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Ouabain (or salt solution lacking potassium) mimics the effects of dark pulses on the circadian pacemaker in cultured chick pineal cells

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Chick pineal cells in static culture display a persistent, photosensitive circadian rhythm of melatonin production and release. Pulses of white light or darkness, in otherwise constant red light, induce phase shifts in subsequent cycles whose magnitude and direction depend on the phase at which the pulse is given. Such 'phase-dependent phase shifts' are mediated by effects on the underlying pacemaker. Here, we describe the effects of ouabain, a specific inhibitor of Na,K-ATPase, and of salt solutions lacking potassium (SS–K), which also inhibit the pump, on the melatonin rhythm. Pulses of ouabain, or of SS–K, induced phase advances and phase delays that were phase and concentration-dependent. The relationship between time of treatment and effect on the subsequent phase of the rhythm (the phase–response curve) for these treatments was essentially the same as that for pulses of darkness.

INTRODUCTION

Circadian rhythms are endogenously generated but can be exogenously regulated[16]. At their 'heart' is a clock-like pacemaker, *from* which there is at least one pathway driving an overt rhythmic function, and *to* which there is at least one pathway conveying environmental information from a photoreceptor[5,20]. Under appropriate conditions, environmental lighting can alter the period and phase of the pacemaker; light cycles can *entrain* circadian rhythms[1].

The overt hormonal and biochemical rhythm that has been studied most intensively is that of melatonin production[2,12]. In the rat (and other mammals), the components of this circadian system are anatomically distinct[13]; the photoreceptors are in the retina, the pacemaker is in the suprachiasmatic nucleus of the hypothalamus, and melatonin is made in the pineal gland. Multisynaptic pathways connect these components. In vitro, the rat pineal is neither rhythmic nor photosensitive, but can be stimulated to make melatonin[12,23]. Addition of ouabain or high K to the medium inhibits the stimulated production of melatonin by the rat pineal[8,15].

In contrast to the rat pineal, the chick pineal in vitro is both rhythmic and photosensitive. Circadian pacemakers[3,11], photosensitivity[4,22], and the mechanisms for regulated melatonin production[2] all reside within the gland. Melatonin production and release continue to go up at night and down in the daytime 'spontaneously' in culture, and the rhythm persists for several cycles even in constant darkness[17] or constant red light[24]. Of the few preparations from multicellular organisms that permit investigation of an entire circadian system in vitro[10], only the chick pineal has thus far been shown to retain these functions in dispersed cell culture[3,17,24].

We have been using chick pineal cells to distinguish perturbations of melatonin output that act through the pacemaker from those acting on the melatonin-synthesizing apparatus. 'Phase-dependent phase shifts' indicate that a stimulus has affected the pacemaker[5,20,21]. Under the conditions used, light or dark pulses induced such phase shifts[24], but several other agents which raised or lowered melatonin

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output did not[25–27]. Here, we describe the effects of ouabain and salt solutions lacking potassium on melatonin output and its rhythm. These agents, which inhibit Na,K-ATPase[9], mimicked the effects of darkness on the circadian pacemaker in chick pineal cells.

MATERIALS AND METHODS

Pineal cell culture

White leghorn chicks were received 0–2 days after hatch from either Bowman's Hatchery (Westminster, MD) or Truslow Farms (Chestertown, MD). Pineal cells were dispersed in trypsin and plated in modified McCoy's 5A Medium (Gibco 380-2230) containing 25 mM HEPES buffer, L-glutamine, penicillin, streptomycin, 10% heat-inactivated fetal bovine serum, and 10% chicken serum as described previously[24]. Each 24 well plate had cells in groups of 4 wells, 1–4 groups per plate. Experiments used cells from up to 100 glands in 60 wells containing about 10^6 cells/well (range among experiments, $0.8–1.2 \times 10^6$ cells/well). Cells were fed by exchange of medium at least daily. Days are numbered successively from the day of plating (day 1), each day starting at the time of onset of white light in the original lighting schedule. Cells were maintained in the plating medium through day 3. On day 4 they were switched to the same medium without added serum and with an additional 10 mM KCl added. Addition of this concentration of KCl increased melatonin output without affecting the period or phase of the rhythm[24]. The effects of feeding schedule, media, sera, and potassium were described previously[24].

Light cycles and drugs

Cells were maintained under 5% CO_2 in air in tissue culture incubators containing red lights, white lights, and timers as described previously[24]. They were exposed to a cycle of 12 h red (R) light and 12 h white (L) light (LR 12:12) through day 5. In this schedule, L acts as 'day' and R acts as 'night'. In some experiments media were collected for assay shortly after onset of white light and shortly before onset of red light. In other experiments cells were switched to constant red light (RR) before 'expected' onset of L at the start of day 6. In these experiments, media were changed and collected at 4 h intervals under red light. Group mean melatonin outputs were plotted against time of collection and phases of control and experimental groups were compared. Magnitudes of phase shifts were determined manually by averaging displacements of peaks and troughs between experimental and control groups on days 7 and 8 as described previously[24].

