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Review article

Cholinergic signal transduction cascades in rat pinealocytes: functional and ontogenetic aspects

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Abstract — In adult rat pinealocytes, acetylcholine activates nicotinic receptors whose stimulation causes a depolarization of the cells, opening of voltage-gated cation channels of the L-type and subsequent increase in the intracellular calcium ion concentration. These events trigger a release of glutamate that, by its action on metabotropic glutamate type 3 receptors, activates an inhibitory cyclic AMP cascade and suppresses norepinephrine-induced melatonin biosynthesis. The nicotinic response is fully developed in the third postnatal week. Prior to this timepoint, rat pinealocytes possess functional muscarinic receptors whose activation causes a rise in the intracellular calcium ion concentration through a calcium release from thapsigargin-sensitive intracellular calcium stores and an opening of store-operated calcium channels. This cascade may influence tissue differentiation and maturation of the melatonin pathway. The demonstration of functional cholinoreceptors and the ontogenetic switch from muscarinic to nicotinic signalling in rat pinealocytes supports the concept that pineal functions in mammals are influenced by neuronal inputs other than the sympathetic innervation which serves as the major regulatory system. © Inra/Elsevier, Paris

pinealocyte (rat) / nicotinic acetylcholine receptor / muscarinic acetylcholine receptor / norepinephrine / glutamate

Résumé — Des cascades de signaux de transduction cholinergique dans les pinéalocytes du rat : aspects fonctionnel et ontogénétique. Dans les pinéalocytes du rat adulte, l'acétylcholine active des récepteurs nicotiniques dont la stimulation induit une dépolarisation cellulaire, l'ouverture des canaux de cations voltage-dépendant du type L et l'augmentation subséquent de la concentration d'ions calcium intracellulaires. Ces événements induisent la libération du glutamate qui, par son action sur des récepteurs métabotropiques de type glutamate 3, active une cascade d'inhibition de l'AMP cyclique et réprime la biosynthèse de la mélatonine induite par la norépinéphrine. La réponse nicotinique est entièrement développée au cours de la troisième semaine postnatale. Avant ce moment, les pinéalocytes du rat possèdent des récepteurs fonctionnels muscariniques dont l'activation induit une hausse de la concentration intracellulaire d'ions calcium via la libération du calcium des réserves intracellulaires sensibles à la thapsigargine et l'ouverture des canaux de calcium actionnée par des

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réserves. Cette cascade peut influencer la différentiation des tissus et la maturation des voies de synthèse de la mélatonine. La démonstration de l'existence de cholinorécepteurs fonctionnels et le changement ontogénétique des signaux muscariniques en signaux nicotiniques dans les pinéalocytes du rat soutiennent l'idée générale que les fonctions pinéales des mammifères sont influencées par des afférences différentes de l'innervation sympathique qui est le système principal de régulation. © Inra/ Elsevier, Paris

pinéalocyte de rat / récepteur d'acétylcholine nicotinique / récepteur de l'acétylcholine muscarinique / norépinéphrine / glutamate

1. INTRODUCTION

The mammalian pineal organ is an important component of the photoneuroendocrine system which rhythmically synthesizes and secretes melatonin during the night in response to photoperiodic stimuli and signals from the circadian clock in the hypothalamic suprachiasmatic nucleus. The melatonin rhythm generation depends on the sympathetic innervation of the pineal gland [12] which rhythmically releases norepinephrine (NE) at the onset of darkness [6]. By stimulating α_1- and β_1-adrenergic receptors, NE causes increases in the intracellular concentrations of calcium ions and cyclic AMP and shapes the melatonin rhythm by regulating the arylalkylamine-*N*-acetyltransferase (AANAT), the key enzyme of the melatonin biosynthesis [14], at transcriptional and post-transcriptional levels. The transcriptional regulation of the AANAT involves activating and inhibitory transcription factors, e.g. phosphorylated CREB and ICER [17, 21, 25, 30, 32–34]; a major post-transcriptional mechanism is the cyclic AMP-dependent rapid and reversible control of selective proteasomal proteolysis [9]. All these data have corroborated the essential role of the sympathetic innervation of the pineal organ and its primary neurotransmitter, NE. In contrast, it is still unclear whether and how non-sympathetic neuronal pathways (see [15, 16, 23]) or hormones are involved in the regulation of melatonin biosynthesis and pineal function in mammals.

