitive potassium (ATP-K^+) channels that are inhibited by increasing cytoplasmic ATP (Noma, 1983) provided a link between the metabolic status of a cell and its membrane potential (Ashcroft, 1988). Similar channels have been found in mammalian brain (Ashford et al., 1988, 1990), and therefore we attempted to record any ion channel activity in SCN neurons that might be sensitive to varying extracellular glucose and to cytoplasmic ATP. After observing such channels in cell-attached and inside-out patch recordings from acutely dissociated SCN neurons, we then sought to determine the role of K^+ channels in the expression of the circadian rhythm. We found that a nonspecific K^+ channel blocker can advance the phase of the expressed circadian rhythm in a manner similar to the high glucose condition.

METHODS

Brain Slice Experiments

Male golden hamsters older than 1 month of age (Charles River Laboratories, Kingston, NY) were housed under a light:dark cycle of 14:10 h (250 μW or 20 lux). Brain slices were prepared between ZTs 0 and 10, with ZT 12 arbitrarily defined as the time of lights off in the animals' housing room. Thus, ZT 0 was 2 h into the light period. Each hamster was administered an overdose of halothane, the brain was quickly dissected, and coronal hypothalamic slices containing the SCN were placed into a gas:fluid interface chamber (Medical Systems Corp., Greenvale, NY). Tissue was maintained at the interface between artificial cerebrospinal fluid (ACSF) containing 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.8 $CaCl_2$, 1.0 $MgSO_4$, 24.8 $NaHCO_3$ (pH 7.4) with either 1, 5, 10, or 20 mM D-glucose (osmolarity balanced with sucrose) and warm humidified 95% O_2:5% CO_2. In some experiments, 1 mM tetraethylammonium chloride (TEA) was added to the ACSF (with 10 mM glucose). ACSF was supplemented with an antibiotic (gentamicin, 50 mg/l) and a fungicide (amphotericin, 2 mg/l) and was maintained at 34.5°C. ACSF osmolarity was measured using a vapor pressure osmometer (Wescor Inc., Logan, UT) to be 313-316 mosm in all conditions. In some preliminary experiments, the ACSF glucose was dropped from 20 to 10 mM, and this was not balanced by added sucrose; in these cases, ACSF osmolarity was 304 mosm. Extracellular single-unit activity was measured using glass micropipettes filled with 2 M NaCl advanced through the coronal slice with a hydraulic microdrive. The signal was amplified, filtered, and discriminated; firing rate was measured using a rate monitor and a computer. The electrode was visually placed into regions of the SCN using a random list of areas defined by a theoretical 3 × 3 grid, with tracks alternating between left and right SCNs. Each cell's spontaneous firing rate was measured for 1 min, and the average firing rate of the cell was plotted against the ZT of recording. Recordings from individual slices were generally conducted for 8 to 12 h per day. Data were analyzed using a 1 h running mean smoother with a 15 min lag, and the time of peak firing rate was calculated as the ZT at the middle of the 1 h bin of

Table 1. Solutions used in patch clamp experiments (millimolar)

	A	*B*	*C*	*D*	*E*	*F*
NaCl	135	—	135	—	135	—
KCl	5	140	5	140	5	140
$CaCl_2$	1	5	1	0.9	5	0.9
$MgCl_2$	1	5	1	1	5	1
HEPES	10	10	10	10	10	10
Glucose	—	—	20	—	—	—
Sucrose	20	—	—	—	—	—
EGTA	—	—	—	1	—	10

highest value. Recording of control slices in this study was interspersed throughout the experimental period.

Patch Clamp Experiments

SCN neurons were acutely dissociated from brain slices of 6- to 20-day-old hamster pups. Animals were overdosed with halothane anesthesia and were decapitated. Brains were quickly dissected in ice-cold Ca^{2+}/Mg^{2+}-free Hanks' solution (Sigma, St. Louis, MO), coronal hypothalamic slices containing the SCNs were prepared and placed in ACSF bubbled with 95% O_2:5% CO_2 at room temperature for 1 h. The SCNs were then carefully cut out with a razor blade in Ca^{2+}/Mg^{2+}-free Hanks' solution (Sigma) and were digested in ACSF containing 1.5 mg/ml protease (Type XIV from bacterial *Streptomyces griseus*, Sigma) at 37°C for 5 min. The tissue was then washed twice in protease-free HEPES-buffered minimum essential medium (MEM, Sigma), and cells were dissociated by gentle trituration in 200 to 300 µl of MEM with a fire-polished Pasteur pipette. The cell suspension was deposited in drops on cleaned glass coverslips (washed in methanol), and cells were allowed to settle for 10 min before flooding with HEPES-buffered MEM. Cells were maintained at room temperature under a 95% O_2:5% CO_2 atmosphere in a sealed chamber and remained viable for 4 to 5 h. Coverslips were transferred to a recording bath in which SCN neurons of 5 to 10 µm diameter (unipolar, bipolar, and multipolar) were identified by size and morphology (see Ito et al., 1991).

Neurons were superfused with a standard saline containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM sucrose, pH 7.4 with NaOH (Solution A in Table 1). Single-channel currents were recorded from cell-attached and isolated inside-out membrane patches using standard techniques (Hamill et al., 1981). Fire-polished borosilicate glass pipettes (World Precision Instruments), fabricated by a two-stage pull (PB-7 puller, Narishage USA, Greenvale, NY) and filled with a solution containing 140 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.4 with KOH (Solution B in Table 1), typically had resistances of 3 to 6 MΩ. To assess effects of glucose on cell-attached recordings, the 20 mM sucrose in the standard saline was replaced with equimolar glucose (Solution C in Table 1). In inside-out patch configuration, the bath solution was exchanged for the recording solution containing 140 mM KCl, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1 mM EGTA (free $[Ca^{2+}]$ of approximately 0.8 µM), 10 mM HEPES, pH 7.2 with KOH (Solution D in Table 1). In experiments investigating ion selectivity, either 135 mM KCl of the pipette solution was replaced with equimolar NaCl (Solution E in Table 1) or, alternatively, currents were recorded in normal standard saline (Solution A with 5 mM KCl).

