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Inducible Cyclic AMP Early Repressor Protein in Rat Pinealocytes: A Highly Sensitive Natural Reporter for Regulated Gene Transcription

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ABSTRACT

Rhythmic activity of arylalkylamine *N*-acetyltransferase (AANAT) determines melatonin synthesis in rat pineal gland. The transcriptional regulation of AANAT involves the activating and inhibiting transcription factors of the cyclic AMP (cAMP)-signaling pathway, cAMP response element-binding protein and inducible cAMP early repressor (ICER), respectively. Activation of this pathway is centered around norepinephrine, stimulating β_1-adrenergic receptors, but various other transmitters can modulate melatonin biosynthesis. To compare the transcriptional impact of norepinephrine with that of other neurotransmitters on melatonin synthesis, we determined ICER protein levels in pinealocytes and, in parallel, hormone secretion. The dose-dependent inductions of ICER protein by norepinephrine, the β_1-adrenergic receptor agonist isoproterenol, vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, and adenosine are correlated to regulatory dynamics in melatonin production. Importantly, ICER protein induction required lower ligand concentrations than the induction of melatonin biosynthesis. Although neuropeptide Y, glutamate, and vasopressin altered norepinephrine-stimulated hormone production without affecting ICER levels, the activation of voltage-gated cation channels increased ICER without affecting hormone synthesis. Sensitivity and versatility of ICER induction in pinealocytes make these neuroendocrine cells a valuable model system in which to study molecular interactions determining a regulated gene expression.

Short-term stimuli can elicit long-term adaptation by affecting gene expression. Within such plasticity, the neurotransmitter-induced synthesis of hormones often functions as an important mediator for stimulus-effector coupling. The mammalian pineal gland serves as an excellent model system for this coupling, as neural signals that code for changes in ambient lighting conditions determine the nocturnally elevated synthesis of the hormone melatonin as a message of darkness for the body (Korf et al., 1998). This day/night rhythm depends on the regulation of the arylalkylamine *N*-acetyltransferase (AANAT), the key enzyme of melatonin biosynthesis, which serves as a molecular interface for all stimuli affecting hormone production (Stehle, 1995; Klein et al., 1996; Foulkes et al., 1997; Maronde et al., 1999).

The most important regulator of AANAT is norepinephrine (NE), which is released in high amounts from sympathetic nerve endings during the dark period (Drijfhout et al., 1996). Via stimulation of β_1-adrenergic receptors, NE activates the cyclic AMP (cAMP)-signaling pathway that influences AANAT levels and activity through transcriptional and post-transcriptional control mechanisms (Stehle, 1995; Klein et al., 1996; Gastel et al., 1998). Transcriptional control involves the post-translational modification of a constitutively expressed activating transcription factor (TF) [i.e., the phosphorylation of cAMP response element-binding protein (CREB); Roseboom and Klein, 1995; Tamotsu et al., 1995; Maronde et al., 1999)] that can bind to the AANAT-cAMP responsive element (CRE; Baler et al., 1997). Nocturnally elevated NE levels also cause a drastic increase in the amount of the mRNA of the inhibitory TF inducible cAMP early repressor (ICER) in rat pineal gland during the second half of the night, whereas transcripts are barely detectable during the progressed light phase (Stehle et al., 1993). The idea that this TF inhibits melatonin synthesis in rat pineal

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ABBREVIATIONS: AANAT, arylalkylamine *N*-acetyltransferase; AC; adenylate cyclase; cAMP, cyclic AMP; ACh, acetylcholine; AVP, arginine-vasopressin; CREB, cyclic AMP response element-binding protein; ICER, inducible cyclic AMP early repressor; ISO, isoproterenol; IR, immunoreaction; NE, norepinephrine; NECA, 5′-*N*-ethylcarboxy-amidoadenosine; NGF, nerve growth factor; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating polypeptide; pCREB, phosphorylated cyclic AMP response element-binding protein; PHE, phenylephrine; PRAZO, prazosin; PROP, propranolol; VP4-9, arginine-vasopressin fragment 4-9; TF, transcription factor; VIP, vasoactive intestinal peptide.

gland via transcriptional mechanisms at the end of the night (Stehle, 1995) is supported by the recent observations that: 1) ICER protein levels in rat pineal gland are strongly elevated during the second part of the night, 2) ICER binds to the AANAT-CRE, and 3) AANAT transcription is disinhibited after transfection of pinealocytes with an ICER antisense construct (Maronde et al., 1999).

In addition, post-translational changes of the AANAT protein seem to be involved in rapid alterations of AANAT enzyme activity and melatonin formation (Gastel et al., 1998; Maronde et al., 1999). Thus, a concerted action of transcriptional and post-transcriptional NE-dependent cAMP-directed mechanisms determines rhythmic hormone production in rat pineal gland.

In addition to the NE/cAMP pathway as the dominant regulator, various neurotransmitters/hormones can modulate melatonin synthesis in rat pineal gland as indicated by pharmacological investigations, binding studies, and data from molecular biology analyses (Stehle, 1995; Korf et al., 1998). To assess whether these modulators affect melatonin synthesis in rat pineal gland at the transcriptional level via ICER, we compared changes in ICER protein levels for each given stimulus with its effect on melatonin biosynthesis and compared these results with those obtained after NE stimulation. We found that NE and other cAMP-elevating agents, like vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and adenosine, always affect ICER protein levels and melatonin synthesis, but the ligand concentrations needed to induce ICER were significantly lower than those required to affect hormone synthesis. Notably, arginine-vasopressin (AVP), glutamate, and neuropeptide Y (NPY) affected NE-induced melatonin synthesis without a change in ICER levels, whereas activation of voltage-gated cation channels induced ICER expression without affecting melatonin synthesis. Our findings show that the TF ICER can be considered as a highly sensitive natural reporter for stimulated signaling pathways in rat pinealocytes. This makes the system a valuable tool to address regulatory mechanisms in neuroendocrine coupling.

Materials and Methods

Animal Maintenance. All experiments were conducted in accordance with the guidelines on the care of experimental animals manifested in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" as approved by the European Communities Council Directive (86/609/EEC).

Male Wistar rats (150–200 g) were raised under a photoperiod of 12 h light/12 h darkness with food and water ad libitum. Animals were sacrificed during the early light phase by decapitation, and the pineal gland was removed and transferred immediately into ice-cold Earle's balanced salt solution medium (for details, see Pfeffer et al., 1998).

Primary Pinealocyte Cultures. Primary pinealocyte cultures were prepared and maintained as described previously (Pfeffer et al., 1998). After 48 h in culture, cells were transferred into fresh medium and stimulated with different substances at various concentrations for up to 24 h. In control preparations, only the dissolving agent was added to the medium. Cell morphology was investigated routinely during and at the end of each experiment by visual inspection, and viability was tested by trypan blue exclusion (data not shown).

To analyze the dose-dependent inducibility of ICER protein, primary pinealocyte cultures were stimulated for 5 h with NE (10^{-5}–10^{-14} M), the β_1-adrenergic agonist isoproterenol (ISO; 10^{-5}–10^{-14} M), VIP (10^{-7}–10^{-12} M), or PACAP (10^{-7}–10^{-12} M). In time course experiments, cells were treated with 10^{-6} M NE or 10^{-6} M ISO for up to 24 h or with 10^{-7} M VIP or 10^{-8} M PACAP for up to 12 h. The doses were selected from the dose-response curves and elicited maximal responses (see below).

To address synergistic effects on the receptor level, cells were incubated with the α-adrenergic agonist phenylephrine (PHE; 10^{-7} M), the α-adrenergic antagonist prazosin (PRAZO; 10^{-6} M), or the β-adrenergic antagonist propranolol (PROP; 10^{-6} M) 20 min before and during a 5-h stimulation with NE (10^{-6} M), ISO (10^{-6} and 10^{-8} M), PHE (10^{-7} M) (all doses according to Vanecek et al., 1985), VIP (10^{-7} M), or PACAP (10^{-7} M) (doses according to Simonneaux et al., 1990, 1993; Chik and Ho, 1995).

Additional cell preparations were treated for 5 h with either AVP (10^{-7} M; Stehle et al., 1991), the proteolytic AVP fragment VP4-9 (10^{-7} M; Stehle et al., 1991), NPY (10^{-6} M; Olcese, 1991), acetylcholine (ACh; 10^{-4} M; Yamada et al., 1998a), glutamate (10^{-3} M; Yamada et al., 1998b), and KCl (60 mM) alone, or glutamate in combination with NE (10^{-6} M).