Ouabain (Ob; Strophanthin G) was purchased from Calbiochem (La Jolla, CA). It was dissolved in medium and filtered before use. Complete salt solutions (SS) were based on the formulation of the enriched medium and contained (in mM): NaCl 110, $NaHCO_3$ 10, $KHCO_3$ 15, NaH_2PO_4 4.2, $MgSO_4$ 0.8, $CaCl_2$ 0.91, glucose 16.7, glutamine 1.5, tryptophan 0.03, NaHEPES buffer 25, penicillin and streptomycin, and 0.3% bovine serum albumin. Low potassium (–K) solutions omitted the $KHCO_3$, substituting an additional 15 mM $NaHCO_3$. Initial pH of these solutions was 7.3.

Assay of [^{14}C]melatonin release

L-[side chain-3-^{14}C]Tryptophan (spec. act. 51.8 mCi/mmol) was purchased from New England Nuclear (Boston, MA). McCoy's 5A medium contains about 0.03 mM tryptophan (of which more than half is contributed by the Bacto-peptone, a proprietary nutrient digest, which it contains). Cells were exposed to [^{14}C]tryptophan for at least 24 h before the start of timed collections. Different experiments

TABLE I

Inhibition of nocturnal [^{14}C]melatonin by various concentrations of ouabain

Chick pineal cells were plated and maintained under LR 12:12 as described in Materials and Methods. Media were collected and replaced at 12 h intervals on day 6 and cells were exposed to various concentrations of ouabain (Ob) during the 'night'. Data are from groups of 4 wells. Blanks (not subtracted) were 2.8 ± 0.1 ($n = 4$) in the units used. [^{14}C]Tryptophan was 0.49 μCi/ml.

Group	*[^{14}C]Melatonin/12 h (0.6×10^{-2} dpm/well)*	
	Day (no drug)	*Night (+ Ob)*
Control	15.2 ± 1.0	53.1 ± 2.0
+ Ob 10^{-6}	16.1 ± 0.7	40.3 ± 1.2
+ Ob 10^{-5}	15.6 ± 0.9	26.8 ± 2.8
+ Ob 10^{-4}	15.4 ± 0.2	13.6 ± 1.0
+ Ob 10^{-3}	14.3 ± 0.3	11.3 ± 0.9

