Low salt mimics effects of dark pulses on circadian pacemaker in cultured chick pineal cells

MARTIN ZATZ AND HIH-MIN WANG
Section on Biochemical Pharmacology, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

ZATZ, MARTIN, AND HIH-MIN WANG. *Low salt mimics effects of dark pulses on circadian pacemaker in cultured chick pineal cells.* Am. J. Physiol. 261 (Regulatory Integrative Comp. Physiol. 30): R1424–R1430, 1991.—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. We reported previously that inhibiting the Na-K-ATPase with ouabain or salt solutions lacking potassium evokes phase shifts with the same phase dependence as those induced by pulses of darkness. One of the consequences of inhibiting the sodium pump is cell swelling. To test the relevance of this effect, we exposed chick pineal cells to pulses of medium containing reduced concentrations of NaCl, which should cause cell swelling. These hypotonic solutions induced phase shifts in the melatonin rhythm with the same phase dependence as those caused by pulses of ouabain or darkness. The size of the phase shifts varied with degree of dilution, and phase shifting was prevented by replacement of NaCl. In view of previous results showing that hypertonic media mimicked the phase-shifting effects of light, these results suggest that cell swelling may mediate the darklike effects of ouabain on the circadian pacemaker in chick pineal cells.

melatonin; cell volume; circadian rhythms

AT THE "HEART" of circadian rhythms lies a clocklike pacemaker (15) 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, 19). Under appropriate conditions, environmental lighting can alter the period and phase of the pacemaker. Circadian rhythms are thus endogenously generated but can be exogenously regulated by light cycles.

The overt hormonal and biochemical rhythm that has been studied most intensively is that of melatonin production (1, 13). In the rat (and other mammals), the components of this circadian system are anatomically distinct (14); 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 (13, 22).

In contrast to the rat pineal, the chick pineal in vitro is both rhythmic and photosensitive. Circadian pacemakers (3, 12), photosensitivity (4, 20), and the mechanisms for regulated melatonin production (1) 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 (28). Of the few preparations from multicellular organisms that permit investigation of an entire circadian system in vitro (11), only the chick pineal has thus far been shown to retain these functions in dispersed cell culture (3, 17, 28).

We have been using chick pineal cells to identify perturbations that act on the pacemaker and to distinguish them from those acting on the melatonin-synthesizing apparatus. "Phase-dependent phase shifts" indicate that a stimulus has affected the pacemaker (5, 19). Under the conditions used, light or dark pulses induced such phase shifts (28), but several other agents that raised or lowered melatonin output did not (23–25). We recently described the effects of high salt and other hypertonic media on melatonin output and its rhythm (29). These agents mimicked the effects of light pulses on the circadian pacemaker in chick pineal cells. We had shown previously that pulses of ouabain or of salt solutions lacking potassium mimicked the effects of dark pulses in phase shifting the melatonin rhythm (26, 27). Ouabain and salt solutions lacking potassium inhibit the Na-K-ATPase (8). One of the consequences of inhibiting the sodium pump is cell swelling (6, 16, 18).

If hypertonic high-salt media, which cause cell shrinkage, mimic the effects of light and ouabain and lack of extracellular potassium, which cause cell swelling, mimic the effects of dark on the circadian pacemaker, then other perturbations that cause cell swelling should also phase shift the pacemaker and show a phase dependence similar to that of dark pulses. We tested this prediction using hypotonic low-salt media. Exposure of chick pineal cells to 4-h pulses of low-salt media did mimic the effects of dark pulses on the circadian pacemaker.

MATERIALS AND METHODS

Pineal cell culture. White Leghorn chicks were received 0–2 days after hatch from Truslow Farms (Chestertown, MD). Pineal cells were dispersed in trypsin and plated in modified McCoy's 5A medium (GIBCO 380-2230) containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-

ethanesulfonic acid (HEPES) buffer, L-glutamine, penicillin, streptomycin, 10% heat-inactivated fetal bovine serum, and 10% chicken serum as described previously (28). Each 24-well plate had cells in groups of 4 wells, 1–4 groups/plate. Experiments used cells from up to 100 glands in 60 wells containing ~10^6 cells/well (range among experiments 0.8–1.2 × 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 (28). The effects of feeding schedule, media, and sera were described previously (28).

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 (28). They were all exposed to a cycle of 12 h red light (R) and 12 h white light (L) (LR 12:12) through *day 4*. In this schedule, L acts as "day" and R acts as "night." Cells were switched to constant red light (RR) before "expected" onset of L at the start of *day 5* or *6*. Media were then collected and replaced 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 displacement of peaks and troughs between experimental and control groups during the last 24 h as described previously (28). Standard errors for phase shifts are not routinely determined. In a typical set of experiments for a phase-response curve, they varied between 15 and 60 min (M. Max, personal communication).

