Biology

Biology fields

Okayama University *Year* 1999

Synaptic-like microvesicles, synaptic vesicle counterparts in endocrine cells, are involved in a novel regulatory mechanism for the synthesis and secretion of hormones

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The Journal of Experimental Biology 203, 117–125 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2333

SYNAPTIC-LIKE MICROVESICLES, SYNAPTIC VESICLE COUNTERPARTS IN ENDOCRINE CELLS, ARE INVOLVED IN A NOVEL REGULATORY MECHANISM FOR THE SYNTHESIS AND SECRETION OF HORMONES

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Accepted 18 October; published on WWW 13 December 1999

Summary

Microvesicles in endocrine cells are the morphological and functional equivalent of neuronal synaptic vesicles. Microvesicles accumulate various neurotransmitters through a transmitter-specific vesicular transporter energized by vacuolar H^+-ATPase. We found that mammalian pinealocytes, endocrine cells that synthesize and secrete melatonin, accumulate L-glutamate in their microvesicles and secrete it through exocytosis. Pinealocytes use L-glutamate as either a paracrine- or autocrine-like chemical transmitter in a receptor-mediated manner, resulting in inhibition of melatonin synthesis. In this article, we briefly describe the overall features of the microvesicle-mediated signal-transduction mechanism in the pineal gland and discuss the important role of acidic organelles in a novel regulatory mechanism for hormonal synthesis and secretion.

Key words: microvesicle, V-ATPase, melatonin, L-glutamate, serotonin, paracrine, autocrine, pinealocyte, endocrine cell.

Introduction

Vacuolar H^+-ATPases (V-ATPases) acidify various kinds of endomembrane systems such as lysosomes and endosomes. Organelles containing V-ATPase are usually called acidic organelles. An acidic pH or an electrochemical gradient of protons across the membrane plays an important role in organellar functions (Nelson and Harvey, 1999).

Endocrine cells contain two important acidic organelles: secretory granules and microvesicles (Regnier-Vigouroux and Huttner, 1993; Thomas-Reetz and De Camilli, 1994; Moriyama et al., 1996). Secretory granules contain hormones, such as insulin and glucagon, and secrete them through exocytosis. The acidic pH is responsible for the storage of monoamines, such as norepinephrine and serotonin, and for the conversion of prohormones (or preprohormones) to their mature form through proteolysis (Johnson, 1988). Microvesicles have recently been identified as novel acidic organelles and are also called synaptic-like microvesicles. Several years ago, Moriyama and Yamamoto (1995a,b) found that pinealocytes, endocrine cells synthesizing and secreting melatonin, contain a large number of microvesicles. Subsequently, combined biochemical and cell biological studies demonstrated that microvesicles contain V-ATPase and accumulate L-glutamate through a mechanism similar to that in synaptic vesicles (Moriyama and Yamamoto, 1995a,b). Microvesicles in pancreatic β and pheochromocytoma PC 12 cells are also acidic and contain γ-aminobutyrate and acetylcholine, respectively (Thomas-Reetz and De Camilli, 1994; Bauerfeind et al., 1993). Upon stimulation, the internal γ-aminobutyrate and acetylcholine are released through regulated exocytosis (Ahnert-Hilger and Weidenmann, 1992; Ahnert-Hilger et al., 1996; Bauerfeind et al., 1993). Thus, endocrine cells can store and secrete classical neurotransmitters through a mechanism similar to that in chemical transduction by neurons.

In this article, we briefly summarize our recent studies on the structure and function of microvesicles in mammalian pinealocytes and also present evidence that the acidic organelles are involved in a novel regulatory mechanism for melatonin synthesis.