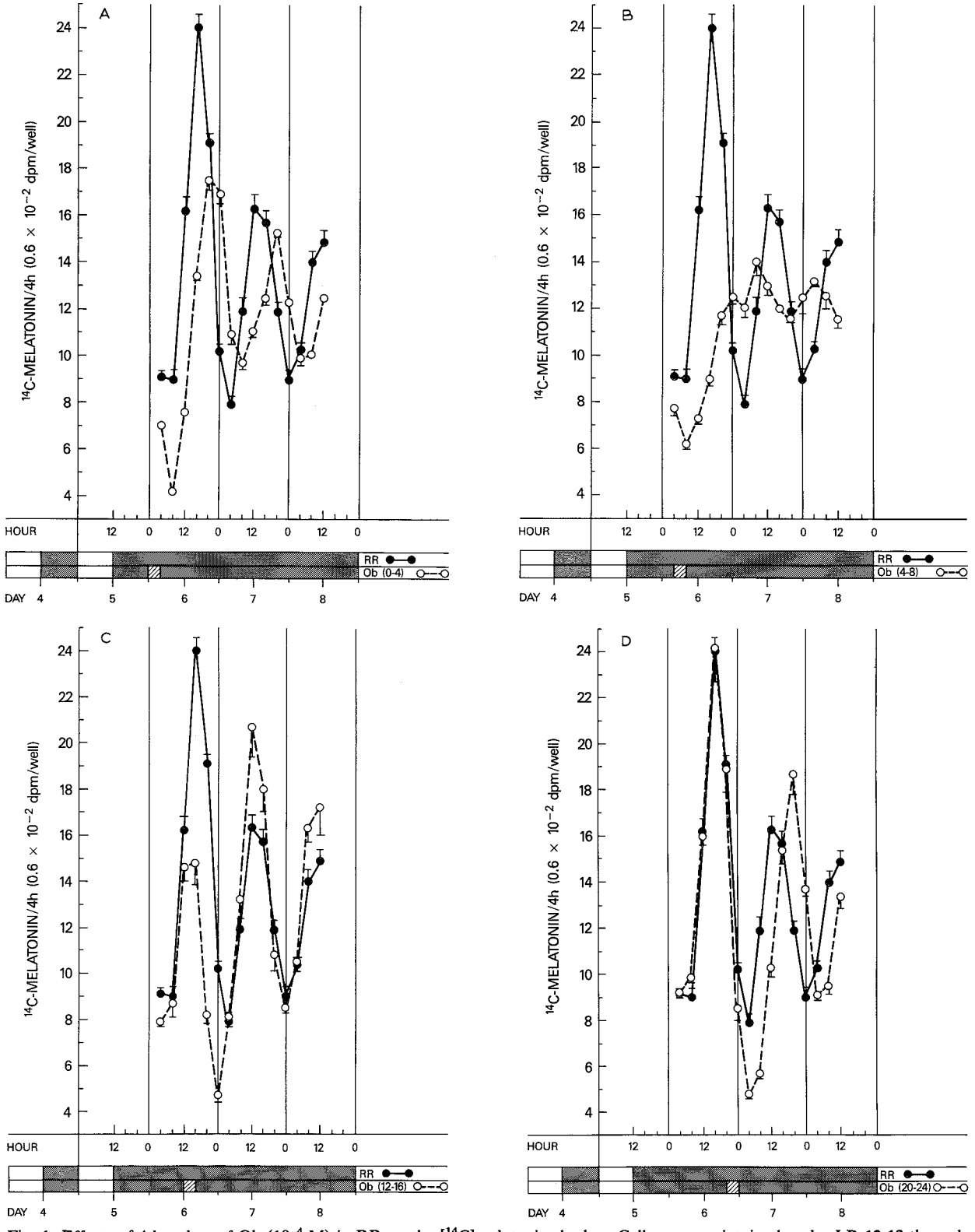

Fig. 1. Effects of 4 h pulses of Ob (10^{-4} M) in RR on the [^{14}C]melatonin rhythm. Cells were maintained under LR 12:12 through day 5 and then switched to RR. Individual groups were exposed to Ob (10^{-4} M) for 4 h during the intervals indicated. Data are means ± S.E.M. for 8 wells (RR controls) or 4 wells, shown at time of collection. Blanks (not subtracted) were 0.9 ± 0.06 (n = 12) and [^{14}C]tryptophan was 0.47 μCi/ml.

TABLE II

Effects of 4 h pulses of ouabain (Ob) at different times on [^{14}C]melatonin release in constant red light (RR)

Cells were maintained under LR 12:12 through day 5 and then switched to RR. Individual groups were incubated in medium containing 10^{-4} M Ob (Expt. 1) or 10^{-5} M Ob (Expt. 2) for 4 h during the intervals indicated. In each experiment, control data are from the same 8 wells for all points, while 'Ob pulse' data are from separate groups of 4 wells, each exposed to Ob only during the interval indicated and otherwise treated like controls. Other results from these experiments are shown in Fig. 1 (Expt. 1), Fig. 2 (Expt. 2), and Fig. 3.

Hour after 'expected' onset of white light (L)	*[^{14}C]Melatonin/4 h (0.6×10^{-2} dpm/well)*			
	Experiment 1		*Experiment 2*	
	RR control	*Ob (10^{-4} M) pulse*	*RR control*	*Ob (10^{-5} M) pulse*
0–4	9.1 ± 0.2	7.0 ± 0.1	11.0 ± 0.3	13.3 ± 0.4
4–8	9.0 ± 0.4	6.2 ± 0.2	10.0 ± 0.2	11.8 ± 0.3
8–12	16.2 ± 0.6	9.2 ± 0.5	15.9 ± 0.3	18.5 ± 0.4
12–16	24.0 ± 0.6	14.8 ± 1.0	24.9 ± 0.4	25.3 ± 0.2
16–20	19.1 ± 0.4	12.1 ± 0.2	21.0 ± 0.6	23.6 ± 0.4
20–24	10.2 ± 0.3	8.5 ± 0.5	11.5 ± 0.3	14.4 ± 0.5

used 0.40–0.50 μCi/ml, present from day 4. In later experiments, stock [^{14}C]tryptophan was washed 4 times with ether before use in order to reduce the blank. When cells were fed daily, they received 1 ml/well. When media were collected for assay of [^{14}C]melatonin at 4 or 12 h intervals, 0.5 ml was used per well. Media were collected into polypropylene test tubes containing 0.05 ml of an indole carrier mix, extracted into 5 ml chloroform, and back-washed with acid and base as described previously[24]. Three ml of the final chloroform phase was transferred to scintillation vials, dried, and counted.

RESULTS

Ouabain (Ob), a specific inhibitor of Na,K-ATPase[9], inhibited the nocturnal release of [^{14}C]melatonin by chick pineal cells (Table I). When cells were exposed to Ob for 12 h during the 'night', in cycle of 12 h white and 12 h red light (LR 12:12), inhibition was observed at all concentrations tested, with about 25% inhibition at 10^{-6} M and complete blockade of the nocturnal increase at about 10^{-4} M. Melatonin output was also inhibited by Ob in the daytime (about 35% at 10^{-5} M) (data not shown).