Low-salt media were made by mixing control medium with "dilution medium" in various proportions. Dilution medium consisted of (in mM) 10 Na-HEPES buffer (pH 7.4), 1 $CaCl_2$, 15 $KHCO_3$, 0.03 tryptophan, and penicillin-streptomycin. Osmolarity of the final media was measured in a vapor pressure osmometer (Wescor 5500).

Assay of [^{14}C]melatonin release. L-[*Side chain*-3-^{14}C]-tryptophan (sp act 51.8–54.8 mCi/mmol) was purchased from New England Nuclear (Boston, MA). McCoy's 5A medium contains ~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 24 h before the start of timed collections. Different experiments used 0.41–0.50 μCi/ml, present from *day 4* or *5*. When cells were fed daily, they received 1 ml/well. When media were collected for assay of [^{14}C]melatonin at 4-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 backwashed with acid and base as described previously (28). Three milliliters of the final chloroform phase was transferred to scintillation vials, dried, and counted.

RESULTS

The rhythm of melatonin output persists when chick pineal cells are switched from a cycle of L to RR (Fig. 1). When cells were exposed to low-salt medium during the first 4 h after expected onset of L, there was a phase delay in subsequent cycles relative to cells maintained throughout in control medium. The phase shift was robust when the osmolarity was reduced by half (~4 h in Fig. 1*A*) and near minimal reliable detectability when the osmolarity was reduced by a third (~2 h in Fig. 1*B*). Similar results were obtained in two other experiments.

Control medium contains a rich assortment of components including salts (predominantly NaCl), amino acids, vitamins, and Bacto-peptone, an unidentified nutrient digest. Initial experiments (not shown) on the effects of low-salt media simply used control medium diluted with water. Later experiments used dilution medium containing buffer, $KHCO_3$, $CaCl_2$, and tryptophan to minimize real and apparent direct effects of reducing these components on melatonin output. Under these latter conditions, there was minimal effect on melatonin output in the 4-h interval when the cells were exposed to the low-salt medium. There was, however, a marked reduction in melatonin output upon return to control medium, reaching its nadir in the second 4-h interval. This effect appeared to be independent of the phase at which the cells were exposed to low-salt medium (e.g., see Figs. 1*A*, 3*A*, and 4*A*). Such same-cycle effects on the overt rhythm cannot be interpreted with regard to the underlying pacemaker (e.g., see Fig. 4*A*).

We showed previously that balanced salt solutions fail to phase shift the pacemaker in chick pineal cells relative to control medium (25). Nonetheless, to determine whether the effect of low-salt medium on the pacemaker was due to the reduction in NaCl concentration rather than to a reduction in concentration of some other component of the control medium, we compared the effects of low-salt medium to those of the same medium with NaCl added to restore osmolarity. The phase delay induced by the low-salt medium (Fig. 2*A*) was abolished by replacement of NaCl (Fig. 2*B*). Thus it was the reduction in NaCl that induced the phase shift. Replacing the NaCl with mannitol (Fig. 2*C*) also essentially blocked the phase delay, indicating that it was the reduction in osmolarity in low-salt medium that induced the phase shift.

The effects of pulses of low-salt medium on subsequent cycles varied with the phase of the melatonin rhythm at which the cells were exposed to the diluted media. When the cells were exposed between 8 and 12 h after expected onset of L, there was an advance of ~3 h in subsequent cycles (Fig. 3*A*) in contrast to the delay seen after exposure between 0 and 4 h (Fig. 1*A*). Similar results were obtained in two other experiments. When the cells were exposed to low-salt medium between 4 and 8 h after expected onset of L in this experiment (Fig. 3*B*) and in another (not shown), the rhythm in the cycles after the perturbation was disrupted and its phase was indeterminate. Such responses have been seen previously, to other agents that perturb the pacemaker, at phases that overlap the transition between phase delays and phase