Out of the variety of putative neuronal and hormonal inputs to the mammalian pineal organ, the cholinergic system appears of particular interest for the following reasons. 1) Several morphological investigations point towards the existence of a parasympathetic innervation of the mammalian pineal gland (for review, see [23]) which may originate from the pterygopalatine ganglion, employ acetylcholine (ACh) as the primary neurotransmitter and antagonize the sympathetic effects. 2) A cholinergic innervation of the mammalian pineal organ has been convincingly demonstrated by immunocytochemistry using antibodies against the vesicular ACh transporter [26]. 3) Biochemical and immunocytochemical results suggest that rat pinealocytes contain ACh and that the ACh content increases tenfold at night [37].

Possible effects of cholinergic agonists on melatonin production and release have been repeatedly investigated (for review, see [19]), but the data are equivocal with regard to the receptor types involved, their location and the functional consequences of their activation. Transpineal in vivo microdialysis has shown that the infusion of the cholinergic agonists carbachol or oxotremorine into the pineal organ of adult rats resulted in a marked decrease in melatonin release during the dark period by inhibiting the NE release from sympathetic nerve fibres [7]. Such data suggest the presence of muscarinic acetylcholine receptors (mAChRs) in a presynaptic location, i.e. on

the sympathetic nerve endings. The existence of nicotinic ACh receptors (nAChRs) in the rat pineal organ was inferred from immunocytochemical investigations [24] and binding studies with radiolabelled specific ligands [31] and it has been suggested that nicotine has an inhibitory effect on pineal melatonin biosynthesis.

In the last 3 years, several investigations have been performed in an attempt to clarify the cholinergic signal transduction mechanisms, using the rat pineal organ as a model. The results of these studies will be reviewed in the present contribution. Moreover, new data will be presented on the ontogenetic development of cholinergic signalling mechanisms in rat pinealocytes.

2. CHOLINERGIC SIGNAL TRANSDUCTION IN PINEALOCYTES OF ADULT RATS

Calcium imaging of isolated, immunocytochemically identified rat pinealocytes has shown that ACh increases the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in more than 90 % of the cells in a dose-dependent manner [29]. All ACh-sensitive pinealocytes also respond to NE with an increase in $[Ca^{2+}]_i$, but the two types of responses are quite different. The ACh-induced rise in $[Ca^{2+}]_i$ is followed by a rapid decrease to basal levels within a few minutes after the onset of the stimulus. This decrease is also seen under a constant exposure to the ligand. The response is mediated by a nicotinic receptor subtype (*figure 1A*) because ACh and nicotine elicit virtually identical effects, and both the ACh- and the nicotine-induced responses are blocked by the specific nicotinic antagonist, *d*-tubocurarine. mAChRs do not play a role in this calcium response because pilocarpine, acting upon all presently known muscarinic receptor subtypes, does not evoke a calcium response, and the muscarinic antagonist atropine does not block the ACh-induced rise in $[Ca^{2+}]_i$. The response to ACh is totally prevented when pinealocytes were kept in calcium-free saline, indicating that the response is based upon a calcium influx.

The components involved in the nicotinic response have been identified using a combination of patch-clamp recordings and calcium imaging [20]. These investigations have shown that the average resting membrane voltage of isolated adult rat pinealocytes is –43 mV, and that the replacement of extracellular NaCl by KCl completely depolarizes the cells. This indicates that the resting membrane voltage is dominated by a K^+ conductance. Single channel recordings reveal the presence of a large conductance, Ca^{2+}-activated, charybdotoxin-sensitive K^+ channel [4, 20]. The application of ACh depolarizes the pinealocytes by an average of 16 mV. The depolarizing effect of ACh is mimicked by nicotine and prevented by tubocurarine. This depolarization is largely abolished in the absence of extracellular Na^+ but is not significantly affected by extracellular Ca^{2+} removal. Removal of extracellular Na^+ also causes a large reduction in the ACh-induced rise in $[Ca^{2+}]_i$. Nifedipine suppresses the ACh-induced increase in $[Ca^{2+}]_i$ by approximately 50 %. The findings indicate that ACh influences adult rat pinealocytes through stimulation of nAChRs which induces a depolarization mainly by a βNa^+ influx through the receptor. The depolarization then activates voltage-gate L-type calcium channels (VCCs) which are responsible for the nifedipine-sensitive portion of the $[Ca^{2+}]_i$ increase.