In additional experiments, cells were incubated with 2-chloroadenosine (10^{-6} M; dose according to Nikodijevic and Klein, 1989), the selective A_2 adenosine receptor agonist 5′-*N*-ethylcarboxy-amidoadenosine (NECA; 10^{-6} M; dose according to Nikodijevic and Klein, 1989), KCl (30 and 60 mM), epidermal growth factor (50 ng/ml), nerve growth factor (NGF; 50 ng/ml), and brain-derived neurotrophic factor (40 ng/ml) (doses of growth factors according to Monaco and Sassone-Corsi, 1997, and the manufacturer).

In all experiments, the concentration of melatonin secreted into the medium by pinealocytes was analyzed by enzyme-linked immunosorbent assay (see below). In one set of experiments, cells were stimulated for 30 min with NE (10^{-6} M), VIP (10^{-7} M), or PACAP (10^{-7} M), and the cAMP content in the medium was subsequently analyzed by radioimmunoassay (see below). All chemicals were obtained from Sigma GmbH (Deisenhofen, Germany) or from Novabiochem (Bad Soden, Germany), unless indicated otherwise.

Melatonin Assay. Melatonin concentration in the medium was measured by means of an enzyme-linked immunosorbent assay based on a commercial radioimmunoassay (Elias, Osceola, WI). The detection limit for melatonin in this assay is 1.5 pg/ml (for details, see Maronde et al., 1999). For all experiments, medium was collected in at least three independent experiments, and samples were assayed in duplicate. Extracellular cAMP was measured based on a commercial radioimmunoassay with slight modifications (Maronde et al., 1999).

Immunocytochemistry. After the indicated stimulation periods, cells were fixed with 4% paraformaldehyde for 10 min and processed as described (Tamotsu et al., 1995; Maronde et al., 1999). Cells were incubated with a rabbit polyclonal ICER antibody (Maronde et al., 1999) in a dilution of either 1:75,000 or 1:100,000 in PBS containing 0.3% Triton X-100 and 1% BSA. In selected experiments, a rabbit polyclonal antibody recognizing Ser^{133} phosphorylated CREB (pCREB; lot 9190/001;1:500; New England Biolabs; Beverly, MA) was used as described (Tamotsu et al., 1995; Maronde et al., 1999). The immunoreaction (IR) was visualized with a biotin-conjugated anti-rabbit IgG (Sigma GmbH) as second antibody, a horseradish peroxidase-conjugated strepavidin antibody (Sigma GmbH) as third antibody, and diaminobenzidine as the chromogen as described (Tamotsu et al., 1995).

Immunoblot Analysis. The immunocytochemical demonstration of ICER protein was complemented by immunoblotting. Proteins from pinealocytes that were unstimulated or stimulated for 5 h with 10^{-6} M NE, 10^{-7} M VIP, or 10^{-7} M PACAP were electrophoresed on a 15% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes (0.45 μM; Bio-Rad Laboratories, Hercules, CA) as described (Wicht et al., 1999). Membranes were incubated with the ICER antibody (1:100,000) or with a total CREB antibody (1:2500; New England Biolabs, Beverly, MA) overnight at 4°C. Signals were detected by chemoluminescence (Pierce Chemical Co., Rockford, IL)

with a horseradish peroxidase-coupled goat anti-rabbit IgG antibody (New England Biolabs).

Data Analysis. Computer-assisted semiquantitative analysis of the immunocytochemically detected ICER signal was performed as described and expressed as the product of density and area of a given signal, corrected for the total area covered by the cells (corrSUMDENS values) (Wicht et al., 1999). Briefly, for each time point, treatment, and dose, three randomly chosen areas covering a total of 100 cells were analyzed. For evaluation of data obtained with one experimental paradigm, the highest corrSUMDENS value from an individual experiment was set as 100%, and all other values were expressed as a percentage of the maximum. Similarly, for comparison of individual experiments, the melatonin content in the medium was expressed as a percentage of the maximal value in a given experiment. In the time course experiments, net melatonin synthesis was calculated between subsequent time points.

Data from at least three independent experiments with two samples each were statistically analyzed with Prism (GraphPAD Prism Software, San Diego, CA; ICER and melatonin, ANOVA with subsequent Bonferroni tests for multiple comparisons with $P < .05$ as the criterion of significance; cAMP, Student's t test) and expressed as mean ± S.E.M.

Immunoblot signals were analyzed semiquantitatively as described (Pfeffer et al., 1998; Maronde et al., 1999; Wicht et al., 1999) with a computer-assisted image analysis system (KS 300; Kontron, Eching, Germany).

Results

β_1-Adrenergic Mechanisms Are Central for Highly Sensitive ICER Protein Induction. In rat pinealocytes, 5-h treatment with NE induced in nearly all cells a nuclear ICER IR that increased dose-dependently. Elevated signals were first observed with a concentration of 10^{-11} M NE, and maximal values in the ICER IR were reached at a concentration of $>10^{-8}$ M (Figs. 1A and 2). In the same preparations, an increase in hormone synthesis was first observed at a concentration of 10^{-9} M NE. Maximal levels of melatonin content in the medium were reached when cells were incubated with a concentration of 10^{-8} M NE (Fig. 1B). The nonreceptor-mediated activation of the adenylate cyclase (AC) by forskolin (10^{-4}–10^{-6} M) induced ICER IR and melatonin synthesis in a similar magnitude as treatment with NE (data not shown).

The β_1-adrenergic agonist ISO induced ICER protein levels and melatonin synthesis in a dose-dependent manner (Fig. 3). After 5 h of stimulation, a significant increase in the ICER IR could be observed at 10^{-9} M ISO (Fig. 3A). The maximal response was achieved at 10^{-7} M ISO (Fig. 3A). An increase in melatonin synthesis was first detectable at a concentration of 10^{-8} M ISO, and maximal values were reached at 10^{-6} M ISO (Fig. 3B). The observed thresholds for NE and ISO to induce ICER were similar to the lowest concentrations inducing CREB phosphorylation (data not shown), as reported earlier (Tamotsu et al., 1995).

To dissect NE effects on ICER protein induction, we stimulated rat pinealocytes with α- and β-adrenergic agonists and antagonists in various combinations. PHE (10^{-7} M; Fig. 4), PROP (10^{-6} M), and PRAZO (10^{-6} M) alone did not affect the ICER protein level and left melatonin synthesis unaltered (data not shown). NE effects on ICER protein level and melatonin synthesis were not influenced by coincubation with 10^{-6} M PRAZO (Fig. 4). In contrast, coincubation with 10^{-6} M PROP diminished NE-induced ICER IR by 41 ± 5% (Fig. 4A) and melatonin levels by 64 ± 4% (Fig. 4B).

ISO concentrations $>10^{-8}$ M led to an ICER protein induction comparable to that seen with NE concentrations of $>10^{-9}$ M (Fig. 4A). The ISO-induced ICER protein level was reduced by 42 ± 8% by the addition of PROP (10^{-6} M; Fig. 4A) to the culture medium. In the same preparations, melatonin synthesis remained slightly elevated, probably due to insufficient blockade of β-adrenergic receptors by PROP (Fig. 4B; Vanecek et al., 1985). Coincubation of ISO-stimulated (10^{-6} M) pinealocytes with PHE (10^{-7} M; Fig. 4) or PRAZO (10^{-6} M; Fig. 4) did not affect ICER protein levels or melatonin synthesis. However, when using a lower concentration of ISO (10^{-8} M) that resulted in a 34 ± 7% reduced ICER IR and a 35 ± 3% reduced melatonin synthesis compared with 10^{-6} M ISO (Figs. 4 and 5D), a potentiating effect of PHE (10^{-7} M; Figs. 4 and 5, D and E) could be observed (ICER, 28 ± 2% increase; melatonin synthesis, 35 ± 5% increase; compared with 10^{-8} M ISO alone; Fig. 4). Although coincubation of cells with PRAZO (10^{-6} M) had no effect on the ISO (10^{-8} M)-induced ICER protein levels or melatonin synthesis, PROP (10^{-6} M) abolished the ISO-induced ICER IR and melatonin synthesis (Figs. 4 and 5F).