Microvesicles in pinealocytes

Microvesicles were originally defined as organelles that are morphologically similar to neuronal synaptic vesicles but distinct from secretory granules (Regnier-Vigouroux and Huttner, 1993; Thomas-Reetz and De Camilli, 1994; Moriyama et al., 1995) (Fig. 1). The detection of synaptophysin, a synaptic vesicle integral membrane glycoprotein, in microvesicles demonstrated that the organelles are distinct from secretory granules (Navone et al., 1986;

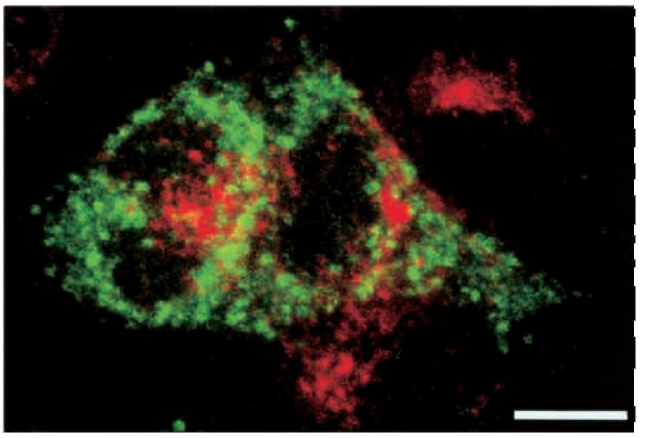

Fig. 1. Immunohistochemical detection of microvesicles and secretory granules in a PC 12 cell. Cultured PC 12 cells were immunostained with antibodies against synaptophysin (red), a marker for microvesicles, and antibodies against chromogranin (green), a marker for secretory granules, and observed by fluorescence microscopy. Scale bar, 10 μm.

Weidenmann et al., 1988; Cutler and Cramer, 1988). Microvesicles have been shown to be widely distributed among various endocrine cells, including pancreatic β cells (Reetz et al., 1991) and pinealocytes (Redecker and Bargsten, 1993).

Pinealocytes provide suitable experimental systems in which to study the role of microvesicles in endocrine cells because they contain a large number of microvesicles (Moriyama et al., 1995; Redecker and Bargsten, 1993; Moriyama and Yamamoto, 1995a). As shown in Fig. 2A, microvesicles are present throughout the cells but are especially abundant in terminal regions, varicosities of the terminal processes and synaptic ribbon regions, where are thought to be the sites of some secretory events. Electron microscopy revealed that microvesicles are small clear vesicles with a diameter of 50–70 nm (Fig. 2B). Biochemical characterization of isolated microvesicles indicated that they are devoid of synapsin I, a marker protein of synaptic vesicles, but contain synaptic vesicle protein 2B (SV2B), another synaptic vesicle protein, indicating that microvesicles are similar to synaptic vesicles but distinct from them (Fig. 2C). Thus, SV2B is also used as a marker protein for microvesicles (Hayashi et al., 1998). Microvesicles contain proteins that are involved in exocytosis, such as synaptotagmin and vesicle-associated membrane protein 2 (VAMP2) (Yamada et al., 1996b; Redecker et al., 1997) (Figs 2C, 3B). Furthermore, V-ATPase is associated with microvesicles, as determined by immunoelectron microscopy and immunoblotting (Moriyama and Yamamoto, 1995a). Like synaptic vesicles, microvesicles may undergo recycling in an area close to cytoplasmic membranes. This process can be monitored by vital staining using a fluorescent dye, FM-1-43 (M. Hayashi, A. Yamamoto and Y. Moriyama, in preparation). The presence of V-ATPase and proteins involved in exocytosis and also the recycling process support the idea that microvesicles are involved in some secretion processes in pinealocytes (see below).

V-ATPase in microvesicles

V-ATPase is a major component of microvesicles (approximately 10 % of their membrane protein) and generates a transmembrane pH gradient (inside acidic) and a transmembrane voltage gradient (inside positive) at the expense of ATP hydrolysis. Cl^- may facilitate the formation of a pH gradient, as in the case of V-ATPases in other endomembrane systems (Moriyama and Yamamoto, 1995b). Analysis of partially purified preparations suggested that the subunit composition of the V-ATPase in microvesicles is similar to that of chromaffin granule ATPase (Moriyama et al., 1995). The acidic pH of microvesicles in living cells can easily be observed by vital staining with Acridine Orange. The acidic internal pH disappears upon the addition of a submicromolar concentration of bafilomycin A_1. Very recently, we determined the internal pH of microvesicles to be approximately 5.0 by means of a 3[2,4-(dinitroanilino)-3′-amino-*N*-methyl-dipropylamine (DAMP) procedure (M. Hayashi, A. Yamamoto, and Y. Moriyama, unpublished data), which was originally developed by Anderson et al. (1984). Upon incubation, DAMP, an amphipathic amine, is accumulated in organelles through the H^+ gradient established across the membranes, as follows:

$$[H^+]_{out}/[H^+]_{in} = [DAMP]_{out}/[DAMP]_{in}\,.$$

The DAMP concentration in the organelles can be determined by immunoelectron microscopy using antibodies against 2,4-dinitrophenol. The internal pH of microvesicles is similar to that of lysosomes and significantly lower than that of isolated microvesicles (approximately 5.5). The potential difference across the microvesicle membranes in living cells remains to be determined.