In experiments assessing the effects of "intracellular" ATP, 3 mM Mg-ATP was added to the recording solution. The Mg^{2+} salt was used to avoid any further Ca^{2+} chelation by ATP. In experiments to reduce "free" $[Ca^{2+}]$ to less than 10 nM at the intracellular membrane surface, a recording solution including 10 mM EGTA was used (Solution F in Table 1). Glybenclamide (Sigma) was dissolved in DMSO and added to the bathing medium, giving a final concentration of 0.01% DMSO, which also was included in control solutions.

All currents were acquired using an Axoclamp 200A interfaced to a computer running pClamp 6.1 software (Axon Instruments, Foster City, CA). Data were filtered at 1 kHz with a low-pass, 8-pole Bessel filter, sampled at 4 kHz and analyzed using pClamp 6.1.

RESULTS

Effects of Glucose on Time of Peak Firing in Brain Slice Experiments

Neuronal firing activity could be recorded from brain slices for up to 4 days in vitro. Typical results are shown in Fig. 1A (Group A in Table 2), with the running mean calculated from an individual slice shown along with the raw data. Time of peak firing rate, as calculated by the time of the maximum of the running mean from individual slices, was advanced in the 20 mM glucose condition (Group B in Table 2) relative to the 10 mM glucose condition (Figs. 1 and 2). When peak times on all days were expressed as a time be-

Table 2. Summary of results across experimental groups for slices recorded on Day 2. Bathing solutions of slices on Days 1 and 2 in vitro are listed along with the average (± *SEM*) time of peak firing on Day 2 and the average firing rate (± *SEM*) of cells recorded between ZTs 24 and 36. Differences in times of peak between groups from ANOVA are noted; no groups were significantly different from Group A in mean firing rate.

Group	*Day 1*	*Day 2*	*Day 2 Time of Peak (zeitgeber time)*	*Day 2 Mean Firing Rate (hertz)*
A	10 mM glucose	10 mM glucose	6.41 ± 0.49	2.91 ± 0.12
B	20 mM glucose	20 mM glucose	3.64 ± 0.55*	2.24 ± 0.16
C	5 mM glucose	5 mM glucose	7.48 ± 0.42	3.13 ± 0.25
D	20 mM glucose	10 mM glucose	6.72 ± 0.28	3.16 ± 0.22
E	5 mM glucose	10 mM glucose	6.19 ± 0.30	3.71 ± 0.09
F	1 mM TEA/ 10 mM glucose	1 mM TEA/ 10 mM glucose	3.03 ± 0.15*	2.94 ± 0.17
G	1 mM TEA/ 10mM glucose	10 mM glucose	6.30 ± 0.45	2.76 ± 0.55

NOTE: ANOVA = analysis of variance; TEA = tetraethylammonium chloride.
*Significantly different from Group A, Kruskal-Wallis ANOVA, $p < .05$.

tween ZTs 0 and 24, peak times in 10 mM glucose ACSF slices averaged ZT 6.350.24 h ($n = 17$), whereas peak times in 20 mM glucose ACSF slices averaged ZT 4.260.29 h ($n = 21$) (see solid lines, Fig. 1B,C). This effect does not appear to be attributable to altered osmolarity given that slices bathed in 10 mM glucose ACSF with the addition of 10 mM sucrose showed a time of peak firing consistent with that of control 10 mM glucose ACSF slices recorded on Day 2 in vitro (10 mM glucose/10 mM sucrose: mean = 6.40, *SEM* = 0.18, $n = 3$; 10 mM glucose: mean = 6.41, *SEM* = 0.49, $n = 8$; Mann-Whitney, n.s.). Under both 10 and 20 mM glucose conditions, slices appeared to maintain a circadian rhythm with a similar period of approximately 24 h (see Fig. 2; differences across days not significant).

The effect of low-glucose bathing media was assessed using 1 mM glucose (balanced with 9 mM sucrose). We were unable to detect any action potentials on Day 2 under these conditions even though slices still were viable, as shown by changing the bathing media to 10 mM glucose ACSF and observing a return of spontaneous firing within 1.5 h ($n = 4$). A less drastic reduction in glucose (5 mM glucose balanced with 5 mM sucrose; Group C in Table 2) slightly delayed the time of peak firing on Day 2 relative to the 10 mM glucose ACSF control (5 mM glucose: mean = 7.48, *SEM* = 0.42, $n = 3$; 10 mM glucose: mean = 6.41, *SEM* = 0.49, $n = 8$; Mann-Whitney, $p < .05$).

Reversibility of Glucose Effect in the SCN Brain Slice

There are two possible interpretations of these results. One possibility is that 20 mM glucose ACSF induces a phase shift in the first cycle but not in subsequent cycles because if 20 mM glucose ACSF induced a circa 2 h phase shift every cycle, then a difference in circadian period would become apparent. A second possibility is that the 20 mM glucose condition alters the expression of the rhythm, the time of peak firing, without altering the underlying oscillation. These two possible interpretations can be distinguished by bathing the slice in 20 mM glucose for the 1st day in vitro but changing the bathing medium to 10 mM glucose on the 2nd day in vitro. If the underlying oscillation is actually phase-shifted by the treatment on Day 1, then the peak on Day 2 will not be at the time observed in control experiments. If, however, only the expression of the rhythm is altered on Day 1, then the slice will show a time of peak on Day 2 similar to that of controls. The experimental results support the second hypothesis (see dashed line in Fig. 1B). Experimental slices bathed in 20 mM glucose ACSF on Day 1 and 10 mM glucose ACSF on Day 2 (Group D in Table 2) showed a time of peak on Day 2 indistinguishable from that of controls (experimental: mean = 6.72, *SEM* = 0.28, $n = 3$; 10 mM glucose: mean = 6.41, *SEM* = 0.49, $n = 8$; Mann-Whitney, n.s.) and different from that of slices bathed in 20 mM glucose ACSF throughout (experimental: mean = 6.72, *SEM* = 0.28, $n = 3$; 20 mM glucose: mean = 3.64, *SEM* = 0.55, $n = 8$; Mann-Whitney, $p < .05$). Similarly, bathing slices in 5 mM glucose ACSF on Day 1 and 10 mM glucose ACSF on Day 2 (Group E in Table 2) produced a time of peak firing on Day 2 indistinguishable from that of controls bathed in 10 mM glucose throughout (experimental: mean = 6.14, *SEM* = 0.31, $n = 2$; 10 mM glucose: mean = 6.41, *SEM* = 0.49, $n = 8$; Mann-Whitney, n.s.).