The fact that nicotinic receptors and VCCs are present in the vast majority of adult rat pinealocytes suggests their important role in the regulation of pineal metabolism. One idea is that ACh may act upon microvesicle-mediated glutamate release from pinealocytes which is elicited by depolarization and activation of VCCs ([39, 40]; *figure 2A*). Glutamate has been shown to suppress the NE-induced melatonin production in the rat pineal organ kept

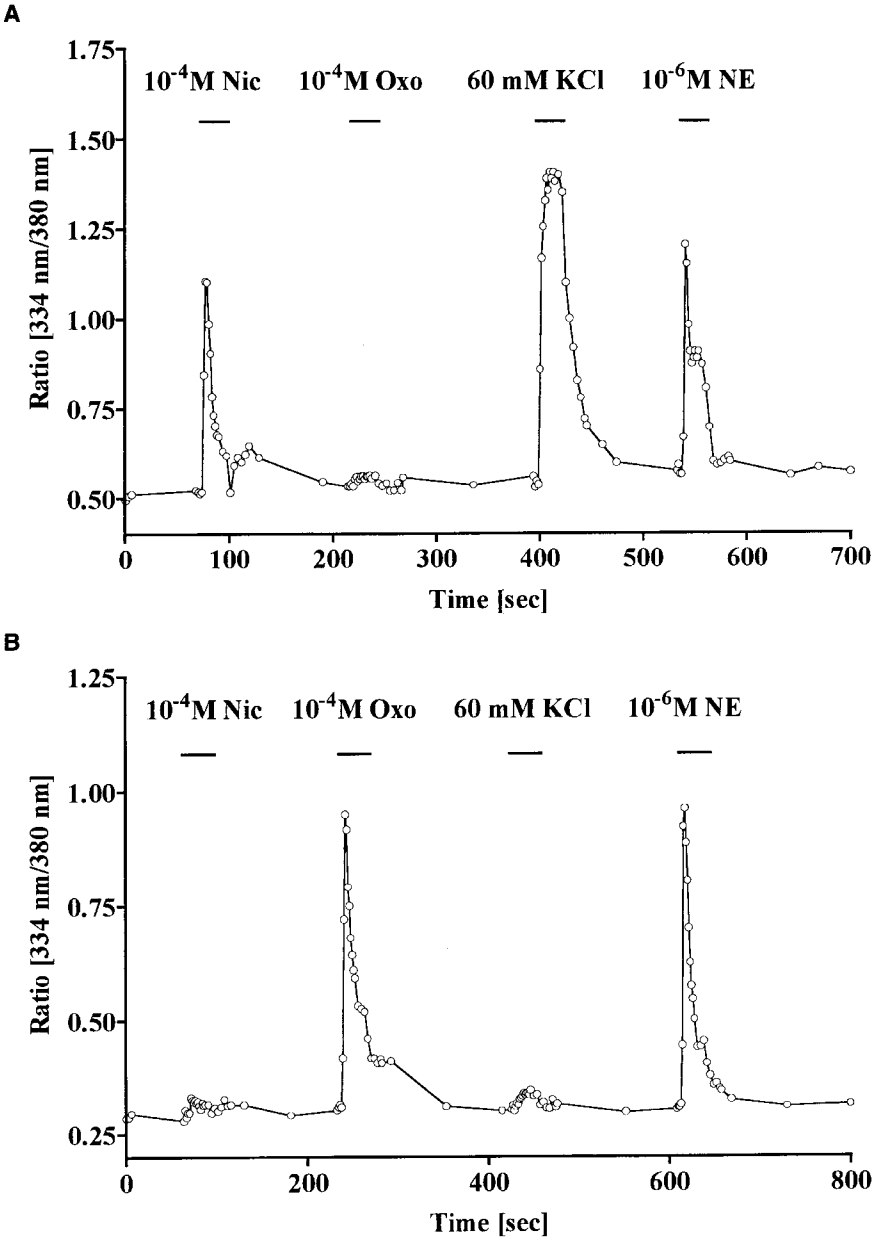

Figure 1. ACh-induced calcium responses recorded from adult and rat neonatal (P0) pinealocytes. **A**) In adult pinealocytes, nicotine, but not oxotremorine-M, induces a rise in $[Ca^{2+}]_i$. Cells showing a nicotinic response also react to NE and 60 mM KCl with an elevation of $[Ca^{2+}]_i$. **B**) In neonatal rat pinealocytes, stimulation with oxotremorine-M (Oxo, 10^{-4}M) elicits a robust calcium signal. Pinealocytes showing a muscarinic response also react to NE with an increase in $[Ca^{2+}]_i$, whereas treatment with the nicotinic receptor agonist nicotine (Nic; 10^{-4}M) or 60 mM KCl does not affect $[Ca^{2+}]_i$.

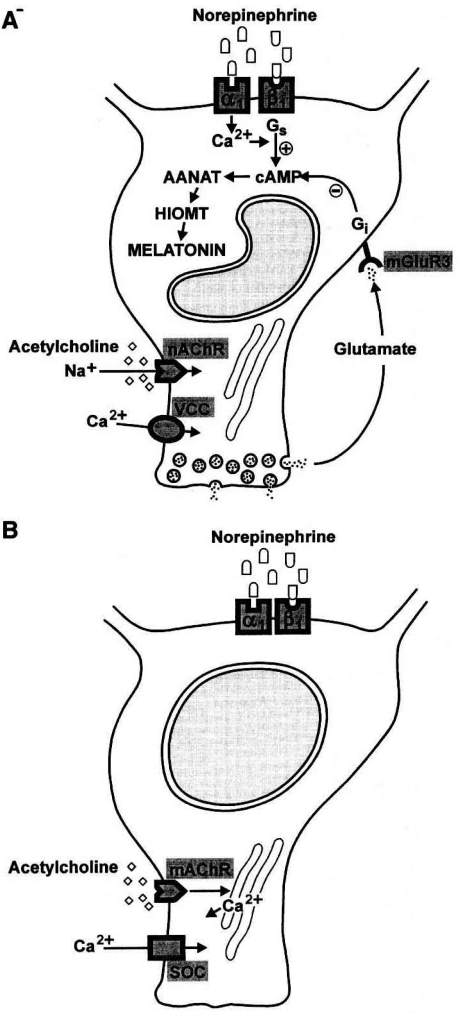