On the basis of the dose-response relationship between NE

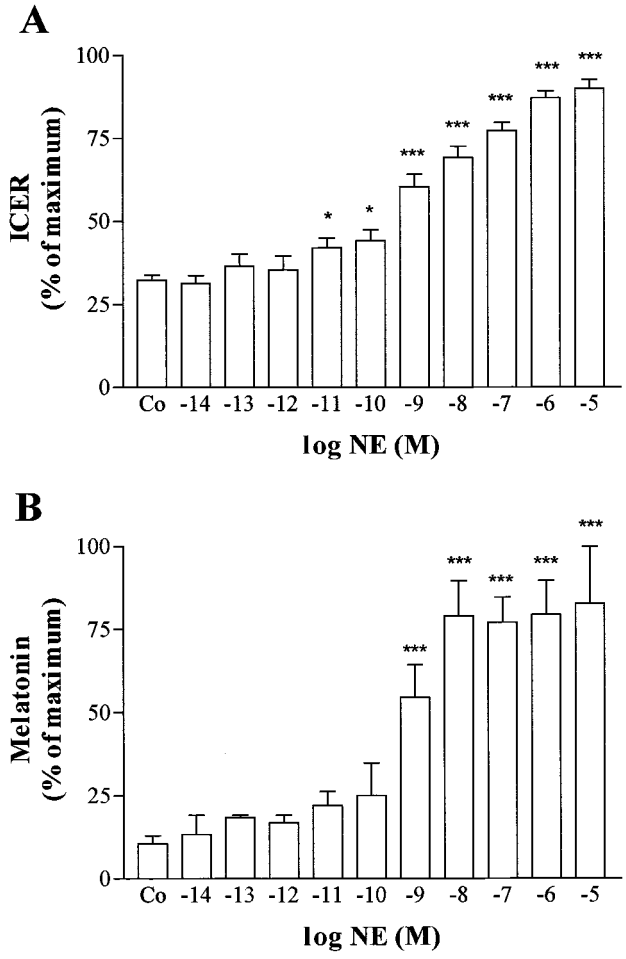

Fig. 1. Dose dependence of norepinephrine-induced ICER IR (A) and corresponding melatonin synthesis (B) in rat pinealocytes. Intensity of ICER IR and melatonin content in the medium are expressed as a percentage of the maximum [$n \geq 4$; *$p < .05$, versus control (Co); ***$p < .001$ versus Co]. Note that a concentration of 10^{-11} M NE induces an ICER signal, but melatonin synthesis rises only with NE concentrations of $>10^{-9}$ M (see also Fig. 2).

concentration and ICER IR, we next investigated ICER dynamics during a 24-h stimulation with 10^{-6} M NE (Fig. 6, A and B). A significant increase in ICER protein levels was first detectable 4 h after the start of NE stimulation. The values in ICER IR peaked after 8 h and declined gradually thereafter. After 22 h, ICER protein levels again had reached background levels, despite a continuous NE stimulation. Net melatonin synthesis increased sharply within the first 8 h of NE stimulation, with the highest synthesis rate between 6 h and 8 h, slowed down during the next 4 h, and ceased after 14 h (Fig. 6A).

In a similar experiment as described above, the effects of 10^{-6} M ISO were investigated. A significant increase in ICER protein levels was detectable 6 h after the addition of ISO (Fig. 6C). ICER IR peaked after 10 h of stimulation and declined gradually thereafter (Fig. 6C). After 16 h, ICER protein levels had reached control levels again. A significant increase in net melatonin synthesis occurred within the first 6 h of ISO stimulation, with peak values occurring after 8 h of stimulation (Fig. 6C). After 12 h of ISO stimulation, no further increase in net melatonin synthesis could be detected. The slower increase and the earlier decline in ICER protein levels and melatonin synthesis induced by ISO stimulation compared with NE effects may be due to the sole activation of β_1-adrenergic receptors by ISO.

Low-Efficiency ICER Protein Induction by VIP and PACAP. Treatment of rat pinealocytes with VIP or PACAP (10^{-7} M) elicited an approximately 2-fold increase in cAMP levels in the medium within 30 min, whereas NE (10^{-6} M) increased cAMP levels 25-fold in the medium (Fig. 7A). Analysis of protein extracts from cells stimulated for 5 h with NE, VIP, or PACAP revealed in the immunoblot the induction of two specific bands, corresponding in size to the ICER and ICERγ isoforms (Fig. 7B). Semiquantitative analysis of induced ICER IR in pinealocytes from parallel experiments revealed a 3.5-fold increase in TF IR after treatment with NE and a 2-fold increase after treatment with VIP or PACAP (Fig. 7C). VIP- and PACAP-induced melatonin synthesis reached approximately 40% of the NE-induced melatonin level (Fig. 7D). In contrast to the ubiquitous ICER induction seen in pinealocytes after NE stimulation, only 40 to 70% of the pinealocytes were ICER immunoreactive after stimulation with VIP or PACAP, supporting the suggested functional heterogeneity of mammalian pineal cells (Korf et al., 1998). Notably, the VIP- and PACAP-induced ICER IR was less intense than the NE-induced signal.

The dose-response analyses revealed that VIP (5 h) induced ICER protein levels in concentrations of $>10^{-10}$ M (Fig. 8A). Plateau levels were reached at a concentration of $>10^{-8}$ M VIP. In the same preparations, a significant increase in melatonin synthesis was first observed at a VIP concentration of 10^{-8} M (Fig. 8B). In parallel experiments, PACAP concentrations $>10^{-10}$ M induced ICER protein (Fig. 8C). In the same preparations, PACAP treatment increased

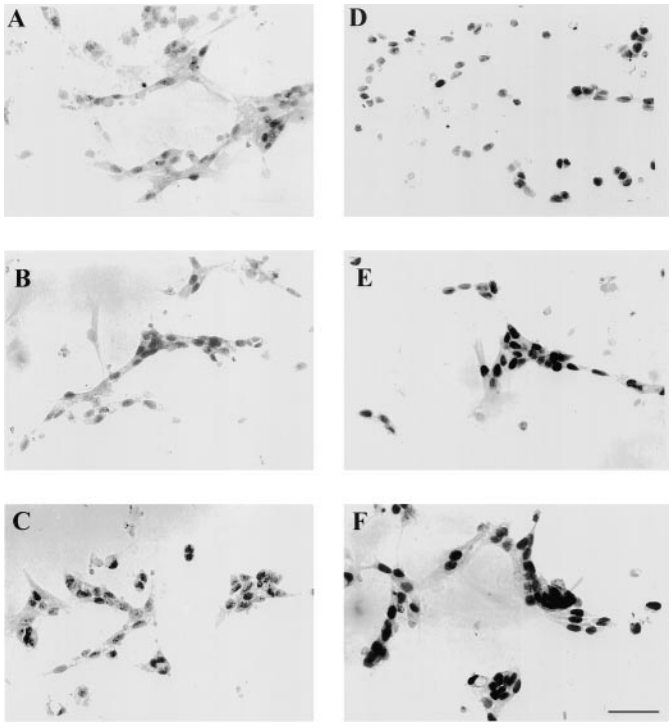

Fig. 2. NE-induced ICER IR in isolated rat pinealocytes. Cells were untreated (A) or stimulated for 5 h with different concentrations of NE (B, 10^{-13} M; C, 10^{-11} M; D, 10^{-9} M; E, 10^{-7} M; F, 10^{-5} M). Note that the ICER IR is restricted to the nucleus (see also Fig. 1). Scale bar indicates 50 μM.