The uptake of neurotransmitters by microvesicles is driven by the electrochemical proton gradient. We found that L-glutamate, an excitatory neurotransmitter, was taken up by pineal microvesicles upon the addition of ATP (Moriyama and Yamamoto, 1995a) (Fig. 3A). No other neurotransmitters, such as γ-aminobutyrate and acetylcholine, were taken up by the vesicles. Both bafilomycin A_1 and proton conductors such as 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile (SF6847) inhibited the uptake completely. Detailed studies on the energetics of the ATP-dependent glutamate uptake demonstrated that the membrane potential (inside positive) is the direct driving force for the uptake of glutamate if a low concentration of Cl^- (2–5 mmol l^{-1}) is present outside the vesicles (Moriyama and Yamamoto, 1995b). The effect of Cl^- may explain the presence of regulatory anion binding site(s) on the transporter, since 4,4′-diisothiocyanatostilbene-disulphonic acid (DIDS), an anion channel blocker, effectively competed for the chloride anions (Moriyama and Yamamoto, 1995b). It is of note that a similar anion channel blocker, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS), does not have such an effect (Y. Moriyama, unpublished observation). Essentially no glutamate uptake was driven by the transmembrane pH gradient. Furthermore, the glutamate transporter shows strict substrate recognition: L-