Effects of Glucose on Cell-Attached Recordings from Dissociated SCN Neurons

To investigate a potential underlying mechanism for the glucose effect on peak firing rate in the slice preparation, we conducted cell-attached patch clamp experiments on isolated SCN neurons to establish whether they demonstrated ion channel activity that was sensitive to varying levels of extracellular glucose. Cell-attached patches were held at 0 mV, and

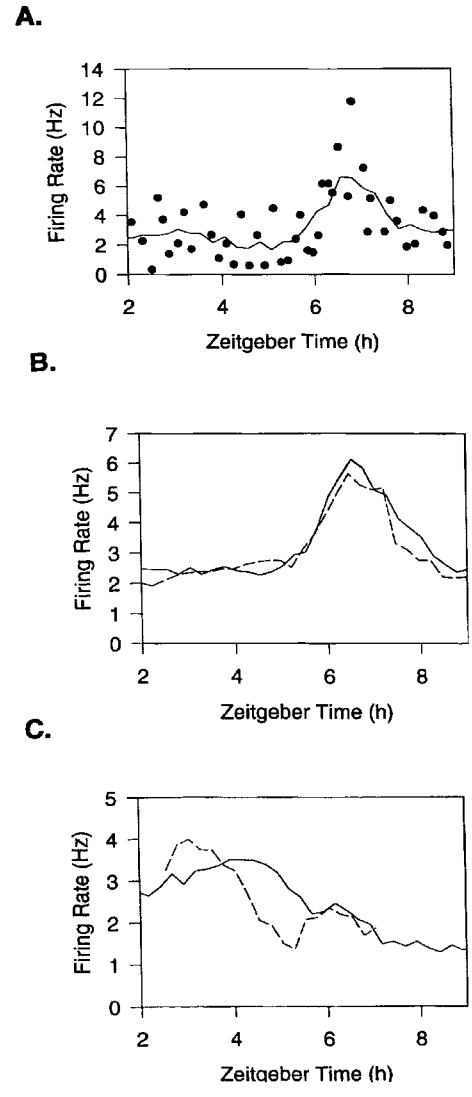

Figure 1. (A) Mean firing rates of individual suprachiasmatic nuclei neurons (closed circles) recorded during the 2nd day in vitro at specific times of day with zeitgeber time (ZT) 12 as the projected time of lights off in the animal's previous housing condition. Running mean (1 h bins, 15 min lag) smoother is shown as a solid line. The maximum of the smoother is taken as the time of peak firing for that slice. Data shown are from one suprachiasmatic nucleus brain slice bathed in artificial cerebrospinal fluid (ACSF) containing 10 mM glucose. (B) Running mean smoother calculated from pooled data collected from slices bathed in 10 mM glucose ACSF (solid line; Group A in Table 2) and from slices bathed in 20 mM glucose during Day 1 in vitro (ZTs 0-24) and 10 mM glucose ACSF on Day 2 in vitro (dashed line; Group D in Table 2). In both cases, cells were recorded during Day 2 in vitro to assess the rhythm in firing rate. The *x* axis shows ZT with ZT 12 being the projected time of lights off on Day 2 in vitro. (C) Running mean smoother calculated from pooled data collected from slices bathed in 20 mM glucose ACSF (solid line; Group B in Table 2) and from slices bathed in 1 mM tetraethylammonium chloride ACSF (dashed line; Group F in Table 2). Recordings were conducted on the 2nd day in vitro.

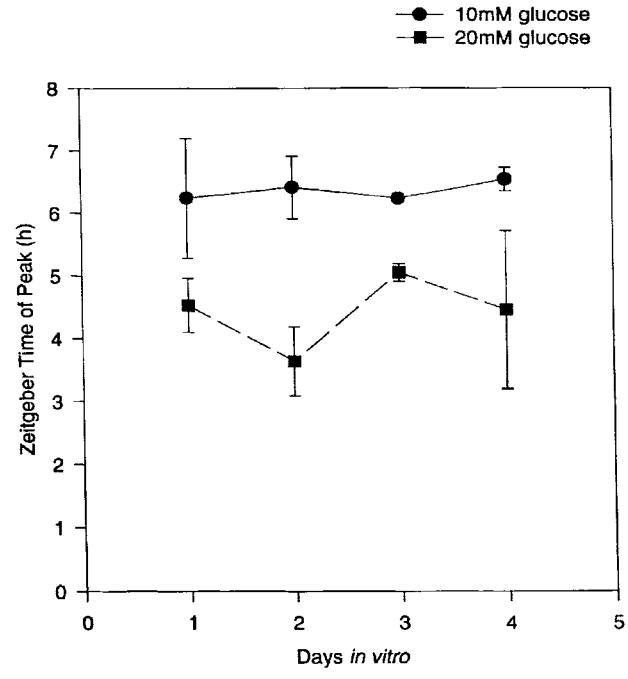

Figure 2. Mean (± *SEM*) zeitgeber times of peak firing rates as calculated by the running mean smoother of individual slices recorded in 10 mM glucose artificial cerebrospinal fluid (ACSF) (circles) or 20 mM glucose ACSF (squares) over 4 days in vitro. ZTs on Days 2 to 4 in vitro were expressed on a scale of 0 to 24 h. The number of individual slices in each group is as follows: 2, 8, 5, and 2 for Days 1, 2, 3, and 4, respectively (10 mM glucose), and 8, 8, 3, and 2 for Days 1, 2, 3, and 4, respectively (20 mM glucose). There was no difference in time of peak across days of recording for either condition (Kruskal-Wallis one-way analysis of variance, n.s.).

inward channel currents (single-channel amplitudes of 1-7 pA) were always observed (n = 23). At this potential with a solution containing 140 mM KCl in the pipette (Solution B), the resting membrane potential largely provided the driving force for K^+ out of the pipette, assuming circa 140 mM intracellular [K^+]. When the bathing saline included 20 mM glucose (Solution C), the activity of the majority of larger conductance (3-7 pA, n = 14) channels was minimal (low probability of opening, P_{open}) compared to recording in 0 mM glucose (Solution A, Fig. 3A, n = 10 of 14 neurons). This effect was fully reversible and was not dependent on the order of initial exposure to either glucose or sucrose media (Fig. 3B-D). Interestingly, in a few recordings where single-channel current amplitudes were very clearly measurable (n = 4), the open event amplitude was larger in the 0 mM glucose condition than in the 20 mM glucose condition (Fig. 3B,C). This indicated that the neurons were more hyperpolarized in 0 mM glucose than in 20 mM glucose, as there evidently was an increased driving force for flow