The rhythm of melatonin output persists when chick pineal cells are exposed to constant red light (RR) (Fig. 1). Four hour pulses of 10^{-4} M Ob, given after switching to RR, lowered the [^{14}C]melatonin output during drug exposure at all phase points (Table II, Exp. 1). This 'acute' inhibition appeared greater at those phases when melatonin output was high. Lower concentrations of Ob (10^{-5} M), given after switching to RR, tended to enhance [^{14}C]melatonin release during drug exposure (Table II, Exp. 2). This 'acute' increase was modest (about 10% of maximal 4 h output), independent of phase (it was seen at all phase points except one), and short-lived. Melatonin output in the interval *after* drug exposure fell below controls (as in Fig. 2A,B).

The effect of Ob pulses on subsequent cycles in RR varied with the phase at which the cells were exposed to the drug. When cells were exposed to 10^{-4} M Ob between 0 and 4 h after 'expected' onset of white light, there was a *delay* of about 6 h in subsequent cycles (Fig. 1A). In contrast, when cells were exposed to 10^{-4} M Ob between 4 and 8 h after 'expected' onset of white light, subsequent cycles were *advanced* relative to controls (Fig. 1B). When cells were exposed to 10^{-4} M Ob between 12 and 16

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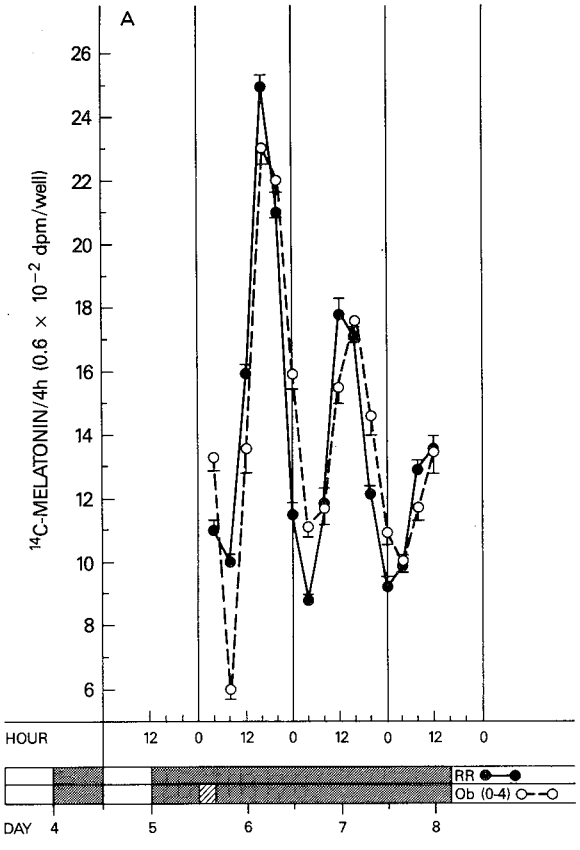
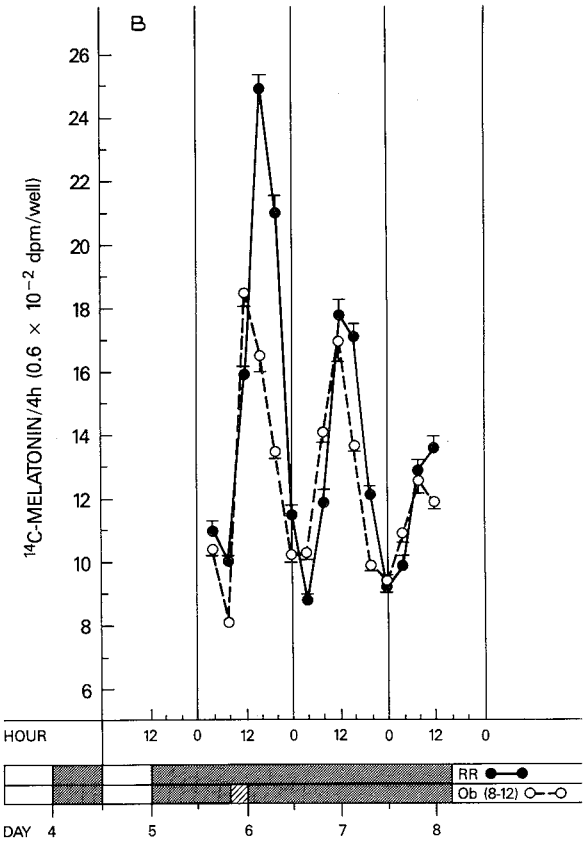
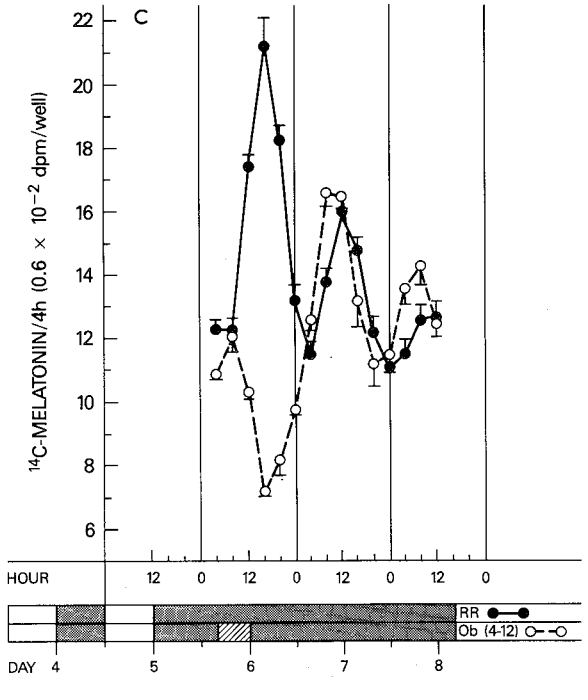
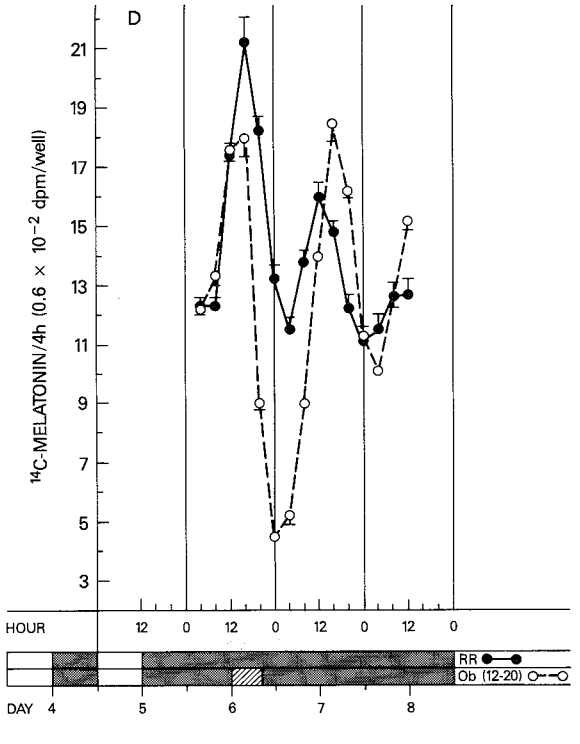
Fig. 2. Effects of pulses of Ob (10^{-5} M) in RR on the [^{14}C]melatonin rhythm. Cells were maintained under LR 12:12 through day 5 and then switched to RR. In A and B, individual groups were exposed to Ob (10^{-5} M) for 4 h, between 0 and 4 h (A) or 8 and 12 h (B) after 'expected' onset of white light. Blanks (not subtracted) were 0.8 ± 0.04 (n = 12) and [^{14}C]tryptophan was 0.46 μCi/ml. In C and D, individual groups were exposed to Ob (10^{-5} M) for 8 h, between 4 and 12 h (C) or 12 and 20 h (D) after 'expected' onset of white light. Blanks (not subtracted) were 1.3 ± 0.05 (n = 12) and [^{14}C]tryptophan was 0.48 μCi/ml. Data are means ± S.E.M. of 8 wells (RR controls) or 4 wells, shown at time of collection.