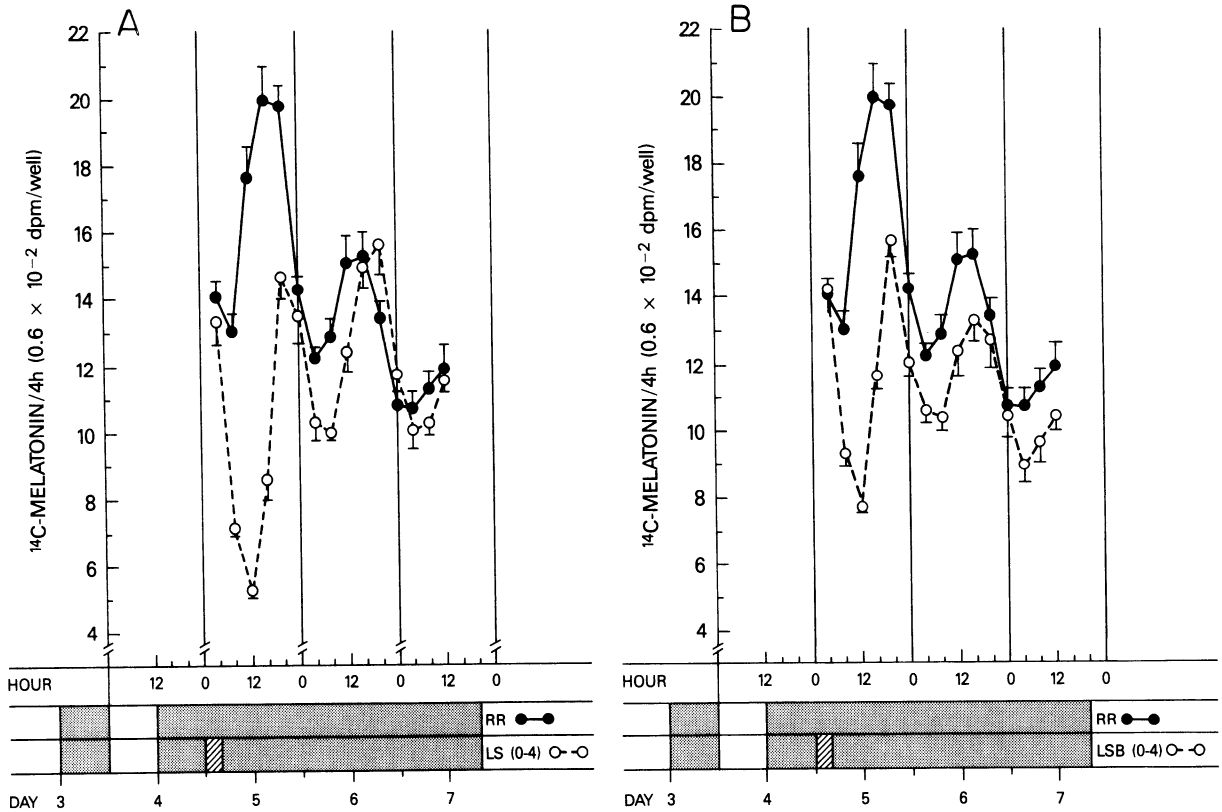

FIG. 1. Exposure to low salt (LS) for 4 h can phase-delay rhythm of [^{14}C]melatonin output in constant red light (RR). Chick pineal cells were maintained under a 12:12 h white light-red light cycle (LR 12:12) through *day 4* and then switched to RR. Individual groups were exposed to LS medium for 4 h between 0 and 4 h after "expected" onset of white light. LS media were made by mixing control and dilution media (see MATERIALS AND METHODS for composition) in various proportions. Osmolarity of control medium was 292 mosM, of the LS medium used in *A* 154 mosM, and of the LS medium used in *B* (LSB) 187 mosM. Data are means ± SE of 8 (RR controls) or 4 wells, shown at time of collection. Blanks (not subtracted) were 1.3 ± 0.2 (n = 12) in units used, and [^{14}C]tryptophan was 0.46 μCi/ml; dpm, disintegrations/min.

advances (27, 28). In another experiment at this phase (Fig. 3*C*), there was much less disruption of the rhythm and a large phase shift, which we interpret as an 8-h advance.

At still another phase (12–16 h after expected onset of L), however, there was virtually no effect on the phase of subsequent cycles after 4 h exposure to low-salt medium (Fig. 4*A*). Similar results were obtained in another experiment. Finally, at 20–24 h after expected onset of L, there was again a phase delay of ~5 h. Similar results were obtained in another experiment. Thus 4-h pulses of low-salt medium induced phase delays, phase advances, or no change in the phase of subsequent cycles, depending on the phase at which the pulse was given.

The relationship between the phase shifts induced by 4-h pulses of low-salt medium and the phase at which the pulse was given (time of pulse) is called a "phase-response curve" (PRC) (5, 11, 15, 19) and is shown in Fig. 5*A*. It has a characteristic shape and orientation. There was a delay in the first interval after expected onset of L, then a sharp transition from maximal delays to maximal advances, then a decline in the size of advances through a "dead zone" with little or no effect on subsequent phase, and finally increasing phase delays. Because the period of the cycle in these experiments is closer to 20 h than to the 24-h period observed in LR 12:12, the cells from 20–24 h after expected onset of L are at essentially the same phase (1 cycle later) as cells from 0–4 h.

There is a striking resemblance between the PRC for 4-h pulses of low-salt medium in RR and that for 4-h pulses of darkness (Fig. 5*B*) (28) or for ouabain (not shown) (26), which is similar to that for darkness. These curves may be compared with the PRCs for 4-h pulses of high NaCl (Fig. 5*A*) (29) and for 4-h pulses of L (Fig. 5*B*) (28).

DISCUSSION

Four-hour pulses of low-salt medium induced phase delays or phase advances or had no effect on the phase of subsequent cycles of the melatonin rhythm displayed by cultured chick pineal cells in RR, depending on the phase at which the cells were exposed to the hypotonic medium. These phase shifts were caused by the reduction in NaCl concentration in the diluted media (Fig. 2). They varied in size with the degree of dilution (Fig. 1).