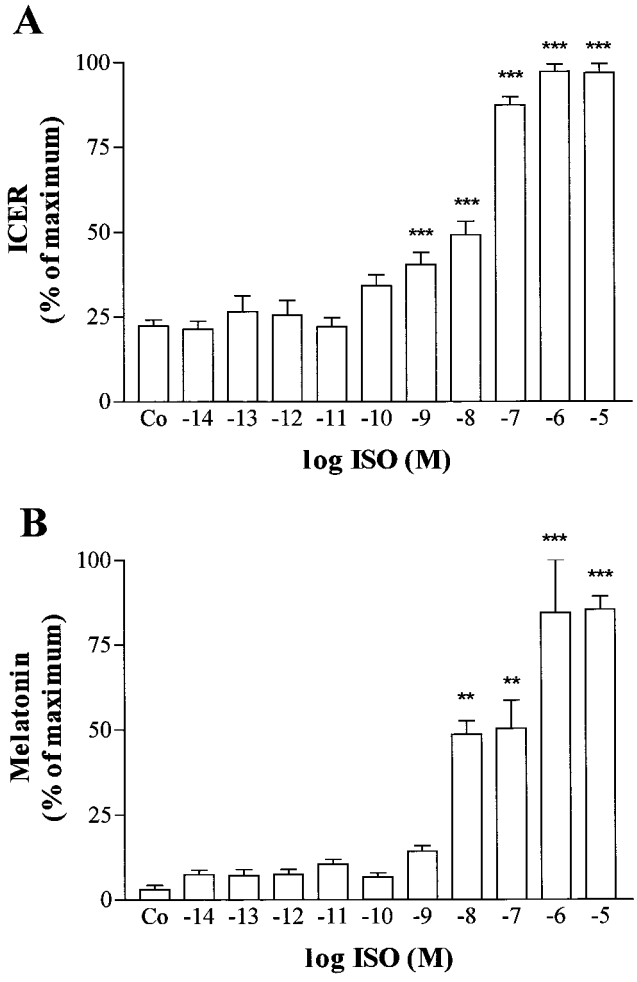

Fig. 3. Dose dependence of isoproterenol-induced ICER IR (A) and corresponding melatonin synthesis (B) in rat pinealocytes. Intensity of ICER IR and melatonin content in the medium are expressed as a percentage of the maximum [$n \geq 4$; **$p < .01$ versus control (Co); ***$p < .001$ versus control). Note that ICER IR is induced at lower doses (10^{-10} M ISO) than melatonin synthesis (10^{-8} M ISO).

melatonin production at concentrations of $>10^{-8}$ M (Fig. 8D). The threshold concentrations for VIP and PACAP to induce an ICER IR also elicited a pCREB IR in pinealocytes (data not shown), as reported earlier (Schomerus et al., 1996).

To investigate the synergistic interactions between adrenergic and peptidergic stimulation in rat pineal gland (VIP, Chik et al., 1988; PACAP, Chik and Ho, 1995), we stimulated cells with 10^{-7} M VIP (Fig. 8E) and 10^{-7} M PACAP (Fig. 8F), alone or in combination with adrenergic agonists and antagonists (Fig. 8, E and F). PHE potentiated VIP- and PACAP-induced ICER IR in rat pinealocytes by 25 ± 10 and 31 ± 7%, respectively (Fig. 8, E and F). Preincubation with PROP or PRAZO had no effects on VIP- and PACAP-induced ICER protein levels (Fig. 8, E and F). In the same preparations, VIP- or PACAP-induced melatonin synthesis was potentiated by PHE by 31.9 ± 9 and 35.8 ± 8%, respectively, whereas PROP and PRAZO had no effect on hormone production (Fig. 8, E and F).

In time course experiments, an increase in VIP-induced ICER IR was first visible after 4 h of a continuous stimulation of rat pinealocytes. ICER IR peaked 8 h after stimulation and subsequently decreased to control levels (Fig. 8G). In the same preparations, an increase of net melatonin synthesis was observed within the first 6 h of simulation, with no further melatonin synthesis thereafter (Fig. 8G).

As seen with VIP, continuous stimulation with PACAP (10^{-8} M) induced ICER protein levels only transiently (Fig. 8H). An induction of an ICER IR was first visible after 6 h of stimulation with PACAP. After 10 h of stimulation, elevated ICER values had decreased to background levels again (Fig. 8H). In the same preparations, an increase in net melatonin synthesis could be observed after 4 h of stimulation with PACAP (Fig. 8H). After 6 h of stimulation, no further increase in net melatonin synthesis could be observed. The time differences in peak values in ICER IR induced by VIP (4 h) or PACAP (6 h) compared with NE (8 h) or ISO (10 h) can be explained by the more pronounced and prolonged enhancement of the ICER IR by adrenergic stimuli compared with peptidergic stimulation (compare Fig. 6, A and C, with Fig. 8, G and H). Interpretation of these data must take into account that the peak values observed for VIP and PACAP

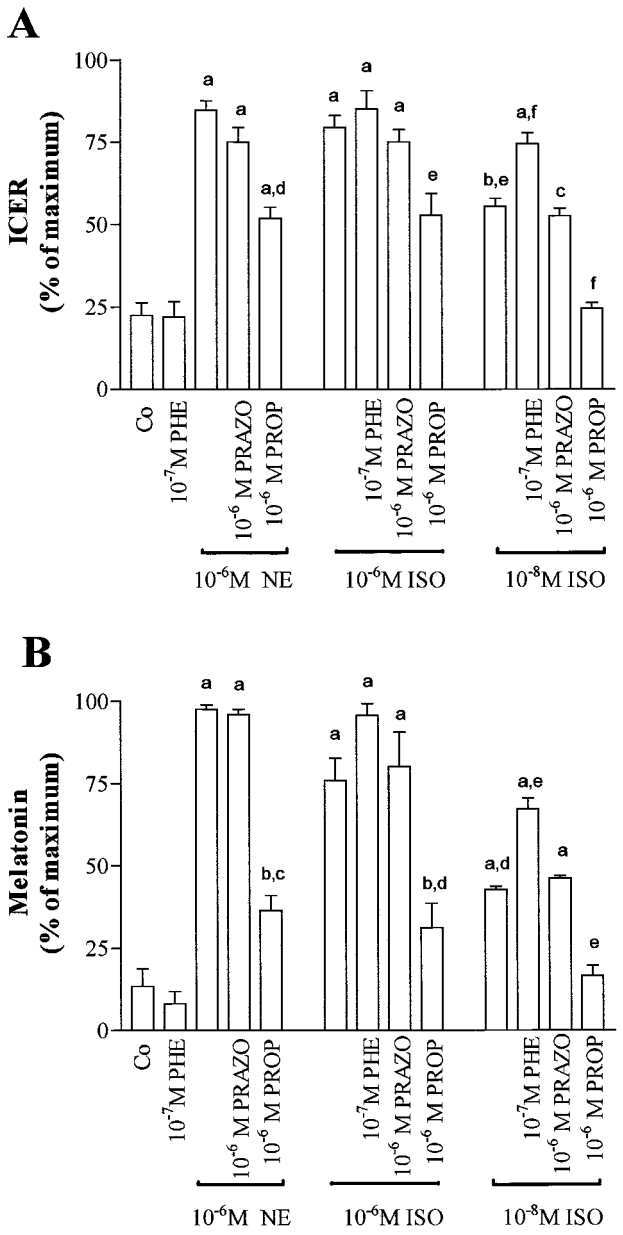

Fig. 4. ICER induction and melatonin synthesis are primarily mediated by β_1-adrenergic mechanisms. Intensity of ICER IR (A) and melatonin content in the medium (B) are expressed as a percentage of the maximum ($n \geq 4$). Note that PHE potentiates induction of ICER IR elicited by low concentrations of ISO. Co, control. A, $^{a}p < .001$ versus control; $^{b}p < .01$ versus control; $^{c}p < .05$ versus control; $^{d}p < .001$ versus 10^{-6} M NE; $^{e}p < .001$ versus 10^{-6} M ISO; $^{f}p < .001$ versus 10^{-8} M ISO. B, $^{a}p < .001$ versus control; $^{b}p < .01$ versus control; $^{c}p < .001$ versus 10^{-6} M NE; $^{d}p < .001$ versus 10^{-6} M ISO; $^{e}p < .001$ versus 10^{-8} M ISO).