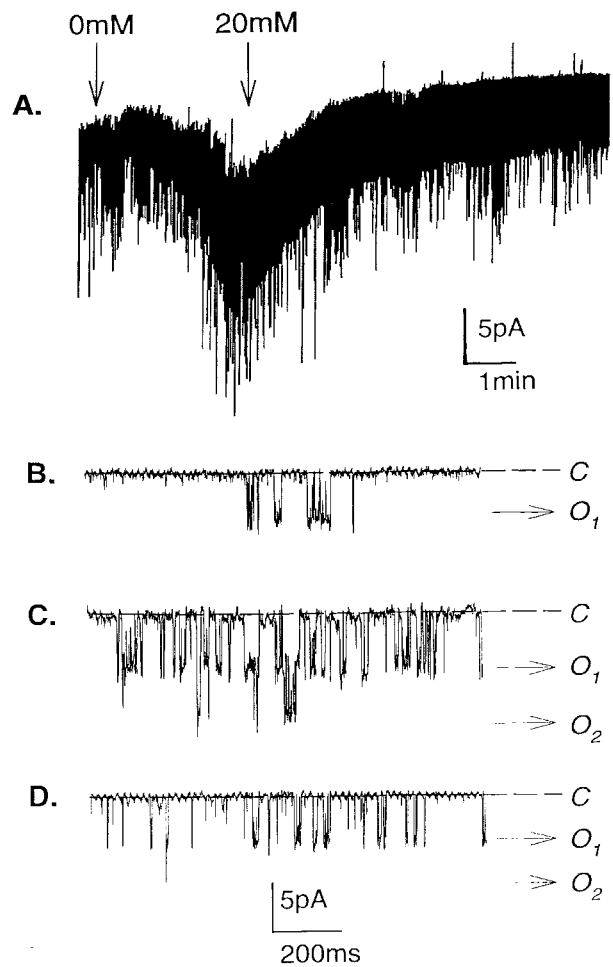

Figure 3. Activation of inward ion channel currents by glucose depletion in cell-attached recordings from acutely dissociated suprachiasmatic nucleus neurons. (A) In this cell-attached patch with the recording pipette containing 140 mM KCl (Solution B in Table 1), inward channel currents were observed when the membrane patch was held at 0 mV (electrical driving force provided by the membrane potential). Initially, the neuron was bathed in standard saline containing 20 mM glucose (Solution C in Table 1). At the first arrow, this solution was exchanged for saline containing 0 mM glucose/20 mM sucrose (Solution A in Table 1). This provoked a large increase in channel activity that diminished after subsequent return to 20 mM glucose solution (second arrow). For the same recording, the increased P_{open} is illustrated in the 1 sec traces with the cell bathed in 20 mM glucose (B), in 0 mM glucose (C), and again in 20 mM glucose (D). Note that the single-channel current amplitudes were greater in 0 mM glucose due to neuronal hyperpolarization providing an increased driving force. C = closed states; O_{1-x} = successive open channel states. Lower scale bar refers to the B, C, and D traces.

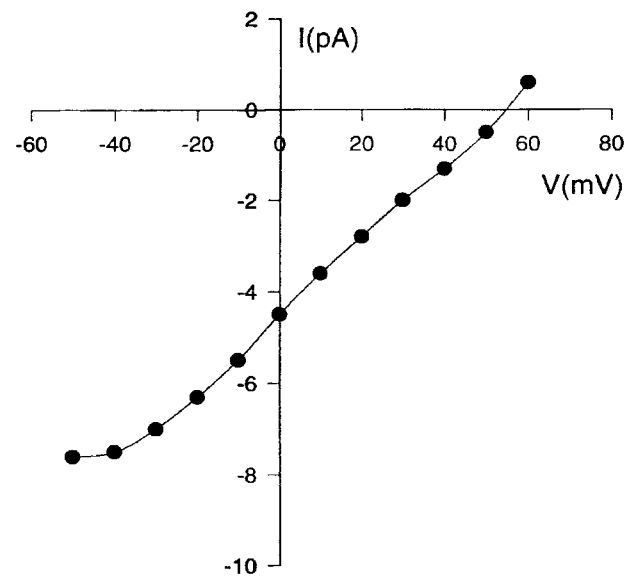

Figure 4. Single-channel current amplitude-voltage relationship for ion channels activated in the absence of glucose. In this example, a cell-attached patch was held at varying potentials with 140 mM KCl in the pipette (Solution B in Table 1), and the neuron was bathed in 0 mM glucose standard saline (Solution A in Table 1). Single-channel current amplitudes were plotted against holding potential. Note that the current reversed at circa +55 mV and became outward at more depolarized potentials. Assuming an approximately similar [K^+] intracellularly as in the recording pipette (140 mM), this relationship suggests that the channels were K^+ selective in a neuron with a resting membrane potential of circa –55 mV.

of K^+ into the cell. Figure 4 illustrates an example of the single-channel current amplitude-holding potential relationship for a channel whose P_{open} value was substantially increased in the absence of extracellular glucose. The current reversed polarity between +50 and +60 mV, which was typical of the glucose-sensitive channels (52 ± 6 mV, $n = 3$). Because we were recording with an estimated symmetrical K^+ gradient, this relationship suggested that these channels were K^+-selective in neurons with resting potentials between –50 and –60 mV. The K^+-selective nature of the glucose-sensitive channels was confirmed in recording in the inside-out patch configuration.