A
^{14}C-MELATONIN/4h (0.6 × 10^{-2} dpm/well)
HOUR
DAY
RR
Ob (0-4)


B
^{14}C-MELATONIN/4h (0.6 × 10^{-2} dpm/well)
HOUR
DAY
RR
Ob (8-12)


C
^{14}C-MELATONIN/4h (0.6 × 10^{-2} dpm/well)
HOUR
DAY
RR
Ob (4-12)


D
^{14}C-MELATONIN/4h (0.6 × 10^{-2} dpm/well)
HOUR
DAY
RR
Ob (12-20)

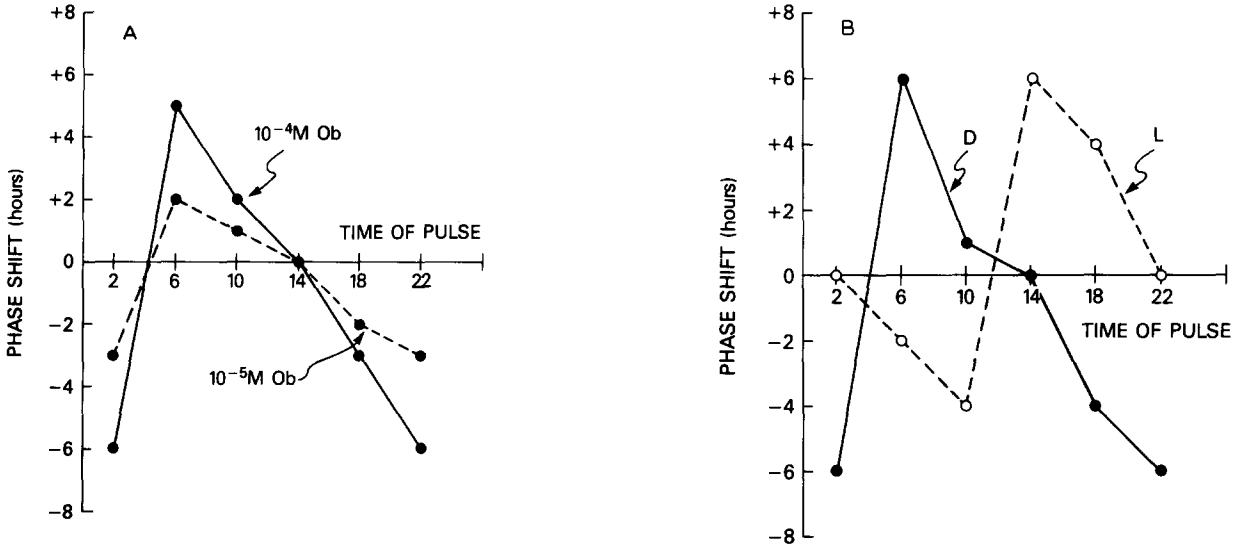


Fig. 3. Phase-response curves for 4 h pulses of ouabain (Ob), white light (L), or darkness (D). A summarizes results of pulse experiments using 10^{-4} M (●——●) or 10^{-5} M (● - - - ●) Ob, portions of which are also depicted in Figs. 1 and 2, respectively. Phase shifts are plotted against the mid-point time of the 4 h pulse. Advances are plotted as positive shifts and delays as negative shifts. Comparison of means for each experimental group exposed to Ob against control means (e.g. Fig. 1B) generates a single point on the phase response curve (e.g. ouabain pulse from 4 to 8 h after 'expected' onset of white light shown in Fig. 1B is the 5 h advance point at h 6). Magnitude of phase shifts was determined manually by averaging displacements of peaks and troughs between experimental and control groups on days 7–8. B summarizes results of experiments using 4 h pulses of L (○ - - - ○) or D (● - - - ●) reported previously[24].

h after 'expected' onset of L, however, there was virtually *no effect* on the phase of subsequent cycles, even though the rise on day 6 was aborted and melatonin output fell 'prematurely' (Fig. 1C). The response of the cells to 10^{-4} M Ob given in the interval from 20 to 24 h after 'expected' onset of L (Fig. 1D) was also a phase delay, similar to that seen when the cells were exposed to Ob from 0 to 4 h (Fig. 1A). Since the period of the cycle in RR is closer to 20 h[24] than to the 24 h period observed in LR 12:12, the cells in Fig. 1D were exposed to Ob at essentially the same phase (one cycle later) as were the cells in Fig. 1A. Thus, 4 h pulses of 10^{-4} M Ob induced phase delays, phase advances, or no change in the phase of subsequent cycles, depending on the phase at which the pulse was given.

Lower concentrations of Ob (10^{-5} M) also induced phase-dependent phase shifts. Phase delays and phase advances caused by 4 or 8 h pulses of 10^{-5} M are illustrated in Fig. 2. These were always smaller than those seen with 10^{-4} M Ob. Eight hour pulses of 10^{-6} M Ob (from 4 to 12 or 12 to 20 h after 'expected' onset of L) failed to cause obvious shifts (data not shown).

The relationships between the phase shifts induced by 4 h pulses of Ob in RR and the phase at which the pulse was given (time of pulse) are shown in Fig. 3A. At any given point, phase shifts induced by 10^{-4} M Ob were larger than those induced by 10^{-5} M Ob. However, the phase response curves for 10^{-4} and 10^{-5} M Ob have similar shapes: each shows phase delays shortly after 'expected' onset of L (0 time), with a sharp transition to phase advances around 'midday', followed by a decline in the size of advances to a point where Ob pulses have no effect on subsequent phase ('dead zone'), and then an increasing phase delay into the next cycle.

There is a striking resemblance between the phase response curves for 4 h pulses of 10^{-4} M Ob (Fig. 3A) and for 4 h pulses of darkness (D) in RR (Fig. 3B), determined previously[24]. These may be contrasted with the phase response curve for 4 h pulses of white light (L), also shown in Fig. 3B.