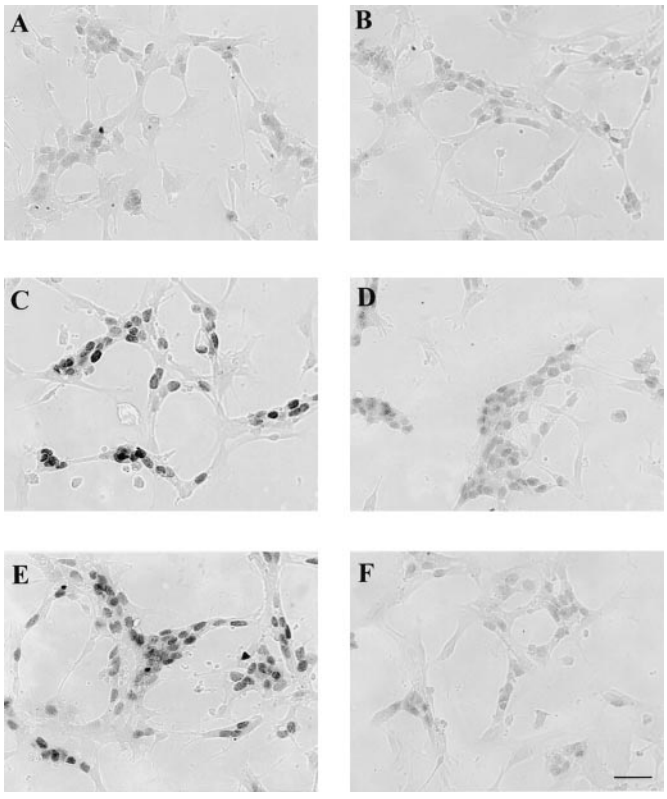

Fig. 5. Adrenergic mechanism inducing ICER IR in isolated rat pinealocytes. Cells were untreated (A) or stimulated for 5 h with PHE (B), NE (C), ISO (10^{-8} M; D), 10^{-8} M ISO plus PHE (E), or 10^{-8} M ISO plus PROP (F). Scale bar indicates 50 μM.

after 4 h are less than half-maximum of the ICER increase after a 4-h NE stimulation. In addition to the lower efficiency of VIP and PACAP, the earlier decline in VIP- or PACAP-induced ICER IR in pinealocytes speaks in favor of a faster desensitization of these receptors.

In addition, we investigated the influence of the neuropeptides AVP, the AVP fragment VP4-9, and NPY on ICER induction and melatonin synthesis. ICER protein levels were not affected by AVP or VP4-9 alone (data not shown) or when applied in combination with NE (Table 1). However, in accordance with an earlier report (Stehle et al., 1991), the NE-induced melatonin synthesis was further enhanced by AVP (23 ± 5%; Table 1).

NPY alone or in combination with NE had no effects on ICER protein levels (Table 1). However, a moderate inhibition of NE-induced melatonin synthesis was observed after a coincubation of pinealocytes with NPY (23 ± 7%; Table 1), as reported earlier (Olcese, 1991).

Adenosine Is a Potent Stimulator for ICER Protein. Stimulation of pinealocytes with adenosine induced ICER protein in virtually all cells, although with a smaller intensity than NE (Table 1). The subsequent analysis of melatonin content in the medium revealed that the effect of adenosine on melatonin levels was only about one third of the NE effect (Table 1). Interestingly, stimulation of pinealocytes with the A_2 adenosine receptor agonist NECA induced ICER IR more efficiently than adenosine (Fig. 9F; Table 1). NECA was more than twice as effective as adenosine in inducing melatonin synthesis (Table 1). Although our data indicate that the effect of adenosine on pineal signaling seems to consist of a predominant A_2 adenosine component eliciting stimulation of AC and a small A_1 adenosine component eliciting inhibition

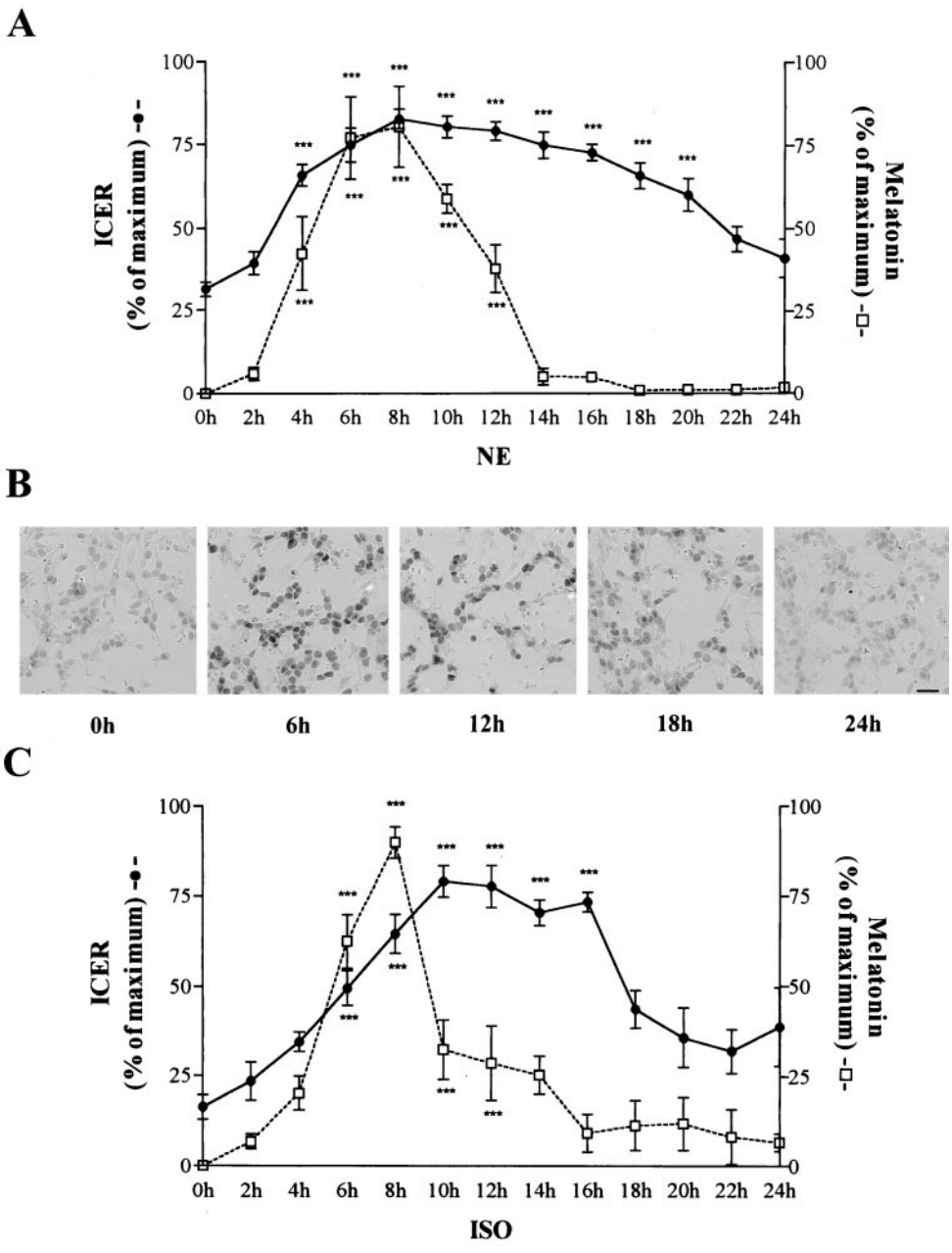

Fig. 6. Dynamics in ICER induction and melatonin synthesis (A and C) after a continuous 24-h stimulation with NE (A and B) or ISO (C). Intensity of ICER IR (A and C) is expressed as a percentage of the maximum ($n \geq 3$). Corresponding melatonin values are expressed as a percentage of the maximum of net synthesis (***$p < .001$ compared with the corresponding control value at the beginning of the experiment). Note the decrease in net melatonin synthesis with increasing ICER protein levels and the decline in ICER protein levels with time because of the described autoregulatory mechanism (Molina et al., 1993). B, the NE-induced ICER IR in pinealocytes at selected time points. Scale bar indicates 50 μM.

of AC (Nikodijevic and Klein, 1989; Stehle, 1995), a more thorough analysis of adenosinergic effects on ICER IR is needed, comparing the potency and effects of selective antagonists.

Depolarization of Pinealocytes Induces an ICER IR. Depolarization of pinealocytes induced by KCl (30, 60 mM) increased ICER protein levels (Fig. 9E; Table 1). In contrast, melatonin synthesis was not affected by KCl-induced depolarization (Table 1). Incubation of pinealocytes with NE and parallel depolarization of cells with 60 mM KCl did not change NE-induced ICER protein levels but reduced NE-induced melatonin synthesis by 44 ± 9% (Table 1). KCl treatment induced a low, but significant, pCREB IR in pinealocytes (data not shown), as reported earlier (Roseboom and Klein, 1995).