ATP-K^+ Channels Recorded in Inside-Out Patches from SCN Neurons

Inside-out membrane patches were readily excised from acutely dissociated SCN neurons, and large-conductance channels (150-300 pS) were repeatedly observed ($n = 30$) in symmetrical K^+ conditions (Solution B: pipette; Solution D: bath). The most fre-

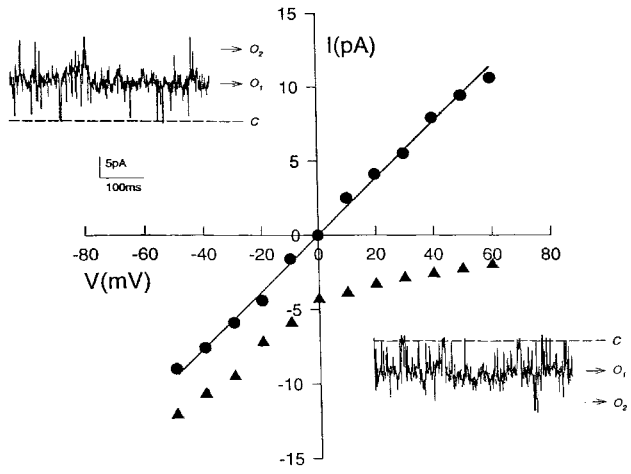

Figure 5. Single-channel current amplitude-membrane potential relationship for ATP/glybenclamide-sensitive channel activity recorded in an inside-out membrane patch. Closed circles indicate the current-voltage relationship in symmetrical K^+ conditions (140 mM K^+) (Solution D in Table 1: bath; Solution B in Table 1: pipette). The currents reversed at 0 mV with a slope conductance of circa 190 pS. The top inset illustrates channel activity recorded at +60 mV, and the lower inset illustrates channel activity recorded at –40 mV (same scale bar for both). Note that there was no voltage dependence to the P_{open} values for this type of channel. Closed triangles describe the channel current amplitude-voltage relationship when this inside-out patch activity was recorded with normal pipette solution (Solution B with 140 mM KCl) and standard saline in the bath (Solution A with 5 mM KCl). Note the shift in the relationship, whereby the reversal potential tended toward very depolarized potentials, as expected for K^+-selective channel activity. C = closed states; O_{1-x} = successive open channel states.

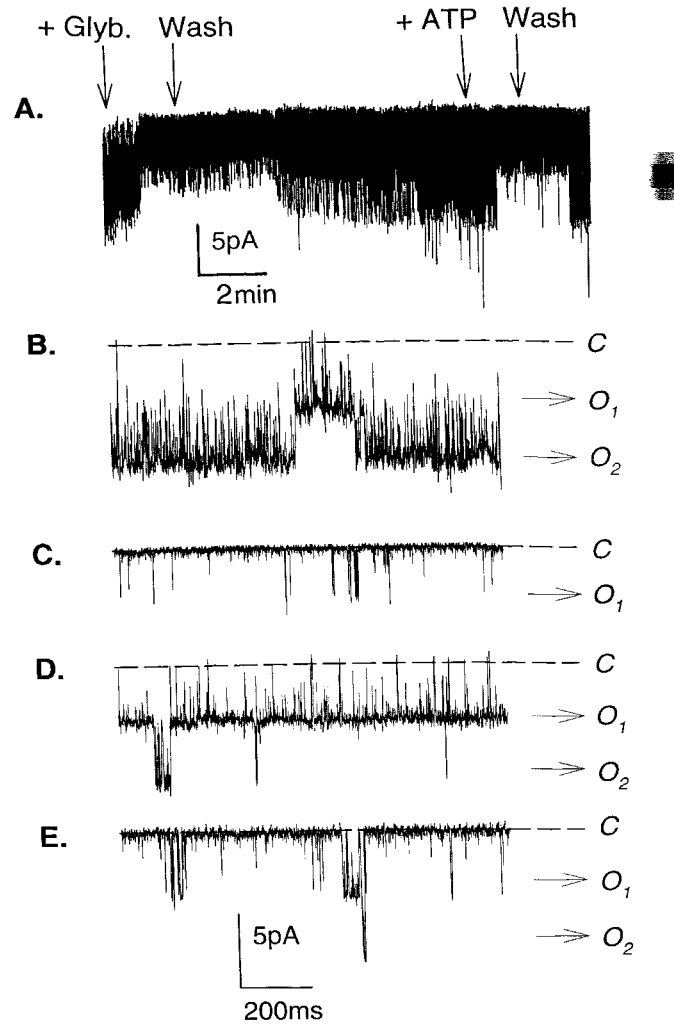

Figure 6. The presence of an ATP-sensitive K^+ channel in an inside-out patch recording from a suprachiasmatic nucleus neuron. (A) In this example, an inside-out patch was excised with a pipette containing standard pipette solution (Solution B in Table 1 with 140 mM KCl) and standard saline (Solution A in Table 1) in the bath and was held at 0 mV. The inward channel currents observed were reversibly blocked by both bath application of 1 μM glybenclamide (onset indicated by arrow) and also by 3 mM ATP. In expanded view, the P_{open} in control saline (B) was clearly reduced by exposure to 1 μM glybenclamide (C). After glybenclamide was washed out (D), the currents subsequently were blocked by ATP (E), also acting at the "intracellular" membrane surface. C = closed states; O_{1-x} = successive open channel states.

quently recorded channel activity (n = 10) had a slope conductance of 176 ± 11 pS (n = 7) with currents reversing at circa 0 mV, close to the expected K^+ equilibrium potential (see Fig. 5). Increased K^+ conductance and neuronal hyperpolarization through decreased extracellular glucose has been demonstrated to result from the relief of inhibition of ATP-K^+ channels by a depletion of intracellular ATP (see Ashford et al., 1990; Spuler et al., 1988). Therefore, we exposed inside-out membrane patches to bath-applied ATP (3 mM, Mg^{2+} salt) and to the sulphonylurea, glybenclamide (1 μM), a known potent blocker of ATP-K^+ channels. Both ATP (n = 10) and glybenclamide (n = 6) reversibly inhibited the predominant channel activity in these inside-out patch recordings (Fig. 6). P_{open} values were reduced by 59 ± 9% for 3 mM ATP and by 68 ± 11% for 1 μM glybenclamide. The K^+ selectivity of this activity was confirmed by recording in an asymmetrical K^+ gradient with standard pipette solution (Solution B) and standard saline in the bath (Solution A, containing 5 mM KCl, in Fig. 5). The ATP/glybenclamide-sensitive activity was voltage independent because