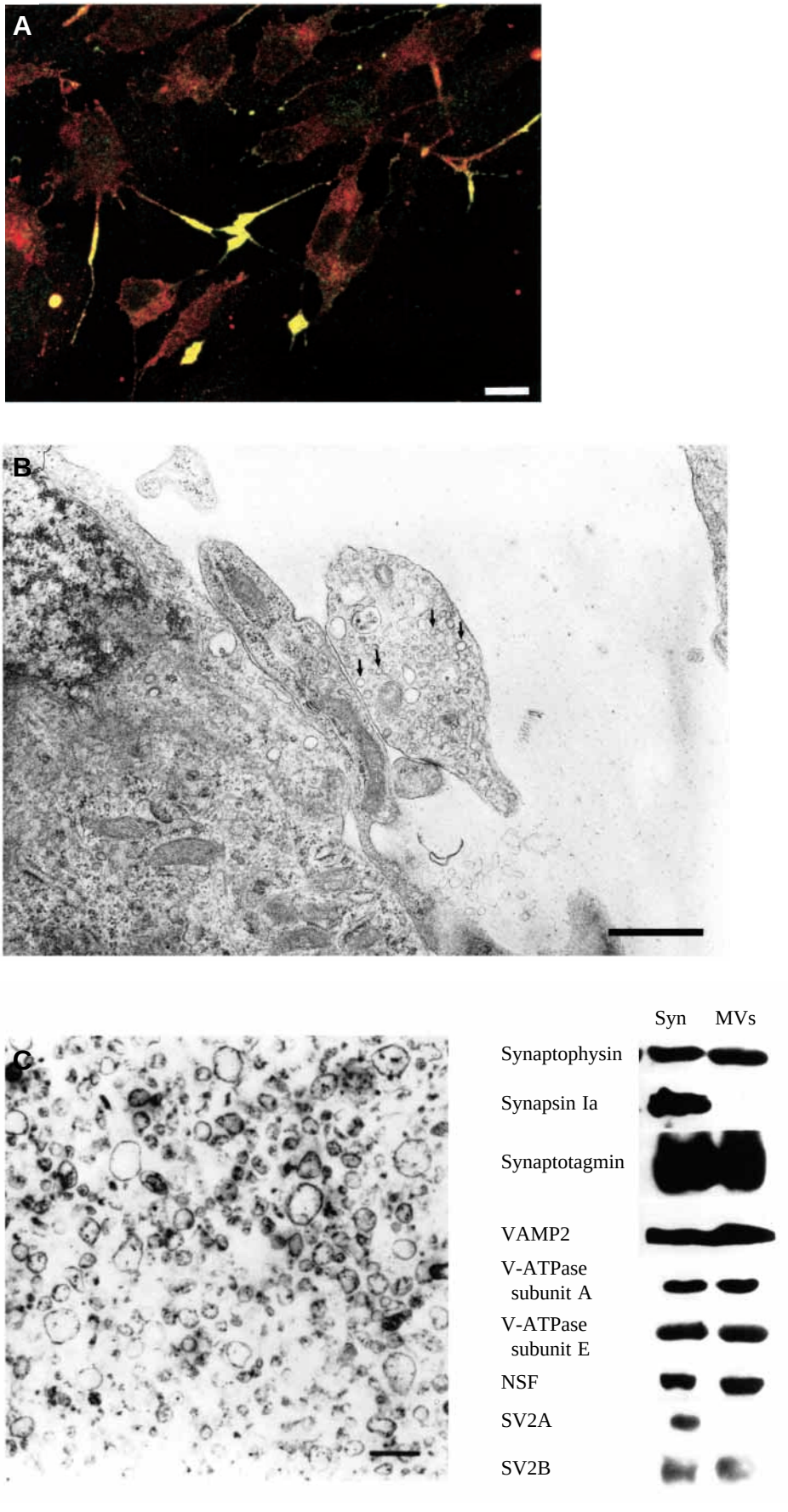

Fig. 2. Pineal microvesicles. (A) Cultured rat pinealocytes were double-immunostained with antibodies against synaptophysin (red) and synaptic vesicle protein 2B (SV2B) (yellow) and observed under a confocal microscope and a Nomarski miscroscope. A superimposed picture is shown. Pineal microvesicles are abundant in the process. Scale bar, 10 μm. (B) Electron micrograph of a vertical section of a process of a rat pinealocyte. Microvesicles are indicated by arrows. Scale bar, 1 μm. (C) Electron micrograph (left) and immunoblot (right) of microvesicles isolated from bovine pineal glands. Syn, synaptic vesicles; MVs, microvesicles; NSF, *N*-ethylmaleimide-sensitive fusion protein. SV2A, synaptic vesicle protein 2A; VAMP2, vesicle-associated membrane protein 2. Scale bar, 200 nm.