Figure 2. Signal transduction mechanisms in pinealocytes from adult and neonatal rats. **A**) In pinealocytes from adult rats, acetylcholine acts upon nicotinic acetylcholine receptors (nAChR) whose stimulation causes the depolarization of the cells through a sodium ion influx, an opening of voltage-gated cation channels (VCC) and a subsequent increase in the intracellular calcium ion concentration. These events trigger a microvesicle-mediated release of glutamate that, by its action on metabotropic glutamate type 3 receptors (mGluR3), activates an inhibitory cyclic AMP cascade and suppresses norepinephrine-induced arylalkylamine-*N*-acetyltransferase (AANAT) activation and melatonin synthesis. **B**) In pinealocytes from neonatal rats, acetylcholine stimulates muscarinic acetylcholine receptors (mAChRs) whose activation causes a rise in the intracellular calcium concentration through calcium release from thapsigargin-sensitive intracellular calcium stores and the opening of store-operated calcium channels (SOC). This cascade may influence tissue differentiation and maturation of the melatonin pathway. α_1- and β_1-adrenergic receptors are already functional (Schomerus, Laedtke and Korf, unpublished results) despite the fact that melatonin is not yet synthesized. HIOMT, hydroxyindole-*O*-methyltransferase; G_i, inhibitory GTP-binding protein; G_s, stimulatory GTP-binding protein.