The activity of Na,K-ATPase is dependent on extracellular potassium[19]. Thus, another way to inhibit its activity is to markedly lower extracellular potassium. We used salt solutions to determine whether omission of potassium from the medium

would cause phase-dependent phase shifts similar to those induced by pulses of Ob or darkness. It did (Fig. 4). Exposing the cells to the complete salt solution (SS) from 0 to 4 h after 'expected' onset of L had no effect on the phase of the subsequent melatonin rhythm (Fig. 4A). Neither did 4 or 8 h

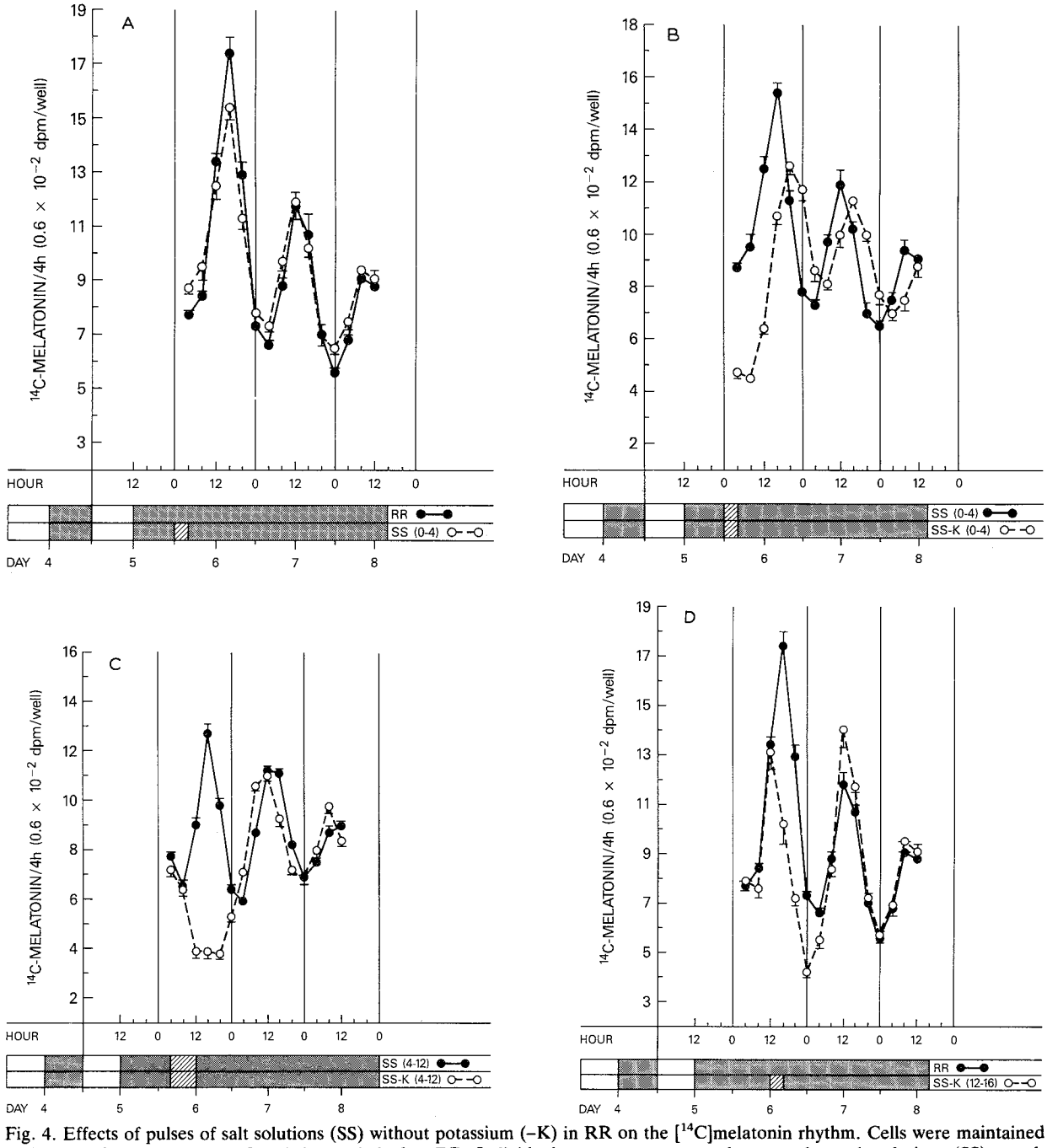


Fig. 4. Effects of pulses of salt solutions (SS) without potassium (−K) in RR on the [^{14}C]melatonin rhythm. Cells were maintained under LR 12:12 through day 5 and then switched to RR. Individual groups were exposed to complete salt solutions (SS) or salt solutions from which potassium was omitted (SS−K) for 4 or 8 h during the intervals indicated. In A, the effect of SS from 0 to 4 h after 'expected' onset of white light is compared to controls. In B, the effect of SS−K is compared to SS during this interval. In C, the effect of SS−K from 4 to 12 h after 'expected' onset of white light is compared to that of SS. In D, the effect of SS−K from 12 to 16 h is compared to controls. A, B and D are from the same experiment. Data are means ± S.E.M. of 8 wells (RR) or 4 wells, shown at time of collection.

pulses of SS at other phase points (data not shown). Exposing the cells to salt solutions lacking potassium (SS–K), however, did induce phase-dependent phase shifts in subsequent cycles. Phase delays, phase advances, and acute effects without subsequent phase shifts were observed (Fig. 4B–D). The direction and phase-dependence of these phase shifts were similar to those obtained with Ob. Their magnitudes were comparable to those obtained with 10^{-5} M Ob, but somewhat variable (presumably because a small amount of potassium-containing medium remained in the wells when the SS–K solution was added).