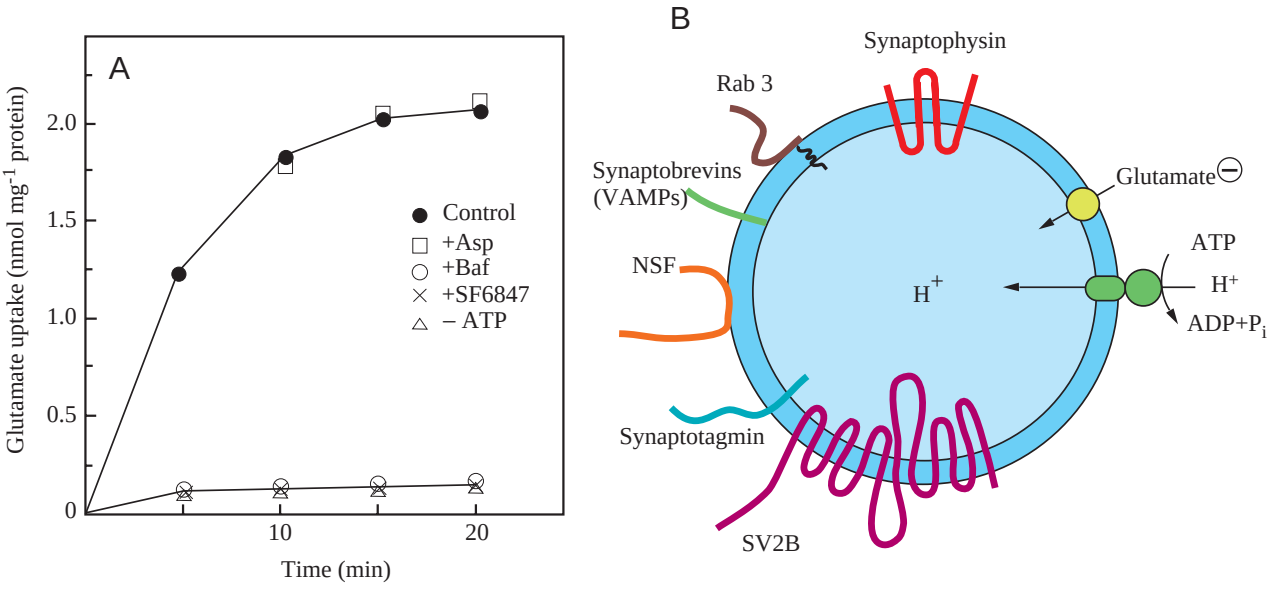

Fig. 3. Time course of ATP-dependent glutamate uptake by microvesicles (A) and a schematic representation of the components of microvesicles (B). Asp, aspartate; Baf, bafilomycin A_1; NSF, *N*-ethylmaleimide-sensitive fusion protein; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; SV2B, synaptic vesicle protein 2B. For details, see Moriyama and Yamamoto (1995a) and Hayashi et al. (1998).

glutamate and only a few cyclic glutamate analogues, such as 1-aminocyclopentane-*trans*-1,3-dicarboxylic acid, were substrates. D-Glutamate is only 15 % effective compared with the L form and no D,L-aspartate is transported. These properties are fully consistent with those of the vesicular glutamate transporter found in synaptic vesicles (Naito and Ueda, 1985; Hartinger and Jahn, 1993). We concluded that a vesicular glutamate transporter operates in pineal microvesicles. This is the first example of the functional operation of a vesicular glutamate transporter outside neurons.

Output of L-glutamate signals

Immunohistochemical studies showed that L-glutamate and synaptophysin are similarly distributed in pinealocytes, indicating that glutamate is accumulated in microvesicles under physiological conditions (Yamada et al., 1996b). Do pinealocytes secrete L-glutamate through microvesicle-mediated exocytosis? Upon stimulation of pinealocytes with KCl, the glutamate concentration in the medium increased in a time-dependent manner, as determined by high-performance liquid chromatography (Yamada et al., 1996b). A loss of activity was also observed on a second successive stimulation, but activity was recovered after 12 h of incubation. KCl-evoked glutamate secretion is also dependent on temperature: it was not observed below 20 °C, appeared gradually with increasing temperature and was maximal at 37 °C. Furthermore, it was inhibited by botulinum neurotoxin types E and A through specific cleavage of synaptic-vesicle-associated protein 25 (SNAP25). Glutamate secretion requires extracellular Ca^{2+}. Various L-type Ca^{2+} channel antagonists, such as nifedipine, inhibited glutamate secretion, but an agonist, 1,4-dihydro-2,6-dimethyl-5-nitro-4[2-(trifluoromethyl)-phenyl]-3-pyridinecarboxylic acid methyl ester (BAY K8644), stimulated secretion. These properties are fully consistent with those of regulated exocytosis, indicating that glutamate secretion is mediated through microvesicle-mediated exocytosis. Entry of Ca^{2+} through voltage-gated Ca^{2+} channels, and the resulting increase in intracellular [Ca^{2+}] in close proximity to the plasma membranes, may trigger microvesicle-mediated exocytosis (Fig. 4).

Because pinealocytes do not form synapse-like structures with neighbouring cells, glutamate secretion may occur in a paracrine-like manner. *In vivo*, microvesicle-mediated exocytosis may occur upon stimulation with acetylcholine (Yamada et al., 1998a), followed by depolarization through the nicotinic acetylcholine receptor (Letz et al., 1997). Thus, it appears that parasympathetic neurons projecting into the pineal gland control melatonin synthesis in a negative manner by way of endogenous glutaminergic systems (Fig. 4). Innervation of the pineal gland by parasympathetic neurons has been demonstrated histologically (for reviews, see Moller, 1992; Laitinen et al., 1995; Schafer et al., 1998).