in vitro [18]. This inhibitory effect of glutamate is due to the activation of metabotropic type 3 glutamate receptors initiating an inhibiting cyclic AMP cascade finally resulting in a decreased NAT activity [42]. The presumed link between the activation of nAChRs, subsequent opening of VCCs, release of glutamate and inhibition of melatonin biosynthesis and release has been experimentally proven by a recent study by Yamada and coworkers [41]. These authors showed that ACh stimulates glutamate release from isolated pinealocytes of adult rats. The removal of calcium ions from the medium reduces ACh-evoked glutamate secretion by 80 %. Blockers of cation channels of the L-type inhibit the ACh-evoked glutamate release, whereas channel agonists stimulate glutamate release. Glutamate secretion is triggered by nicotine, but not by muscarine. The nicotine- or ACh-evoked glutamate secretion is inhibited by *d*-tubocurarine, a competitive inhibitor of the nAChR, whereas alpha-bungarotoxin, a selective

inhibitor of some subtypes of nAChR and the muscarinic antagonists scopolamine and atropine are ineffective. These data show that an alpha-bungarotoxin-insensitive nAChR is responsible for glutamate secretion from adult rat pinealocytes. Both nicotine and ACh also strongly inhibit the NE-induced activation of AANAT and melatonin biosynthesis. This inhibition is prevented by tubocurarine and a specific antagonist of the class II metabotropic-type glutamate receptor.

The precise mechanism through which ACh inhibits NE-induced AANAT activation remains to be elucidated. As mentioned above AANAT can be regulated at the transcriptional and post-transcriptional level. The transcriptional regulation involves phosphorylation of the activating transcription factor which, however, is not affected by ACh, nicotine or *L*-glutamate (*figure 3A, B*). These results suggest that the cholinergic effects on AANAT activity are exerted at the post-transcriptional rather than the transcriptional level.

3. ONTOGENETIC DEVELOPMENT OF CHOLINERGIC SIGNAL TRANSDUCTION IN RAT PINEALOCYTES

To study the ontogenetic development of cholinergic signal transduction mechanisms we have investigated pinealocytes isolated from newborn, 1-, 2-, 3-, 4-, 5, 7-, 10-, 14- and 21-day-old rats. ACh at a concentration of $\geq 10^{-6}$ M induces a robust biphasic rise in $[Ca^{2+}]_i$ in approximately 90 % of neonatal pinealocytes (P0). A transient maximum in $[Ca^{2+}]_i$ is followed by a sustained elevation of $[Ca^{2+}]_i$ which finally drops to basal levels upon removal of the stimulus. Calibration of the semiquantitative ratio data revealed that, after ACh stimulation, $[Ca^{2+}]_i$ increases from a basal level of approximately 40 ± 10 nM to a maximum value of approximately 400 ± 150 nM. The ACh-responsive cells also react to NE with a rise in $[Ca^{2+}]_i$. Like ACh, the muscarinic agonists muscarine (non-selective) and oxotremorine-M (relatively M1-selective) and the non-specific cholinergic agonist carbachol elicit a calcium signal in 90 % of the cells at concentrations of $\geq 10^{-6}$ M. In contrast, nicotine (10^{-4} M) induces a weak calcium signal in less than 10 % of cells only (*figure 1B*). The muscarinic antagonists atropine (non-selective) and pirenzepine (M1-selective) totally block the ACh-evoked calcium signal at a concentration of $\geq 10^{-8}$ and $\geq 10^{-7}$ M, respectively. In contrast, the nicotinic antagonist *d*-tubocurarine (10^{-4} M) only partially inhibits the ACh-evoked rise in $[Ca^{2+}]_i$. In the second postnatal week, the percentage of cells which clearly respond to 10^{-4} M nicotine with a rise in $[Ca^{2+}]_i$ increases to approximately 10 %. These cells usually also react to ACh and oxotremorine-M at concentrations of $\geq 10^{-6}$ M. After the second postnatal week, the percentage of cells responding to muscarinergic stimuli decreases from 90 % to approximately 50 %. By the end of the third postnatal week oxotremorine and muscarine at concentrations of up to 10^{-4} M are basically without effect in most pinealocytes. In contrast, a robust calcium signal is elicited in 90 % of the cells by ACh and nicotine when applied at 10^{-4} M. This type of pinealocyte is prevailing in adult rats (*figure 1A*).

These findings show that the cholinergic receptor types in rat pinealocytes undergo a striking transformation from mAChRs in neonatal animals to nAChRs in adult rats. They conform to binding studies showing that the amount of muscarinic ACh binding sites in the rat pineal organ declines in the course of ontogeny from relatively high levels early in development [27, 28] to low levels in adulthood [8, 19, 35].