DISCUSSION

Pulses of Ob induced phase-dependent phase shifts in the circadian rhythm of melatonin output displayed by cultured chick pineal cells in RR. These phase shifts were concentration dependent. Ob is a specific and well-known inhibitor of Na,K-ATPase which pumps Na out of, and K into, cells[9,18,19]. There are at least two forms of this enzyme, with differing affinities for Ob[7]. Since Ob binding to the enzyme is inhibited by moderately high concentrations of extracellular K (as used here), no attempt is made to infer the dominant subtype from the concentration dependence in these experiments.

Phase-dependent phase shifts indicate an effect on the underlying circadian pacemaker[5,20,21]. A mechanistic pathway must exist from the stimulus to a component of the pacemaker (an entrainment pathway). The phase–response curve for pulses of Ob is strikingly similar to the PRC obtained previously[24] for pulses of darkness (D). This similarity suggests that Ob and D have the same (or equivalent) ultimate effects on the oscillator; that their entrainment pathways converge. Whether the sequence of events initiated by pulses of Ob or D converge proximal to, or within, the pacemaker is unknown.

It is presumed that the effects of Ob on the circadian pacemaker in chick pineal cells are mediated by its inhibition of the Na,K-ATPase. This presumption is supported by the effects of salt solutions lacking K. The Na,K-ATPase requires extracellular K for its activity[9,19], and is markedly inhibited at K concentrations below 1 mM. Salt solutions lacking K induced phase shifts similar to those obtained with Ob.

The early consequences of inhibiting the Na, K-ATPase are changes in the distribution of Na and K across the cell's plasma membrane[18]. Both intracellular levels and ratios (inside to outside, Na to K) of these ions can change markedly. The aspect of these initial changes whose consequences lead to the pacemaker is unknown. The ability of Ob to induce phase shifts has been reported previously in an invertebrate system[6]. There, the depolarizing effect of Ob on membrane potential has been emphasized and proposed to mediate its effects on the pacemaker. In chick pineal, however, moderate changes in membrane potential caused by 3-5-fold changes in extracellular K concentration failed to induce apparent phase shifts in the melatonin rhythm[24]. A 10-fold change (from 5 to 50 mM) in extracellular K also failed to induce a phase shift or to block a phase advance induced by a pulse of white light (Robertson and Takahashi, personal communication). Thus, although a role for depolarization in the effects of Ob on the circadian pacemaker in chick pineal cells has not been ruled out (perhaps Ob induces a depolarization greater than has been obtained by manipulation of extracellular K in these experiments), available evidence does not support this hypothesis. Membrane potential is determined largely by the ratio of intracellular to extracellular K concentrations. Perhaps it is the changes in, for example, K or Na *levels* that are most important for the effects of Ob on the circadian pacemaker in chick pineal cells.

Inhibition of the Na,K-ATPase has diverse secondary consequences, ionic and metabolic[18]. It has been shown in several systems[10], now including chick pineal cells[14], that protein synthesis inhibition can induce phase shifts with a PRC similar to that obtained for Ob. In two experiments, we found that a 4 h pulse of 10^{-4} M Ob (in RR from 0 to 4 h after 'expected' onset of L) *did* inhibit total protein synthesis (about 35%) relative to controls. However, 10^{-5} M Ob, which also induces obvious phase shifts under these conditions (Fig. 2A), did *not* inhibit total protein synthesis. It would appear, then, that inhibition of protein synthesis is not *required* for Ob to affect the pacemaker. Nevertheless, protein synthesis inhibition may *contribute* to the effects of Ob on the circadian pacemaker in chick pineal cells.

The initial effect of Ob is to inhibit Na,K-

ATPase[9], resulting in increased intracellular Na, decreased intracellular K, and consequent changes in membrane potential and ionic ratios. These changes, in turn, affect other ionic and metabolic regulatory processes[18]. At least one of these consequences of inhibiting Na,K-ATPase is, or leads to, a change in a component of the circadian pacemaker. There are few stimuli known to lead to the pacemaker in chick pineal cells. The consequences of Ob's action, and its interaction with other entrainment pathways (particularly that of light) are worthy of further investigation[28].

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