Termination of glutamate signals

Secreted L-glutamate is rapidly taken up by pineal cells, reducing the cytotoxicity of glutamate (Yamada et al., 1997; Yatsushiro et al., 1997). Glutamate uptake is driven by Na^+ and Cl^-. A plasma-membrane-type Na^+-dependent glutamate transporter is responsible for glutamate uptake. Among several isoforms, pinealocytes predominantly express the GLT-1-type transporter at their plasma membranes (Yamada et al., 1997) (Fig. 4). Although the pineal gland contains several cell

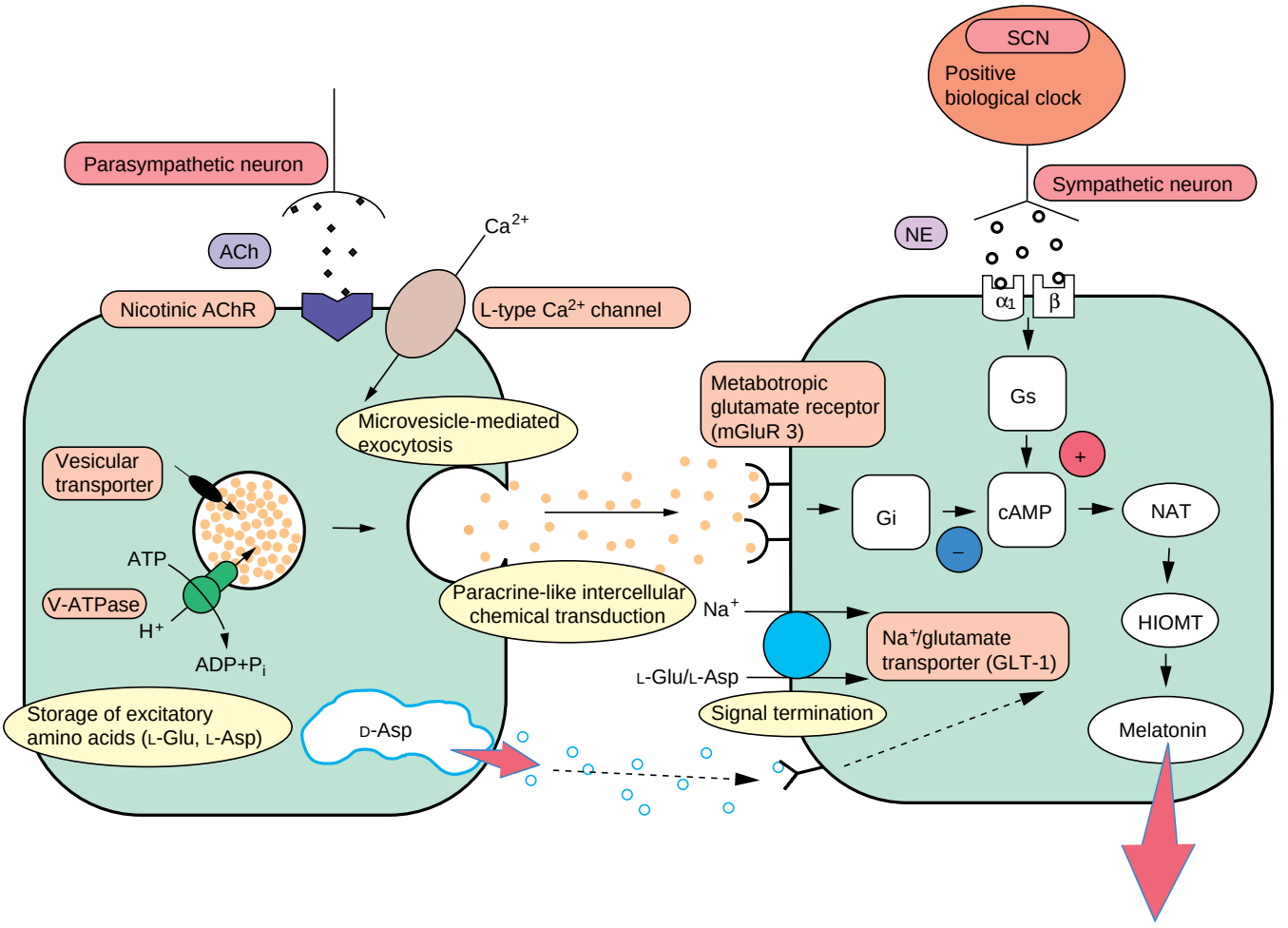

Fig. 4. Endogenous glutaminergic systems in pinealocytes. This illustration emphasizes the mechanism by which pinealocytes use L-glutamate as an intercellular signalling molecule. It is notable that each pinealocyte possesses all the machinery for glutamate signal output (microvesicles and proteins involved in exocytosis), input (receptors and proteins involved in intracellular signal transduction) and termination (Na^+-dependent re-uptake transporters). Thus, glutamate may function either as a paracrine or as an autocrine chemical mediator. For details, see the text. Furthermore, pinealocytes contain a high concentration of D-aspartate in their cytoplasm. Pinealocytes may secrete D-aspartate and use it as a negative regulator for melatonin synthesis in a receptor-mediated manner. For details, see Ishio et al. (1998) and Yatsushiro et al. (1997). ACh, acetylcholine; AChR, acetylcholine receptor; Gi, inhibitory G-protein; Gs, guanidine-nucleotide-binding protein; NAT, *N*-acetyltransferase; HIOMT, hydroxyindole-o-methyltransferase, NE, norepinephrine; SCN, suprachiasmatic nucleus; α_1, β, α_1- and β-adrenergic receptors.

species, no other cell types, including glial-like cells, express these transporters. Although further studies are necessary, the plasma-membrane-type glutamate transporter may function as a re-uptake system that terminates glutamate signalling, as stated above. The presence of a re-uptake system for L-glutamate also supports the mechanistic similarity between this endogenous glutaminergic system and neuronal chemical transduction.

Input of glutamate signals

What is the role of released glutamate? Does glutamate play an important role in pineal functions, e.g. in the synthesis and secretion of melatonin? Melatonin is a hydrophobic hormone that affects many physiological functions, such as the circadian rhythm and seasonal reproduction (Axelrod, 1974; Klein, 1985; Reiter, 1981). In mammals, melatonin synthesis is under photoperiodic control by way of the suprachiasmatic nucleus (SCN) of the hypothalamus. At night, the SCN sends stimulatory