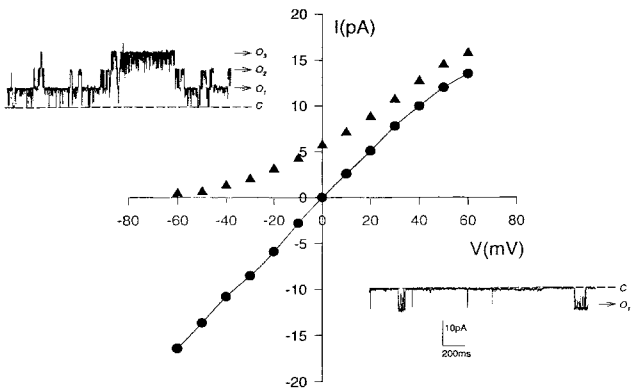

Figure 7. An example of the single-channel current amplitude-voltage relationship for a large conductance, voltage-dependent K^+ channel in an excised inside-out patch from a suprachiasmatic nucleus neuron. Closed circles represent current amplitudes recorded in symmetrical K^+ conditions (140 mM) (Solution D in Table 1: bath; Solution B in Table 1: pipette; $n = 5$). In an asymmetric K^+ gradient (Solution D in Table 1: bath; Solution E in Table 1: pipette), the current-voltage relationship (closed triangles) and the reversal potential were shifted to more negative potentials ($n = 3$). Insets at the top left and bottom right show the voltage dependence of the activity recorded in symmetrical K^+ at +30 and –30 mV membrane potentials, respectively (scale bar applies to both). P_{open} values for this recording were 0.55 and 0.02 at the +30 and –30 mV potentials, respectively (assuming there are three active channels in the patch). C = closed states; O_{1-x} = successive open channel states.

P_{open} values did not vary significantly over a broad range (–60 to +60 mV) of membrane potentials (Fig. 5, $n = 7$).

A Ca^{2+}-Dependent Channel Also Inhibited by Intracellular ATP

Inside-out patch experiments revealed another channel type that also was sensitive to exposure to ATP at the cytoplasmic surface. The activity of this channel was voltage dependent ($n = 6$) with substantially higher P_{open} values at depolarized potentials (see insets in Fig. 7). It had a slope conductance of 244 ± 14 pS ($n = 3$) when recorded in a symmetrical K^+ gradient (Solution B: pipette; Solution D: bath). If the pipette solution was changed to give an asymmetric K^+ gradient (Solution E: pipette), then the reversal potentials of these voltage-dependent currents were shifted to more negative potentials (< –60 mV), also indicative of K^+ selectivity. When ATP (3 mM) was added to the

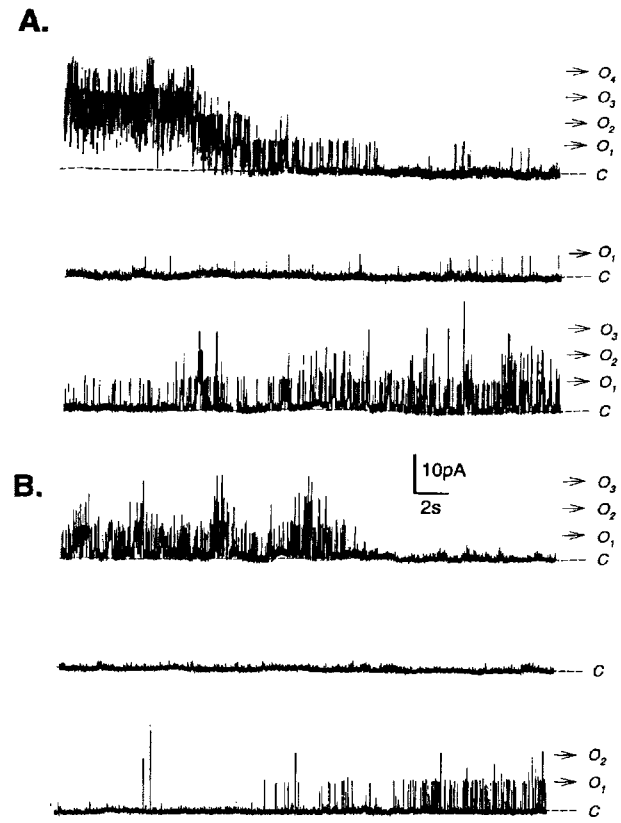

Figure 8. A Ca^{2+}-dependent K^+ channel also sensitive to "cytoplasmic" ATP. Channel activity was recorded from an inside-out patch held at a membrane potential of +30 mV in symmetrical K^+ conditions (140 mM) (Solution D in Table 1: bath; Solution B in Table 1: pipette). (A) These traces demonstrate inhibition by 3 mM ATP of this activity. ATP was introduced 10 sec before the start of the top trace. The middle trace was recorded in the continued presence of ATP. The lower trace began after 45 sec washout with control recording solution and shows recovery in activity. (B) These traces illustrate the Ca^{2+} dependence of the activity recorded from the same inside-out patch. A solution containing a high EGTA concentration (10mM, Solution F in Table 1) was introduced at the beginning of the top trace. In the continual presence of high EGTA (middle trace), activity was completely abolished. Channel activity returned after washout for 1 min in control recording solution (1 mM EGTA, Solution D). This channel activity also demonstrated marked voltage-dependence and was more active at depolarized potentials (not shown). C = closed states; O_{1-x} = successive open channel states.

recording solution, after a brief delay (5-30 sec), this voltage-dependent activity was largely inhibited (83 ± 6% reduction in P_{open}, $n = 4$, Fig. 8A). The inhibition was reversible as activity recovered after washout of the

ATP. Furthermore, to establish whether these currents were Ca^{2+}-dependent, the EGTA concentration was raised to 10 mM in the recording solution (Solution F), thereby lowering the "free $[Ca^{2+}]$" to less than 10 nM. This reduction in intracellular free $[Ca^{2+}]$ abolished the activity (n = 4), and again currents recovered after washout in low (1 mM) EGTA solution (Fig. 8B). Bath-applied glybenclamide (1 μM) had no effect on the Ca^{2+}-dependent K^+ channel activity (n = 2, not shown).

Neurons of the SCN, therefore, possessed two distinct large conductance K^+-selective channel activities that were both sensitive to intracellular ATP: (1) voltage-independent, 190 pS, glybenclamide-sensitive (ATP-K^+) and (2) voltage dependent, 260 pS, and Ca^{2+}-dependent. Other channel types were present in some of recordings, notably a 150 pS, Ca^{2+}-dependent K^+ channel that was not inhibited by intracellular ATP (n = 2) or glybenclamide (1 μM, n = 1) and a smaller conductance (40 pS) channel that also was Ca^{2+}-dependent (ATP not tested, n = 1).