Phase-dependent phase shifts indicate an effect on the underlying circadian pacemaker (5, 11, 19). A mechanistic pathway must exist from the stimulus to a component of the pacemaker itself (an entrainment pathway). We recently showed (29) that the PRC for pulses of high salt (or other hypertonic media) is strikingly similar to the PRC for pulses of L (Fig. 5). This similarity suggests that hypertonic solutions and L have the same (or equiv-

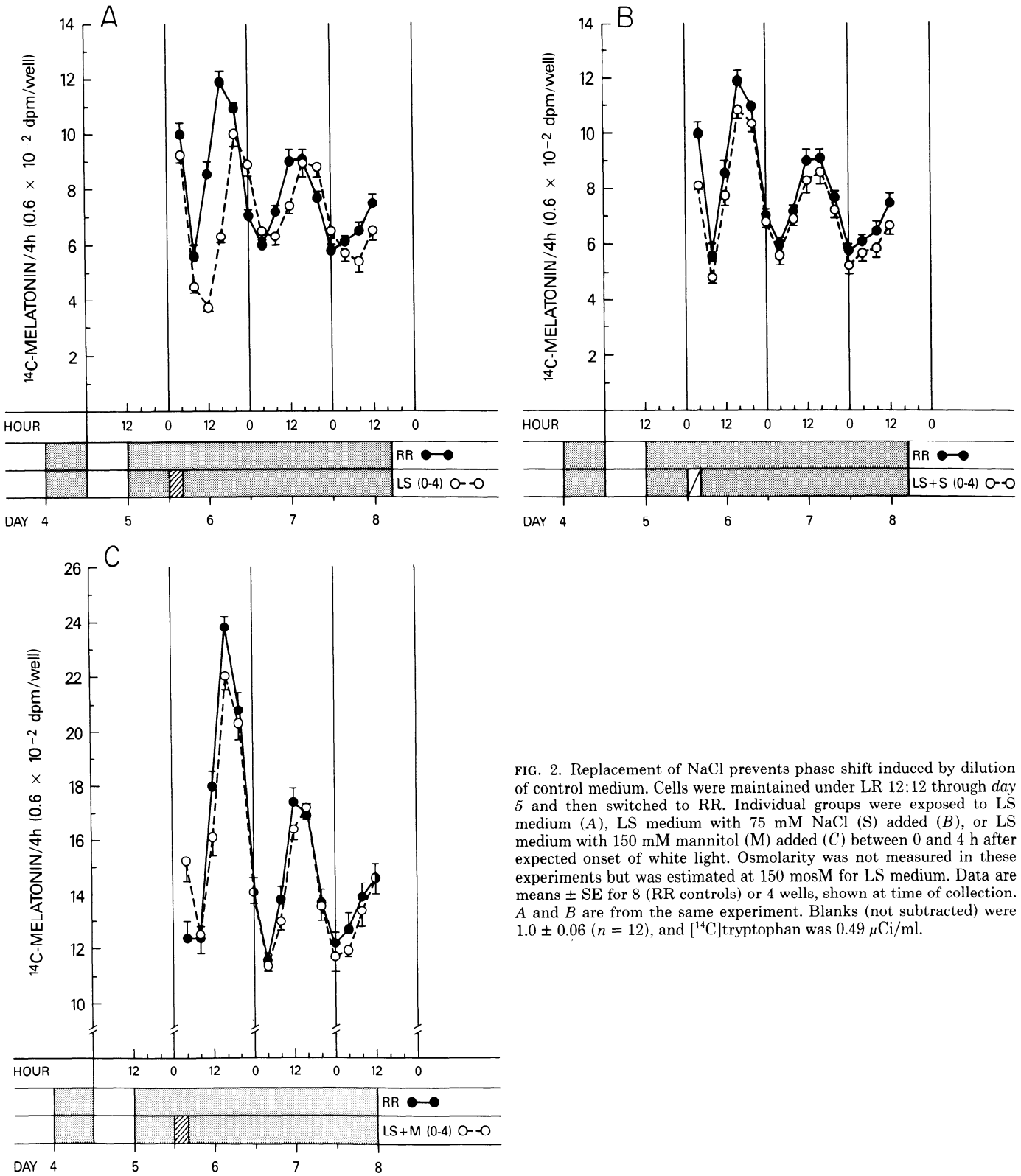

FIG. 2. Replacement of NaCl prevents phase shift induced by dilution of control medium. Cells were maintained under LR 12:12 through *day 5* and then switched to RR. Individual groups were exposed to LS medium (*A*), LS medium with 75 mM NaCl (S) added (*B*), or LS medium with 150 mM mannitol (M) added (*C*) between 0 and 4 h after expected onset of white light. Osmolarity was not measured in these experiments but was estimated at 150 mosM for LS medium. Data are means ± SE for 8 (RR controls) or 4 wells, shown at time of collection. *A* and *B* are from the same experiment. Blanks (not subtracted) were 1.0 ± 0.06 (n = 12), and [^{14}C]tryptophan was 0.49 μCi/ml.

alent) ultimate effects on the oscillator, i.e., that their entrainment pathways converge. Whether the sequences of events initiated by pulses of high salt or L converge proximal to or within the pacemaker is unknown.