signals to the pineal gland through sympathetic neurons. Norepinephrine released from nerve endings binds to the adrenergic receptors on the plasma membranes of pinealocytes and activates adenylate cyclase through a heterotrimeric guanine-nucleotide-binding protein. The resultant increase in the concentration of cyclic AMP stimulates the transcription of the serotonin *N*-acetyltransferase gene, causing stimulation of melatonin output (Foulkes et al., 1997). Thus, melatonin synthesis is positively controlled by sympathetic neurons. In contrast, exogenous L-glutamate inhibits serotonin-*N*-acetyltransferase activity (Govitrapong and Ebadi, 1988) and the synthesis (Yamada et al., 1996a,b) and secretion (Kus et al., 1994; van Wyk and Daya, 1994) of

melatonin, suggesting a negative regulatory role for L-glutamate.

To define the role of glutamate in pinealocytes, the signal-transduction pathway by which glutamate inhibits melatonin synthesis was investigated. It was found that pinealocytes express functional metabotropic glutamate receptor type 3 (mGluR3) (Yamada et al., 1998b). This class of receptor is known to be coupled with an inhibitory G protein and decreases cyclic AMP concentration upon stimulation (Riedel, 1996). As expected, stimulation of the receptor decreased the cyclic AMP concentration and inhibited serotonin *N*-acetyltransferase activity and melatonin synthesis (Yamada et al., 1998b). The glutamate-evoked inhibition of melatonin synthesis occurred even in the presence of norepinephrine, suggesting that glutamate signalling overcomes the sympathetic control. The concentration of cyclic AMP is, therefore, an important factor in the regulation of melatonin synthesis. This study was the first to report the presence of functional mGluRs in endocrine cells.

A second example of the expression of functional mGluRs in endocrine cells also involves pinealocytes. We showed very recently that mGluR5, one of the class I mGluRs, is also functionally expressed in pinealocytes (Yatsushiro et al., 1999). This class of receptor is known to be linked to phosphatidylinositol hydrolysis/Ca^{2+} signal transduction and uses quisqualate as a selective agonist (Riedel, 1996). We measured intracellular [Ca^{2+}] in Fura-2-loaded pinealocytes and found that 200 μmol l^{-1} quisqualate increased intracellular [Ca^{2+}] by 245±42 nmol l^{-1} (mean ± S.E.M., N=265) even in the absence of extracellular [Ca^{2+}], and that the effect of quisqualate was blocked by methylcarboxyphenylglycine (MCPG), an effective class I mGluR antagonist. The physiological role(s) of mGluR5 in pinealocytes is unknown at present.