The developmental switch of the cholinoceptor type of rat pinealocytes is paralleled by a change in the signalling cascade distal to the receptors. As mentioned above, the nicotinic calcium response of adult rat pinealocytes involves the induc-

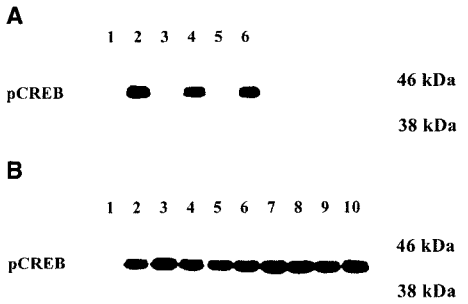

Figure 3. Immunoblot for pCREB in pineal glands from adult rats. For immunoblotting, the pineal glands were kept in an organ culture for 24 h prior to the experiments, stimulated with drugs, and then homogenized in the sample buffer by sonification. Pineal protein extracts were electrophoresed and blotted onto nitrocellulose membranes that were incubated with a polyclonal antibody against pCREB. Membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. Binding of the antibody was detected by chemiluminescence (UltraSignal, Pierce, Rockford, IL, USA) on autoradiographic films. Data obtained by immunoblots were analysed semiquantitatively using a computer-assisted image analysis system (KS 400, Kontron, Eching, Germany). Equal loading was ensured by incubating the membranes with a polyclonal antibody against total CREB that remained constant under the various treatment conditions. Each experiment was performed in triplicate. A) In unstimulated pineal glands, no pCREB immunoreactivity is detectable (lane 1). Treatment of glands with NE (10^{-7} M) for 30 min induces pCREB immunoreactivity (lane 2). Application of ACh (10^{-4} M; lane 3) or *L*-glutamate (10^{-4} M; lane 5) for 30 min does not cause pCREB immunoreactivity. Furthermore, NE-induced pCREB immunoreactivity is not significantly affected by ACh or *L*-glutamate, when cultured glands are pretreated with ACh or *L*-glutamate and then stimulated with NE (30 min; 10^{-7} M) in the presence of ACh (10^{-4} M; lane 4) and *L*-glutamate (10^{-4} M; lane 6), respectively. **B**) pCREB Immunoreactivity is not detectable in unstimulated glands (lane 1) and is induced by the treatment of glands with NE for 30 min (lane 2), 2 h (lane 3), 4 h (lane 4), 6 h (lane 5) and 8 h (lane 6). The amount of NE-induced pCREB immunoreactivity is not affected by *L*-glutamate as shown in glands pretreated with NE (10^{-7} M) for 30 min and then treated with a combination of *L*-glutamate (10^{-4} M) and NE (10^{-7} M) for 1.5 h (lane 7), 3.5 h (lane 8), 5.5 h (lane 9) or 7.5 h (lane 10).

tion of a depolarizing Na^+ influx and the subsequent activation of VCCs leading to a transient rise in $[Ca^{2+}]_i$ which is followed by a gradual drop to basal levels in the presence of the stimulus [20]. The muscarinic calcium response in neonatal pinealocytes consists of a primary phase mainly associated with the mobilization of Ca^{2+} from thapsigargin-sensitive intracellular stores and a secondary phase associated with Ca^{2+} entry into the cell (*figure 2B*). Interestingly, these thapsigargin-sensitive calcium stores also contribute to the calcium signal elicited by stimulation of α_1-adrenergic receptors. When neonatal pinealocytes are kept in Ca^{2+}-free saline and pretreated with NE, they exhibit a transient increase in $[Ca^{2+}]_i$ due to the release of calcium from intracellular compartments, but do not respond to subsequent stimulation with ACh in calcium-free saline. Vice versa, the depletion of these stores by ACh application prevents the response to NE. The calcium channels mediating the influx of Ca^{2+} in the secondary phase of the muscarinic response have not yet been identified and it remains to be established whether the calcium entry mechanism in the secondary phase of the muscarinic calcium signal is identical to that mediating the calcium influx in response to NE stimulation.

VCCs are apparently not yet developed in neonatal pinealocytes since treatment with depolarizing concentrations of KCl (*figure 1B*) or the agonist Bay K 8644 is without an effect on $[Ca^{2+}]_i$. Accordingly, specific L-type channel antagonists (nifedipine, verapamil) do not affect the ACh-evoked, muscarinic calcium signalling.