Pulses of darkness, ouabain, or salt solutions lacking potassium also induce phase-dependent phase shifts in the melatonin rhythm (26, 28). The PRCs for these perturbations are very similar to each other but differ from the PRC for L. We interpreted (27) these relationships as reflecting a similar effect of ouabain or darkness on a critical component of the pacemaker, which effect is opposite to that of L on the same component. These conjectures cannot yet be tested directly but provide a framework for understanding, integrating, and designing experiments.

One of the consequences of inhibiting the sodium

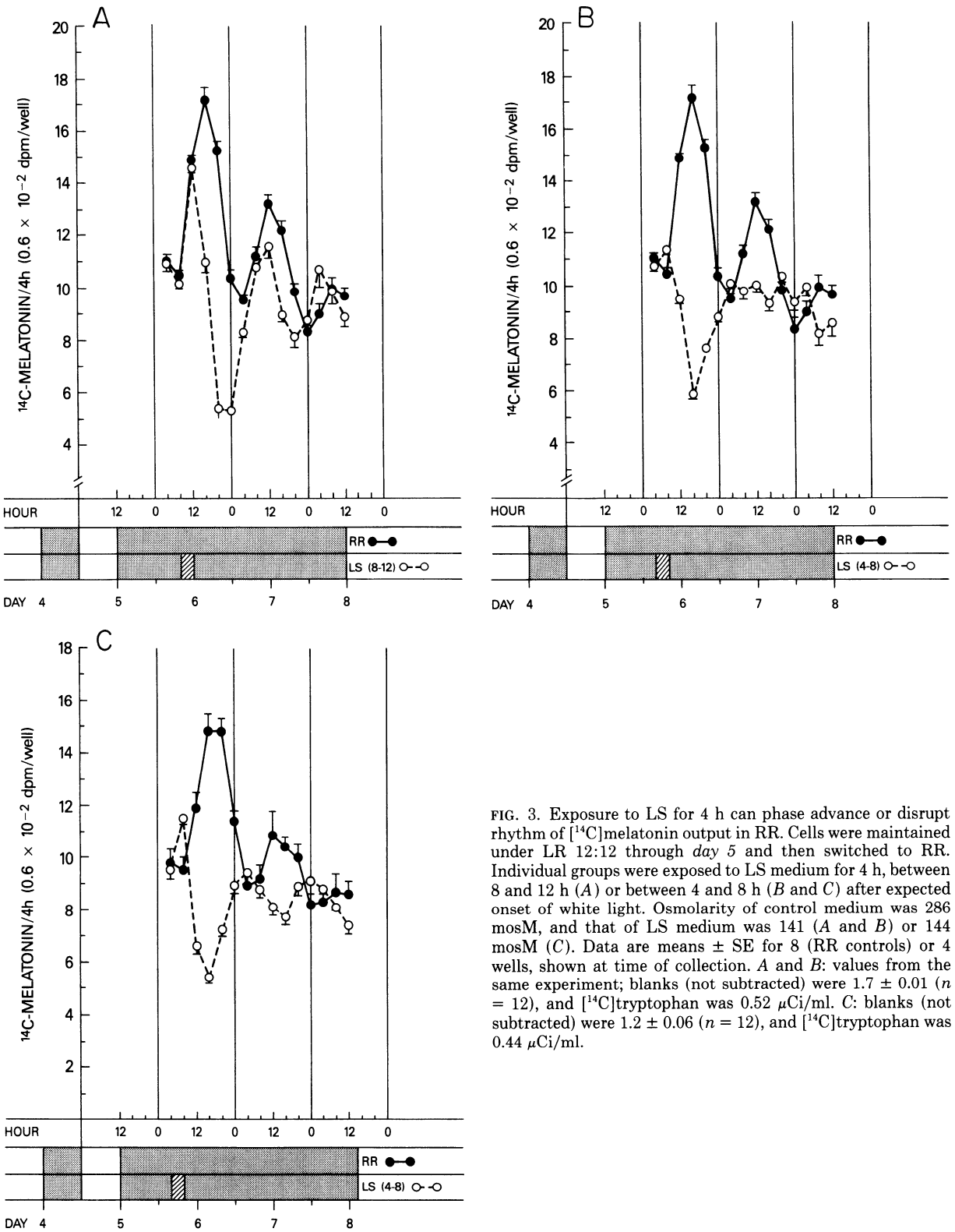

FIG. 3. Exposure to LS for 4 h can phase advance or disrupt rhythm of [^{14}C]melatonin output in RR. Cells were maintained under LR 12:12 through *day 5* and then switched to RR. Individual groups were exposed to LS medium for 4 h, between 8 and 12 h (*A*) or between 4 and 8 h (*B* and *C*) after expected onset of white light. Osmolarity of control medium was 286 mosM, and that of LS medium was 141 (*A* and *B*) or 144 mosM (*C*). Data are means ± SE for 8 (RR controls) or 4 wells, shown at time of collection. *A* and *B*: values from the same experiment; blanks (not subtracted) were 1.7 ± 0.01 (n = 12), and [^{14}C]tryptophan was 0.52 μCi/ml. *C*: blanks (not subtracted) were 1.2 ± 0.06 (n = 12), and [^{14}C]tryptophan was 0.44 μCi/ml.