ACh and Glutamate Do Not Affect ICER IR. Incubation of pinealocytes with ACh or glutamate alone did not induce an ICER IR (Table 1) or a pCREB IR (C. Schomerus and H.-W.K., unpublished observations). Also, the NE-induced ICER IR was not affected by coincubation of cells with ACh and glutamate (Table 1). However, glutamate reduced NE-induced melatonin synthesis to 52 ± 2%, whereas ACh did not influence the NE-induced hormone production (Table 1).

Incubation of pinealocytes for 5 h with the growth factors NGF, epidermal growth factor, and brain-derived neurotrophic factor did not induce ICER protein or melatonin synthesis (data not shown). In PC12 cells, at least NGF was able to induce ICER mRNA by activation of a Ras-dependent signaling pathway and subsequent phosphorylation of CREB (Monaco and Sassone-Corsi, 1997). Because this ICER induction was abolished on neuronal differentiation of the PC12 cells, we suggest that the lack of effects of growth factors on ICER expression in cultured pinealocytes reflects the postmitotic differentiation state of these cells.

Discussion

In rat pineal gland, ICER can be considered as a natural reporter for stimuli that increase cAMP levels and affect melatonin biosynthesis transcriptionally. NE, the primary neurotransmitter shaping melatonin production in rat pineal gland, is also the most potent enhancer of ICER protein levels, providing further evidence for the role of the inhibi-

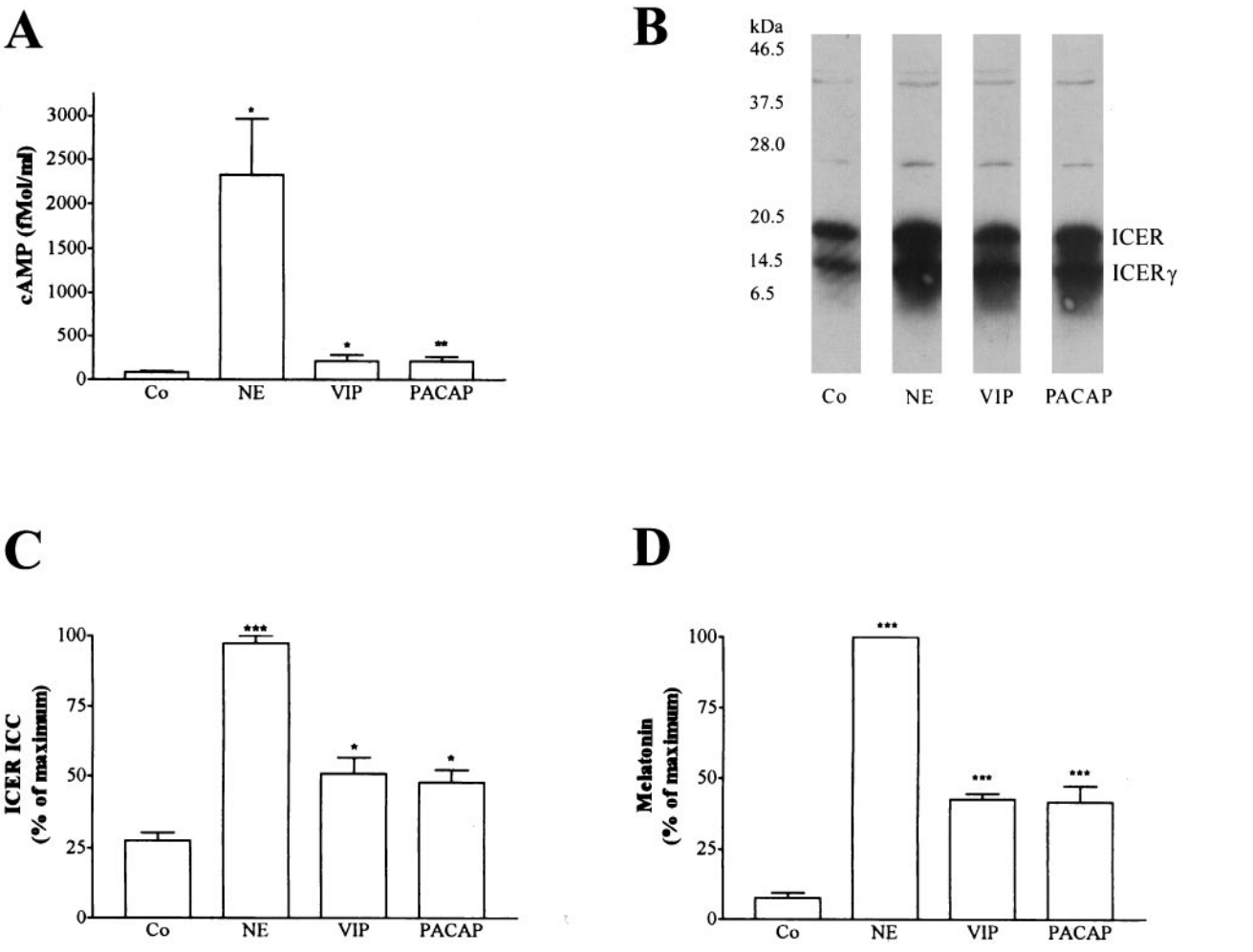

Fig. 7. Characterization of effects of VIP and PACAP on ICER IR and melatonin synthesis. Comparison of effects induced by NE, VIP, or PACAP on cAMP content as assessed by cAMP concentration in the medium (A), on the ICER IR detected in immunoblot (B) or immunocytochemical preparations (C), and on melatonin synthesis (D). cAMP levels were measured 30 min after stimulation, ICER IR and melatonin synthesis after 5 h. Note the different efficiency of VIP and PACAP to NE-effects in all parameters analyzed. The two specific bands in B correspond in size to the ICER and ICERγ isoforms (Maronde et al., 1999).