Altered Time of Peak Firing in the SCN Brain Slice by a K^+ Channel Blocker

To test the hypothesis that inhibition of a K^+ channel might account for the altered peak time observed in high-glucose ACSF, we bathed slices in ACSF with 1 mM TEA, a K^+ channel blocker. Slices bathed in 1 mM TEA ACSF (10 mM glucose; Group F in Table 2) showed a peak time earlier than that of control slices, similar to times resulting from high-glucose ACSF (1 mM TEA: mean = 3.03, *SEM* = 0.15, n = 3; 10 mM glucose: mean = 6.41, *SEM* = 0.49, n = 8; Mann-Whitney, $p < .05$). This apparent phase shift in the time of peak firing in response to 1 mM TEA on Day 1 was not maintained when the slice was bathed in 10 mM glucose ACSF without TEA on Day 2 (Group G in Table 2) (1mM TEA Day 1 only: mean = 6.3, *SEM* = 0.45, n = 3; no TEA exposure: mean = 6.41, *SEM* = 0.49, n = 8; Mann-Whitney, n.s.). Figure 1C summarizes these results.

Effects of Glucose Not Reliably Correlated with Changes in Mean Firing Rate

These effects on time of peak firing appear to be independent of a general effect on level of firing. The firing rate of cells in slices bathed in the various experimental ACSF solutions did not differ from that of cells in slices bathed in 10 mM glucose ACSF throughout the experiment (Kruskal-Wallis analysis of variance; see Table 2). We conducted preliminary experiments testing SCN cells for alterations in spontaneous firing rate in response to altered glucose levels (0-20 mM) in the bath of a submersion-type brain slice chamber. Most cells showed little response to an 8 to 10 min application of ACSF with reduced glucose (34 cells tested).

DISCUSSION

The brain slice results demonstrate that the time of hamster SCN peak firing rate varies with glucose concentration. The change in time of peak firing does not appear to be attributable to a permanent phase shift of the circadian clock or a change in circadian clock period. Slices bathed in 20 mM glucose ACSF on Day 1 in vitro showed an advanced time of peak firing. When bathing medium was changed back to 10 mM glucose ACSF on Day 2, peak firing reverted to the time observed in control 10 mM glucose slice recordings. The effects reported here appear to be due to altered glucose concentration and not to altered osmolarity, because addition of 10 mM sucrose to 10 mM glucose ACSF did not produce similar effects to 20 mM glucose ACSF.

Glucose effects could potentially be mediated via elevated intracellular ATP concentration and subsequent inhibition of ATP-sensitive K^+ channels as described in other brain areas (Ashcroft, 1988; Ashford et al., 1988, 1990; Fellows et al., 1993). We conducted patch clamp experiments on acutely dissociated SCN neurons from neonatal hamsters to establish whether any channel activity recorded from cell-attached membrane patches was sensitive to varying extracellular glucose and whether inside-out membrane patch activity was indeed sensitive to intracellular ATP. In cell-attached recordings, some large conductance channels (3-7 pA at 0 mV, approximately symmetrical $[K^+]$) were affected by varying extracellular glucose. In zero glucose, this activity was maximal and channel current amplitudes were greater due to a hyperpolarization of the recorded neuron. In high (20 mM) glucose, the neuron was relatively depolarized and the P_{open} for the channels was diminished (see Fig. 3). Current amplitude relationships for cell-attached patches suggested that the glucose-affected channels were K^+ selective and that resting membrane potentials for SCN neurons were circa –55 mV (see Fig. 4).

Two types of K^+ channels were observed in SCN neurons, both of which were inhibited by intracellular

ATP. From inside-out recordings, the glucose-sensitive activity was demonstrated to result predominantly from classical ATP-K^+ channels that were reversibly inhibited by intracellular ATP and the sulphonylurea, glybenclamide. In symmetrical 140 mM KCl conditions, the voltage-independent ATP-K^+ channels recorded in the SCN had a slope conductance of 176 ± 11 pS. This value is significantly larger than the 150 pS ATP-K^+ channel activity recorded from rat ventromedial hypothalamic neurons (Ashford et al., 1990), possibly due to species/location-specific differences. However, the percentage reduction of the ATP-K^+ P_{open} values by 3 mM ATP were similar for both studies: 68 ± 11% for SCN, 57 ± 6.7% for rat ventromedial hypothalamic neurons (Ashford et al., 1990).

Another ATP-sensitive channel type was identified in these neurons, as there also was a substantial and reversible reduction in the activity of a larger conductance (250 pS at +60 mV) voltage-dependent K^+-selective channel current (see Fig. 7) when 3 mM Mg-ATP was applied to the intracellular membrane surface (see Fig. 8). The magnesium salt of ATP was used to prevent any further Ca^{2+} chelation by the ATP (Klöckner and Isenberg, 1992). These voltage-dependent currents were shown to be Ca^{2+} dependent as they were reversibly inhibited by reducing internal calcium to less than 10 nM. A similar Ca^{2+}-dependent and ATP-sensitive, large conductance (250 pS) K^+ channel has been reported in neurons from rat ventromedial hypothalamic neurons (Treherne and Ashford, 1991). In the ventromedial hypothalamus, this channel was observed only occasionally (< 5% of recordings), whereas it was recorded more frequently in hamster SCN (in 6 of 30 active patches). The inhibition of the SCN channel by 3 mM ATP (83 ± 6%) was more pronounced than that achieved by the same concentration of ATP in rat ventromedial hypothalamic recordings (51 ± 11%) (Treherne and Ashford, 1991). A large conductance Ca^{2+}-dependent K^+ channel recorded from an insulin-secreting cell line was only inhibited by changes in ATP levels resulting from exposure to high (10 to 20 mM) extracellular glucose concentrations (Ribalet et al., 1988). This may be similar to the channel observed in the SCN where peak firing time was altered by increasing glucose concentrations within this range. In vivo brain glucose concentration has been estimated at approximately 3 mM glucose in cerebrospinal fluid (Reid et al., 1988) and approximately 0.5 mM glucose in extracellular fluids (Fellows et al., 1992). Our study suggests that applications of 10 to 20 mM glucose, although standard for brain slice studies (Reid et al., 1988), may constitute a considerable increase in glucose availability, thus partially (or completely) inhibiting any K^+ channels that are sensitive to intracellular ATP.