pump is cell swelling (6, 16, 18). The "convergence hypothesis" suggests that if cell swelling is part of the mechanistic pathway by which ouabain phase shifts the pacemaker, then at least some other perturbations that cause cell swelling should also phase shift the pacemaker and generate a PRC similar to that for ouabain. Because high-salt media (which cause cell shrinkage) induce light-like phase shifts, the "hypothesis of opposites" also predicts darklike effects of cell swelling. We showed here that hypotonic low-salt media induced phase shifts in the melatonin rhythm with a PRC similar to that for darkness or ouabain and "opposite" to that for L or high salt (Fig. 5).

The convergence of these entrainment pathways would be most interesting if cell shrinkage were an effect of L necessary for entrainment or if cell volume changes were

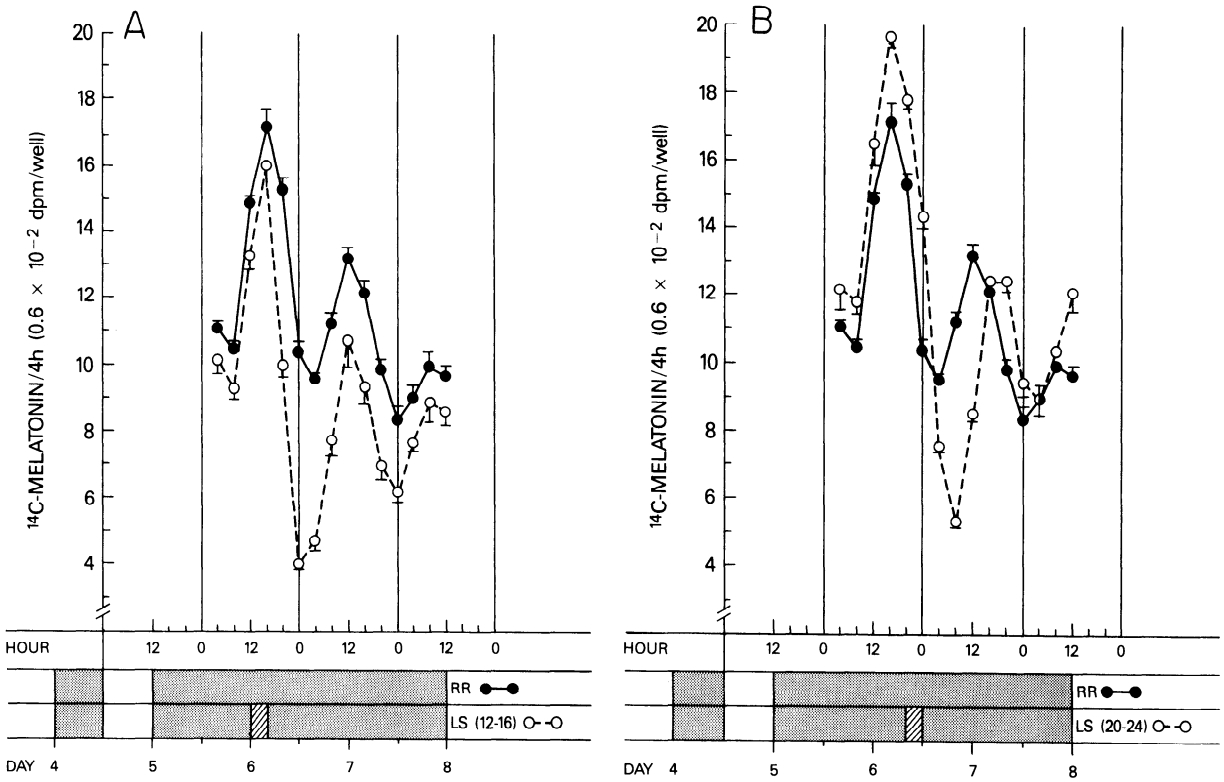

FIG. 4. Effect of 4 h exposure to LS on pacemaker at 12–16 or 20–24 h after expected onset of white light. Cells were maintained under LR 12:12 through *day 5* and then switched to RR. Individual groups were exposed to LS medium for 4 h between 12 and 16 h (*A*) or between 20 and 24 h (*B*) after expected onset of white light. Osmolarity of control medium was 286 mosM, and that of LS medium was 141 mosM. Data are means ± SE of 8 (RR controls) or 4 wells, shown at time of collection. Other results from this experiment are shown in Fig. 3, *A* and *B*.