The transformation of the cholinergic signalling system during the development of rat pinealocytes strikingly changes the spatial and temporal patterns of the ACh-evoked calcium response. Since these patterns are considered important determinants which enhance the flexibility of Ca^{2+} to regulate diverse cellular processes [2, 5, 10, 22], the biphasic response evoked via

mAChRs in neonatal pinealocytes is suited to promote the activation of Ca^{2+}-sensitive events different from those activated via nAChRs in adult pinealocytes. A direct impact of muscarinic signalling on the regulation of melatonin production can be excluded since melatonin is not yet synthesized in the first postnatal week [13]. More likely, activation of mAChRs may play a regulatory role in the development of the pineal gland, in general, and in the maturation of the melatonin-generating system, in particular. The activation of mAChRs has been shown to promote cellular proliferation in neuronal and non-neuronal cells [1, 11]. In the retina, signalling via mAChRs has been proposed to be related to morphogenesis [43] and proliferation [38]. Interestingly, retinal cells also appear to express different types of cholinergic receptors during development which, it is proposed, have diverse and temporally regulated roles in their differentiation [38]. Similar differentiation processes may be controlled via mAChRs in the developing pineal organ which shares the diencephalic origin as well as photoreceptive and phototransducing properties with the retina [17]. The muscarinic response disappears concomitantly with the completion of mitosis [36]. This raises the possibility that the stimulation of mAChRs may also promote cell division in the pineal gland. Alternatively, muscarinic cholinergic signalling may be related to the expression of phototransduction molecules many of which are present at high levels in neonatal rat pineal organs and at low levels in adult rat pineal organs [3].

Pinealocytes lose the capacity to respond to muscarinic stimuli by the third postnatal week when the rhythmic melatonin synthesis is fully developed. The loss of the 'muscarinic phenotype' may be caused by a decrease in receptor number, decreased affinity between receptor and ligand, and/or actions distal from the mAChRs. Concomitantly, pinealocytes gain responsiveness to ACh via nAChRs. The factors responsible for the differential maturation of cholinergic calcium signalling mechanisms remain unclear. The switch is obviously not caused by cell death of those pinealocytes which are endowed with a muscarinic signalling cascade since the decrease in sensitivity to ACh and muscarinic agonists is paralleled by an increase in responsiveness to nicotine in one and the same cell. Interestingly, the functional maturation of VCCs precedes the development of a calcium response mediated by nAChRs. This raises the interesting hypothesis of whether the development of VCCs may induce the maturation of the 'proximal' nicotinic receptor.

4. CONCLUSIONS

Cholinergic signal transduction cascades operate in rat pinealocytes at all postnatal stages. In adult pinealocytes ACh acts upon nAChRs whose stimulation causes depolarization of the cells, opening of VCCs and a subsequent increase in the intracellular calcium ion concentration [20, 29]. These events trigger the release of glutamate that, by its action on metabotropic glutamate type 3 receptors, activates an inhibitory cyclic AMP cascade and suppresses NE-induced NAT activation and melatonin synthesis [41, 42]. In adult rats, mAChRs are absent from the pinealocyte membrane, but are present on sympathetic nerve fibres. Activation of these receptors blocks NE release from sympathetic nerve terminals, thus leading to a decreased melatonin output [7]. Thus, ACh appears to employ two different pathways to inhibit NE-induced melatonin synthesis and release in adult rats. The nicotinic response of rat pinealocytes is fully developed in the third postnatal week. Prior to this timepoint, rat pinealocytes express functional mAChRs whose activation causes a rise in the intracellular calcium ion concentration through a calcium release from thapsigargin-sensitive intracellular calcium stores and the opening of store-operated calcium channels. The functional significance of the muscarinic signal transduction cas-

cade in pinealocytes during early postnatal development remains to be clarified. It may be assumed that this cascade influences tissue differentiation and maturation of the melatonin pathway. The demonstration of functional cholinoreceptors and the ontogenetic switch from muscarinic to nicotinic signalling in rat pinealocytes supports the concept that pineal functions in mammals are modulated by a variety of inputs that may fine-tune the signals from the major regulatory system, the sympathetic innervation.

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