The inhibition of K^+ channels might account for the altered peak time observed in high glucose ACSF. When we bathed slices in ACSF with 1 mM TEA, a K^+ channel blocker, slices showed a peak time earlier than that of control slices, similar to results using high-glucose ACSF. The apparent phase shift in the time of peak firing was not maintained when the slice was bathed in ACSF without TEA on Day 2, indicating that the effect of TEA also was on the output of the clock, not the underlying oscillation. Thus, effects of TEA were similar to those from experiments using 20 mM glucose ACSF.

Neurons of the SCN show a circadian rhythm in glucose use that is maintained in vitro, with peak levels in the mid-subjective day (Newman et al., 1992). If ATP were responsible for inhibiting a K^+ channel in SCN neurons, then it might be expected that peak firing time would coincide with a high level of free intracellular ATP. One study indicates that levels of ATP in the SCN also show a circadian rhythm, with peak levels in the late subjective night out of phase with the subjective day peak in glucose use and peak firing (Yamazaki et al., 1994). It is possible that, in vivo, at times of peak firing when ATP levels are low, levels of glucose could limit the cell's activity.

An alternative mechanism by which glucose might modulate the time of peak firing in SCN slices is by effects on excitatory local circuit activity. In one study, reduction of glucose in the medium bathing rat SCN brain slices was shown to reduce both spontaneous and evoked excitatory postsynaptic potentials while not changing the resting membrane potential (Shoji et al., 1992). However, time of peak firing in rat SCN brain slices apparently is not affected by glucose levels. Studies using 19 to 25 mM glucose have reported peak times varying across the full range (ZTs 5.0-8.0 [e.g., Gillette, 1986; Rangarajan et al., 1994]), and one study using 10 mM glucose did not report an unusual time of peak firing (ZT 6.5 [Shirakawa and Moore, 1994]). Thus, it appears that rats may be insensitive to the effects of glucose described here for the hamster, although this has not been tested directly.

Our results indicate that the time of peak firing of SCN neurons is responsive to changes in the bathing solution. Our findings do not alter the interpretation of previous studies of phase shifts of the SCN firing rate rhythm (e.g., Cote and Harrington, 1993; Gillette,

1991; Prosser and Gillette, 1989; Shirakawa and Moore, 1994; Starkey et al., 1995) because in these studies bathing solutions were kept constant, with the exception of a brief drug application on Day 1 in vitro. In such experiments, there is no reason to assume that the measured Day 2 phase shifts to brief Day 1 drug applications are not reflecting phase shifts of the underlying pacemaker. Thus, although we report apparent phase shifts that are only alterations in output, these are responses to tonic levels of bath constituents.

The firing rate rhythm is simply an output of the circadian clock and is no more a direct measure of pacemaker phase than are other observed rhythms (e.g., locomotor activity rhythms). Phase shifts of locomotor activity rhythms often take several days to express themselves fully, with partial phase shifts on intervening days labeled "transients." The results of our study do not apply to the question of transients because there is no evidence of a true phase shift. Whether the SCN firing rate rhythm also expresses transients while phase shifting to neurochemicals may be best explored by a brief drug application on Day 1 in vitro, followed by recording Days 3 and 4 as well as the commonly recorded Day 2.

The effects observed here do not appear to be due to "masking," in which circadian output is altered in direct response to a stimulus (Mistlberger and Rusak, 1994), because a constant level of a masking stimulus would simply alter the overall level of output (e.g., when locomotor activity is suppressed by light). It remains possible that the various glucose or TEA solutions may have actually shifted the phase of the underlying oscillator and that the transitions back to control solutions then shifted the clock back to its original phase. A more likely scenario is that the bathing solution can affect membrane properties and thereby alter the time at which the cell reaches conditions required for peak firing.

It may be helpful to think of the membrane as remaining coupled to the dominant oscillator, but altered levels of K^+ channel activation may alter the phase angle of entrainment of the membrane to the nuclear/cytoplasmic oscillator. Alternatively, the cell membrane may contain a slave oscillator, which can become uncoupled from the dominant oscillator in response to bathing solutions that alter levels of K^+ channel activation. If this explanation is valid, then the membrane oscillator can generate a rhythmic output for at least four cycles in vitro; however, when released from the altered bath conditions after 1 day in vitro, the slave reverts to the phase of the dominant oscillator presumably located in the nucleus and/or cytoplasm. The idea that the membrane is not an integral component of the intracellular oscillator is supported by our results showing that blockade of most K^+ channels for 1 day does not alter the period of the dominant pacemaker as well as results from others showing that long-term blockade of sodium channels (Schwartz et al., 1987) and calcium channels (Prosser et al., 1992) do not alter the period of the rodent circadian clock. A major challenge is to discover how SCN neurons are able to maintain synchronization with other SCN neurons under conditions of drastically altered membrane properties. Synchronization of SCN neurons with external stimuli probably does not arise from the sole signal of hyperpolarization or depolarization of the membrane. This already has been suggested in the rat SCN brain slice, where both K^+ channel activation and increased cAMP activity are required for the phase-shifting effect of serotonin (Prosser et al., 1994).

Drastic reduction of bath glucose to 1 mM completely suppressed SCN firing in hamster brain slices. Thus, output from the SCN could be reduced or eliminated in cases of major glucose depletion. This might account for changes in hormonal rhythms and reproductive function following nutritional constraints (Wade and Schneider, 1992).

A small body of work indicates a role of the SCN in glucose homeostasis through the sympathetic nervous system (reviewed in Nagai et al., 1994). The SCN is thought to regulate the pineal gland via the superior cervical ganglia (Illnerova, 1991), and photic effects on activity of the vagal and splanchnic nerves are dependent on the SCN (Niijima et al., 1992, 1993). These studies may point to a role of SCN neurons as regulators of the sympathetic nervous system in addition to functioning as the circadian pacemaker. Glucose may act as a modulatory or feedback signal to SCN regulation of the sympathetic nervous system as well as modulating the output of the circadian clock.

Although the implications of the diverse functional roles of SCN cells have yet to be elucidated, our results indicate a new direction for further study. The output of the hamster circadian clock, as expressed via the rhythm in firing rate in vitro, responds to glucose in a novel way. The clock is not reset, but the phase of the

output is altered. Determining the functional significance of these responses will be a future challenge.

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