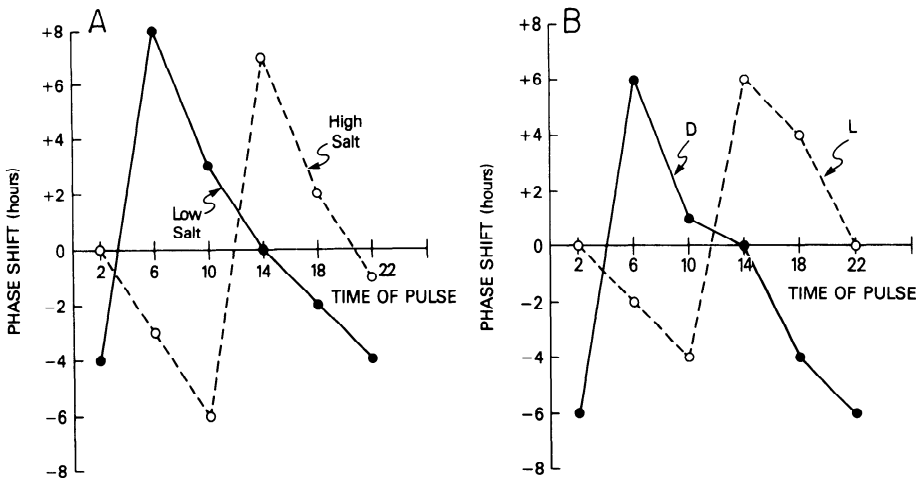

FIG. 5. Phase-response curves for 4-h pulses of high salt, LS, white light (L), or darkness (D) in RR. *A*: summary of results of pulse experiments using LS medium, portions of which are also depicted in Figs. 1, 3, and 4. Phase shifts are plotted against midpoint time of 4-h pulse. Advances are plotted as positive shifts and delays as negative shifts. Comparison of means for each experimental group exposed to LS medium against control means (e.g., Fig. 3*A*) generates a single point on phase-response curve (e.g., LS pulse from 8 to 12 h after expected onset of L shown in Fig. 3*A* is 3-h advance point at *hour 10*). Magnitude of phase shifts was determined manually by averaging displacements between experimental and control groups of last peaks and troughs. Phase-response curve for pulses of 75 mM NaCl added to control media, reported previously (29), is also shown. *B*: summary of results of experiments using 4-h pulses of white light or darkness reported previously (28).

closely linked to a component of the pacemaker itself (21). There is presently no evidence favoring either of these possibilities. Effects of light on the volume of isolated rod outer segments (2) and their discs (shrinkage, blocked by ouabain) (9) have been reported, as has light activation of the sodium pump in blowfly photoreceptors (7), but the relevance of these findings is moot. Cell volume regulation is complex (10), with different mechanisms (channels, transporters, exchangers) involved in different cell types and both reciprocal and

nonreciprocal processes involved in expanding and contracting cell volume, respectively. In addition to changes in rates of ongoing processes, such as the Na-K-ATPase, there are also compensatory mechanisms evoked by changes in cell volume that are quiescent in the absence of perturbation. Furthermore, a number of metabolic and physiological processes are affected by changes in cell volume (21). Thus the entrainment pathways for hypotonic and hypertonic solutions might converge with that for light several steps downstream from changes in cell volume. Notwithstanding these considerations, the mechanisms and circadian consequences of cell volume changes in chick pineal cells and their interactions with other entraining stimuli are worthy of further investigation.

We thank Deborah Mullen for editorial assistance.

Address for reprint requests: M. Zatz, Bldg. 36, Rm. 2A-17, LCB, NIMH, Bethesda, MD 20892.

Received 19 February 1991; accepted in final form 8 July 1991.