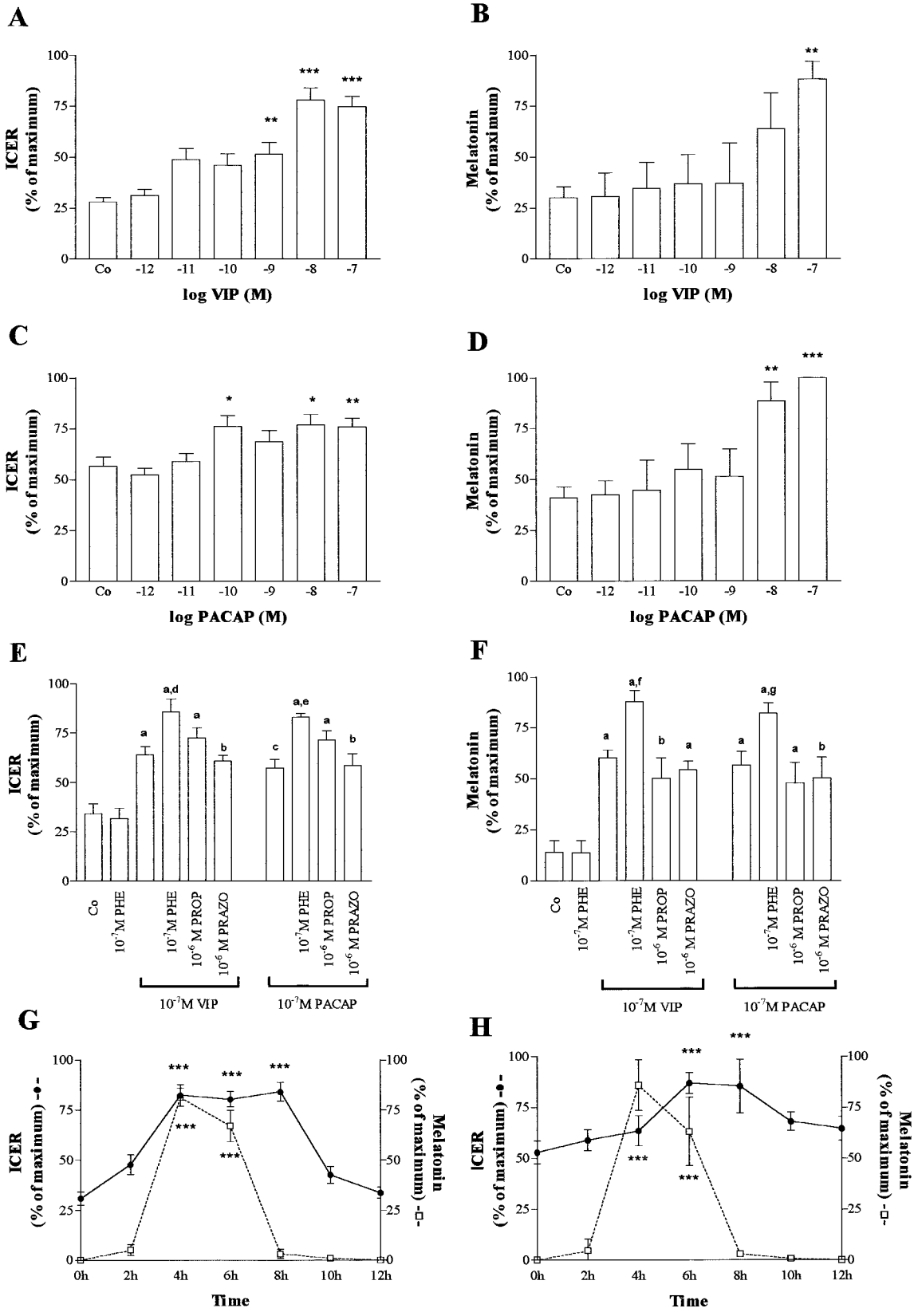

Fig. 8. Dynamics in VIP- and PACAP-induced ICER protein levels and melatonin synthesis. Dose dependence of VIP- and PACAP-induced ICER IR (A and C) and melatonin synthesis (B and D). Note that the threshold for ICER IR induction is lower than that for melatonin synthesis. Analysis of VIP- and PACAP-effects on ICER IR (E) and melatonin synthesis (F). Note that PHE potentiates the VIP- and PACAP-induced ICER IR in rat pinealocytes [$^{a}p < .001$ versus control (Co), $^{b}p < .01$ versus control, $^{c}p < .05$ versus control, $^{d}p < .05$ versus VIP, $^{e}p < .01$ versus PACAP, $^{f}p < .01$ versus VIP, $^{g}p < .05$ to PACAP]. ICER IR and melatonin are from the same preparations and are expressed as a percentage of the maximum ($n \geq 5$ for all experiments; $*p < .05$, $**p < .01$, $***p < .001$). Dynamics in ICER induction and melatonin synthesis after a continuous 12-h stimulation with VIP (G) or PACAP (H). Note the decrease in net melatonin synthesis with increasing ICER protein levels. Intensity of ICER IR and net melatonin synthesis are expressed as percentage of the maximum ($n \geq 4$; $*p < .05$, $**p < .01$, $***p < .001$ versus control values at the beginning of the experiment).

tory TF ICER in melatonin synthesis in vivo (Maronde et al., 1999). The superior sensitivity of the ICER induction and the dynamics in TF protein abundance demonstrate that a transmembrane stimulation of AANAT transcription simultaneously provides the basis for a subsequent controlled termination of enhanced gene expression. Consequently, ligands like glutamate and NPY that do not affect ICER protein levels in rat pinealocytes seem to impinge on melatonin synthesis post-transcriptionally, or they might affect AANAT transcription through cAMP-independent mechanisms only.

TABLE 1
Induction of ICER IR and melatonin production by NE, other neurotransmitters, and drugs

Additions	ICER IR	Melatonin Production
	% of maximum	
Control	26.0 ± 1.4	18.2 ± 1.9
10^{-6} M NE	89.1 ± 2.4[a]	77.7 ± 2.0[a]
10^{-6} M AVP	25.3 ± 2.2	23.3 ± 5.2
10^{-6} M NE/10^{-6} M AVP	78.3 ± 5.6[a]	100 ± 0.0[a,b]
10^{-6} M NPY	18.6 ± 2.2	18.6 ± 2.2
10^{-6} M NE/10^{-6} M NPY	82.2 ± 5.1	56.1 ± 6.2[a,d]
Control	32.8 ± 1.8	19.2 ± 2.0
10^{-6} M NE	89.3 ± 1.4[a]	95.2 ± 2.1[a]
10^{-6} M ADO	52.9 ± 2.8[a]	35.9 ± 2.1[c,d,f]
10^{-6} M NECA	66.9 ± 3.8[a,d,e]	74.2 ± 5.9[a]
Control	19.7 ± 3.4	9.3 ± 2.4
10^{-6} M NE	83.8 ± 3.8[a]	98.0 ± 2.0[a]
10^{-4} M ACh	25.4 ± 3.2	6.4 ± 1.0
10^{-6} M NE/10^{-4} M ACh	85.4 ± 3.6[a]	88.5 ± 9.4[a]
10^{-3} M glutamate	27.0 ± 4.9	9.6 ± 5.5
10^{-6} M NE/10^{-3} M glutamate	84.1 ± 3.4[a]	47.7 ± 2.1[a,b]
60 mM KCl	40.5 ± 4.4	13.0 ± 2.1
10^{-6} M NE/60 mM KCl	87.0 ± 3.9[a]	56.0 ± 8.7[a,d]

Semiquantitative densitometric analysis of ICER IR and melatonin synthesis expressed as percentage of maximum ($n \geq 3$).
[a] $p < .001$ versus control; [b] $p < .01$ versus NE; [c] $p < .05$ versus NE; [d] $p < .001$ versus NE; [e] $p < .05$ versus NECA; [f] $p < .001$ versus NECA.

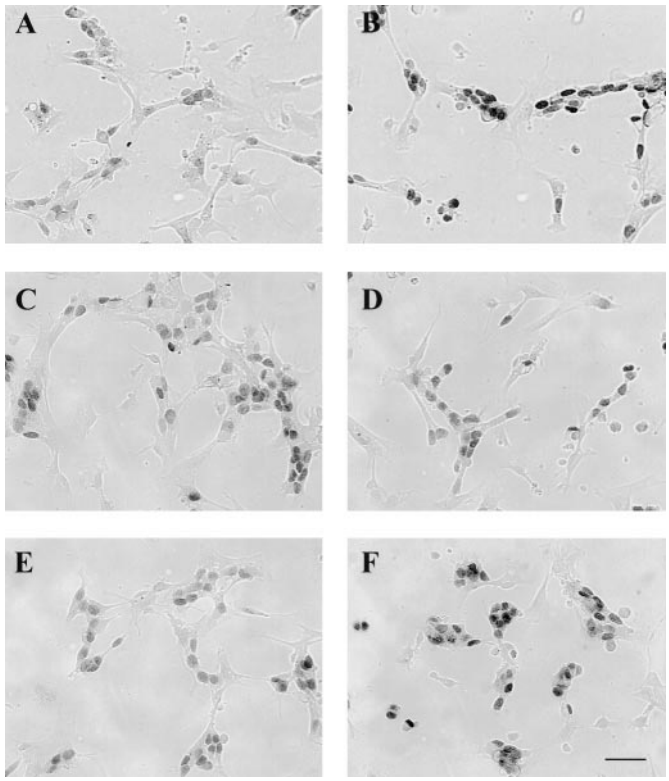

Fig. 9. ICER IR in untreated isolated rat pinealocytes (A) or cells stimulated for 5 h with NE (10^{-6} B), VIP (10^{-7} C), PACAP (10^{-7} D), KCl (60 mM E), and NECA (10^{-6} F).