REFERENCES

1. BINKLEY, S. Pineal biochemistry: comparative aspects and circadian rhythms. In: *The Pineal Gland, Anatomy and Biochemistry*, edited by R. J. Reiter. Boca Raton, FL: CRC, 1981, vol. 1, p. 155–172.
2. BOWNDS, D., AND A. E. BRODIE. Light sensitive swelling of isolated frog rod outer segments as an in vitro assay for visual transduction and dark adaptation. *J. Gen. Physiol.* 66: 407–425, 1975.
3. DEGUCHI, T. A circadian oscillator in cultured cells of chicken pineal gland. *Nature Lond.* 282: 94–96, 1979.
4. DEGUCHI, T. Rhodopsin-like photosensitivity of isolated chicken pineal gland. *Nature Lond.* 290: 706–707, 1981.
5. ESKIN, A. Identification and physiology of circadian pacemakers. *Federation Proc.* 38: 2570–2572, 1979.
6. FARBER, J. L., O. O. HOLOWECKY, A. SERRONI, AND G. D. V. VAN ROSSUM. Effects of ouabain on potassium transport and cell volume regulation in rat and rabbit liver. *J. Physiol. Lond.* 417: 389–402, 1989.
7. HAMDORF, K., P. HOCHSTRATE, G. HOGLUND, B. BURBACH, AND U. WIEGAND. Light activation of the sodium pump in blowfly photoreceptors. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 162: 285–300, 1988.
8. HANSEN, O. Interaction of cardiac glycosides with ($Na^+ + K^+$)-activated ATPase. A biochemical link to digitalis-induced inotrophy. *Pharmacol. Rev.* 36: 143–164, 1984.
9. HELLER, J., T. J. OSTWALD, AND D. BOK. Effect of illumination on the membrane permeability of rod photoreceptor discs. *Biochemistry* 9: 4884–4889, 1970.
10. HOFFMAN, E. K., AND L. O. SIMONSEN. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* 69: 315–382, 1989.
11. JACKLET, J. W. Neural organization and cellular mechanisms of circadian pacemakers. *Int. Rev. Cytol.* 89: 251–294, 1984.
12. KASAL, C. A., M. MENAKER, AND J. R. PEREZ-POLO. Circadian clock in culture: *N*-acetyltransferase activity in chick pineal glands oscillates in vitro. *Science Wash. DC* 203: 656–658, 1979.
13. KLEIN, D. C. Photoneutral regulation of the mammalian pineal gland. In: *Photoperiodism, Melatonin and the Pineal Gland. Ciba Foundation Symposium 117*, edited by D. Evered and S. Clark. London: Pitman, 1985, p. 38–56.
14. MOORE, R. Y. Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. *Federation Proc.* 42: 2783–2789, 1983.
15. PITTENDRIGH, C. S. Circadian systems: general perspective. In: *Handbook of Behavioral Neurobiology: Biological Rhythms*, edited by J. Aschoff. New York: Plenum, 1981, vol. 4, p. 57–80.
16. PROVERBIO, F., T. PROVERBIO, R. G. MATTEO, T. M. PERRONE, AND R. MARIN. Na-pump activity in rat kidney cortex cells and its relationship with the cell volume. *FEBS Lett.* 236: 318–320, 1988.
17. ROBERTSON, L. M., AND J. S. TAKAHASHI. Circadian clock in cell culture: I. Oscillation of melatonin release from dissociated chick pineal cells in flow-through microcarrier culture. *J. Neurosci.* 8: 12–21, 1988.
18. ROSSIER, B. C., K. GEERING, AND J. P. KRAEHENBUHL. Regulation of the sodium pump: how and why? *Trends Biochem. Sci.* 12: 483–487, 1987.
19. TAKAHASHI, J. S., AND M. ZATZ. Regulation of circadian rhythmicity. *Science Wash. DC* 217: 1104–1111, 1982.
20. WAINWRIGHT, S. D., AND L. K. WAINWRIGHT. Regulation of cycle in chick pineal serotonin *N*-acetyltransferase activity in vitro by light. *J. Neurochem.* 35: 451–457, 1980.
21. WATFORD, M. A "swell" way to regulate metabolism. *Trends Biochem. Sci.* 15: 329–330, 1990.
22. ZATZ, M. The role of cyclic nucleotides in the pineal gland. In: *Cyclic Nucleotides II: Physiology and Pharmacology*, edited by J. W. Kebabian and J. A. Nathanson. New York: Springer, 1982, p. 691–710.
23. ZATZ, M., AND D. A. MULLEN. Norepinephrine, acting via adenylate cyclase, inhibits melatonin output but does not phase-shift the pacemaker in cultured chick pineal cells. *Brain Res.* 450: 137–143, 1988.
24. ZATZ, M., AND D. A. MULLEN. Photoendocrine transduction in cultured chick pineal cells (II): effects of forskolin, 8-bromocyclic AMP and 8-bromocyclic GMP on the melatonin rhythm. *Brain Res.* 453: 51–62, 1988.
25. ZATZ, M., AND D. A. MULLEN. Does calcium influx regulate melatonin production through the circadian pacemaker in chick pineal cells? Effects of nitrendipine, Bay K 8644, Co^{2+}, Mn^{2+}, and low external Ca^{2+}. *Brain Res.* 463: 305–316, 1988.
26. ZATZ, M., AND D. A. MULLEN. Ouabain (or salt solution lacking potassium) mimics the effects of dark pulses on the circadian pacemaker in cultured chick pineal cells. *Brain Res.* 501: 37–45, 1989.
27. ZATZ, M., AND D. A. MULLEN. Photoendocrine transduction in cultured chick pineal cells (III): ouabain (or dark) pulses can block, overcome, or alter the phase response of the melatonin rhythm to light pulses. *Brain Res.* 501: 46–57, 1989.
28. ZATZ, M., D. A. MULLEN, AND J. R. MOSKAL. Photoendocrine transduction in cultured chick pineal cells: effects of light, dark, and potassium on the melatonin rhythm. *Brain Res.* 438: 199–215, 1988.
29. ZATZ, M., AND H.-M. WANG. High salt mimics effects of light pulses on circadian pacemaker in cultured chick pineal cells. *Am. J. Physiol.* 260 (*Regulatory Integrative Comp. Physiol.* 29): R769–R776, 1991.