NE at concentrations of $>10^{-8}$ M induces a maximal ICER IR that does not exceed the forskolin-induced ICER IR. Because an ISO effect can be potentiated only when concentrations of $<10^{-7}$ M are used, it can be concluded that the α-adrenergic potentiation of the β-adrenergic ICER IR is masked at NE concentrations commonly used ($>10^{-7}$ M). Similarly, the ISO-induced phosphorylation of CREB could not be potentiated by PHE (Tamotsu et al., 1995; Maronde et al., 1999). These observations support the suggested β_1-adrenergic/cAMP/pCREB/ICER link for a regulated pineal melatonin synthesis (Stehle et al., 1993; Stehle, 1995; Foulkes et al., 1997; Maronde et al., 1999). Furthermore, like the phosphorylation of the transcriptional activator CREB (Tamotsu et al., 1995), protein levels of the inhibitory TF ICER can be induced at low nanomolar NE concentrations. This indicates a causal molecular dependence of ICER transcription on CREB phosphorylation. A comparative threshold analysis reveals that both the phosphorylation of CREB and ICER protein induction are at least 100 times more sensitive to NE than activation of AANAT transcription (Roseboom et al., 1996), AANAT enzymatic activity (Klein and Weller, 1973), and melatonin synthesis (Simonneaux et al., 1989; this study). We account the different thresholds for activation of cAMP-inducible genes in rat pineal gland to differences in promoter structure. In molecular terms, the chance for pCREB to replace ICER from one of the four CREs on the ICER gene (Molina et al., 1993) and thus to shift transcriptional inhibition toward activation is four times more likely compared with competitive dynamics involving one CRE present on the AANAT gene (Foulkes et al., 1996; Baler et al., 1997). The suggestion that transcriptional induction depends on CRE structure gains support because the structurally different CREs on the ICER gene seem to be differentially accessible (Monaco and Sassone-Corsi, 1997). This different affinity of TFs to various CREs may explain why despite the high sensitivity of the ICER gene toward cAMP challenges, this TF needs several hours to top the transcriptional impact of rapidly elevated levels in pCREB: it is only after this lag period that accumulating ICER protein eventually displaces pCREB from the AANAT-CRE, causing a cessation of AANAT transcription. Our present in vitro findings match our recent in vivo analysis showing that the balance between pCREB and ICER protein in rat pineal gland shifts in parallel with the rhythm of melatonin synthesis (Maronde et al., 1999).

It is not surprising that VIP- and PACAP-stimulated cAMP accumulation in the medium reaches only about 10% of the NE-induced level because only a distinct subpopulation of pinealocytes is equipped with receptors for VIP or/and PACAP (Masuo et al., 1992). In addition, the efficiency of the stimulation with VIP or PACAP on cAMP-dependent gene expression, as monitored in the ICER IR and in melatonin synthesis, is less than half-maximum and of shorter duration compared with effects elicited by NE. The submaximal activation of the cAMP-signaling pathway by VIP or PACAP allows for an α-adrenergic potentiation of cAMP accumulation (VIP, Chik et al., 1988; PACAP, Chik and Ho, 1995), CREB phosphorylation (E.M. and H.-W.K., unpublished observations), AANAT enzymatic activity (VIP, Yuwiler 1987; PACAP, Yuwiler, 1995), melatonin synthesis (VIP, Yuwiler,

1995; PACAP, Simonneaux et al., 1993; Chik and Ho, 1995), and ICER protein levels (present study). It seems that once NE has exceeded a certain threshold of AC activation, it masks a modulatory impact by other stimuli on the basal transcriptional rhythm. Reduced dynamics in VIP- and PACAP-induced transcriptional events match with dynamics observed in cAMP accumulation (VIP, Chik et al., 1988; PACAP, Chik and Ho, 1995), CREB phosphorylation (VIP and PACAP, Schomerus et al., 1996), AANAT transcription (VIP, Roseboom et al., 1996), AANAT enzymatic activity (VIP, Yuwiler 1987; PACAP, Yuwiler, 1995), and melatonin synthesis (VIP, Simonneaux et al., 1990; PACAP, Simonneaux et al., 1993; present study). Similar to thresholds observed in NE-induced signaling events, the concentrations of VIP or PACAP required to induce an ICER IR are lower compared with those needed to induce melatonin synthesis. However, the low threshold concentrations inducing the ICER IR match with threshold concentrations that induce CREB phosphorylation (Schomerus et al., 1996). Thus, the temporal and dose-dependent effects of VIP or PACAP on the transmembrane cascade transcriptionally regulating melatonin synthesis in rat pinealocytes are stringently mirrored in the intensity of the ICER IR and in ICER protein dynamics.

The data obtained with adrenergic and peptidergic stimulation indicate that ICER protein can be viewed as a highly sensitive reporter of cAMP-induced transcriptional events in rat pinealocytes. This view is supported by our results obtained with adenosine. The efficient stimulation of ICER expression and melatonin synthesis by adenosine conforms to the high abundance of adenosine receptors on pinealocytes, most of which represent A_2 adenosine receptors stimulating the AC (Stehle et al., 1992; Stehle, 1995), whereas A_1 adenosine receptors, inhibiting the AC (Nikodijevic and Klein, 1989), are less abundant (Stehle, 1995). Our finding highlights that adenosine, as the substrate for the AC to generate cAMP and as a major breakdown product of this second messenger, also functions in rat pinealocytes to modulate the efficiency of cAMP signaling by an impact on ICER abundance.

Stimulation of receptors for AVP, NPY, ACh, and glutamate affects melatonin biosynthesis without influencing ICER protein levels. This observation and the fact that none of these substances induce CREB phosphorylation indicate that these ligands act on pineal melatonin synthesis independent of cAMP signaling.

First, AVP potentiates NE-induced AANAT activity (Stehle et al., 1991) and melatonin synthesis (present study). The V_1-vasopressinergic receptor is highly expressed in pineal tissue (Ostrowski et al., 1994) and is coupled to the phosphoinositol system. However, vasopressinergic signaling events that elevate intracellular calcium concentration do not induce CREB phosphorylation (C. Schomerus and H.-W.K., unpublished observations) and seem to be insufficient to increase ICER protein levels.

Second, the NPY-mediated decrease in NE-induced cAMP levels (Olcese, 1991) seems to be too weak or may occur too late to affect the NE-initiated increase in ICER protein levels. It can therefore be reasoned that the inhibition of NE-induced AANAT activity and melatonin synthesis by NPY (Olcese, 1991; present study) must occur downstream of the transcriptional induction of AANAT and ICER, might involve post-translational modifications of melatonin-synthesizing enzymes, or might be mediated by unknown inhibitory transcriptional events.

Last, the glutamatergic activation of metabotropic glutamate 3 receptors inhibits a stimulated melatonin synthesis by affecting the AC (Yamada et al., 1998b; present study). This effect is initiated by a cholinergic activation of L-type calcium channels that leads to a Ca^{2+} influx from extracellular sources (Schomerus et al., 1995; Letz et al., 1997) to initiate glutamate release. However, neither ACh nor glutamate was able to affect the NE-induced increase in ICER IR in rat pinealocytes. Interestingly, ACh and glutamate also had no effect on the phosphorylation of CREB (M.P. and J.H.S., unpublished observations).

Surprisingly, depolarization of rat pinealocytes with KCl (e.g., the activation of voltage-sensitive calcium channels) induced ICER protein expression without affecting melatonin synthesis on its own. It seems that the calcium-mediated transactivation of pineal genes depends on the recruiting mechanism for this second messenger and/or the degree of up-regulation in intracellular calcium concentration. Interestingly, a weak CREB phosphorylation could also be elicited by the KCl-induced depolarization of pinealocytes (M.P. and J.H.S., unpublished observations). Such delicate mechanisms of Ca^{2+} recruitment leading to the differential activation of the protein kinase A or the Ca^{2+}/calmodulin-dependent protein kinases were demonstrated in hippocampal neurons (Bading et al., 1993). Thus, we and others (Roseboom and Klein, 1995) suggest that KCl activates in pinealocytes through a cAMP-independent mechanism the phosphorylation of CREB and, subsequently, ICER.

The induction of ICER protein levels in rat pinealocytes stringently follows the physiological need of these neuroendocrine cells, demanding inhibition of cAMP-directed gene expression with prolonged stimulation period. The high sensitivity of the stimulus-induced activation of the inhibitory TF ICER can be interpreted as a protective mechanism, by which the cell is prepared to limit mRNA accumulation of CRE-bearing genes. This anticipation may play a particular role under in vivo conditions, as we observed in rat pineal gland the persistent presence of a considerable amount of ICER protein throughout the 24-h light/dark cycle (Maronde et al., 1999). In this line of evidence fall the observations that the developmental maturation of the ICER-generating system in rat pineal gland (Stehle et al., 1995) coincides with the decline in mRNA levels of the β_1-adrenergic receptor (Pfeffer et al., 1998) and, notably, also of AANAT transcription (Pfeffer and Stehle, 1998). Our data support the concept that ICER acts in the rat pineal gland as a very sensitive transcriptional inhibitor for elements involved in melatonin synthesis (Stehle, 1995; Foulkes et al., 1997) and emphasizes the fundamental and important biological role of this TF in neuroendocrine signal transduction.

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