Pinealocytes also express ionotropic glutamate receptors (iGluRs). Among the three subfamilies identified in neuronal tissues, the (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptor is functionally expressed in pinealocytes (S. Yatsushiro, H. Yamada, M. Hayashi, A. Yamamoto and Y. Moriyama, in preparation). This class of receptor functions as a Na^+ channel and depolarizes the membrane potential through the entry of Na^+ (Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). Similarly, the addition of AMPA to pinealocytes causes membrane depolarization, increasing intracellular [Ca^{2+}] through L-type Ca^{2+} channels and resulting in an increase in microvesicle-mediated glutamate exocytosis.

We should emphasize that a single pinealocyte possesses all the systems required for the output, termination and input of glutamate signalling, as stated above. Therefore, once a pinealocyte has been stimulated by acetylcholine, which comes from parasympathetic neurons, melatonin synthesis should be inhibited through mGluR3. Simultaneously, the same pinealocyte can secrete glutamate through activation of iGluRs followed by activation of L-type Ca^{2+} channels. The released glutamate transmits the inhibitory signals (e.g. glutamate) to neighbouring cells. Thus, glutamate may affect pinealocyte function through at least two alternative pathways. Although further studies are necessary, alternative glutamate signalling pathways may explain the mechanism by which glutamate signalling spreads through pineal glands.

Serotonin as another secretory product of pinealocytes

Serotonin, a precursor for melatonin, is known to be another secretory product of pinealocytes (Sugden, 1989). Previous studies indicated that a high concentration of serotonin is present in vesicles as well as in the cytoplasm (Juillard, 1979; Juillard and Collin, 1980). Upon stimulation by norepinephrine, serotonin is released internally (Aloyo and Walker, 1988) and then stimulates serotonin *N*-acetyltransferase activity *via* the S2 receptor, resulting in increased melatonin output (Olcese and Munker, 1994; Sugden, 1990; Miguez et al., 1997). Thus, it seems likely that serotonin has a positive modulatory effect on melatonin synthesis, although the mechanisms underlying the storage and release of serotonin are unknown.

Very recently, we found that only a limited population of pinealocytes (less than 10 %) express functional vesicular monoamine transporter type 1 (VMAT1) (M. Hayashi, A. Yamamoto, M. Haga and Y. Moriyama, in preparation). VMAT2, another isoform of VMAT (Schuldiner et al., 1995), is not expressed in the pineal gland. VMAT1 is present in vesicles containing serotonin (Schuldiner et al., 1995). Our preliminary experiments suggested that the vesicles correspond to dense-cored vesicles and that the internal serotonin is secreted upon stimulation of pinealocytes with norepinephrine (H. Yamada and Y. Moriyama, unpublished observations). It is possible that pinealocytes use serotonin as a positive regulator for melatonin synthesis through a paracrine-like mechanism. The internal pH of the dense-cored vesicles is also maintained at an acidic value by V-ATPase, as determined by the DAMP method described above, and the acidity is responsible for the accumulation of serotonin in the vesicles (M. Hayashi, A. Yamamoto and Y. Moriyama, in preparation).

Proposed mechanism for melatonin synthesis through a paracrine network

Pinealocytes may secrete L-glutamate through microvesicle-mediated exocytosis and inhibit norepinephrine-stimulated melatonin synthesis through an mGluR-mediated signalling pathway (Fig. 4). Glutamate secretion can be triggered either by acetylcholine, through a nicotinic receptor, or by glutamate, through an iGluR-mediated signalling pathway. Acetylcholine originates from the nerve endings of parasympathetic neurons. Some pinealocytes may secrete serotonin upon stimulation with norepinephrine, which comes from the nerve endings of sympathetic neurons. Norepinephrine itself has a direct stimulatory effect on melatonin synthesis through an adrenergic receptor. On the basis of the above observations, we propose a working hypothesis for the regulation of melatonin synthesis by glutamate and serotonin. In the model,

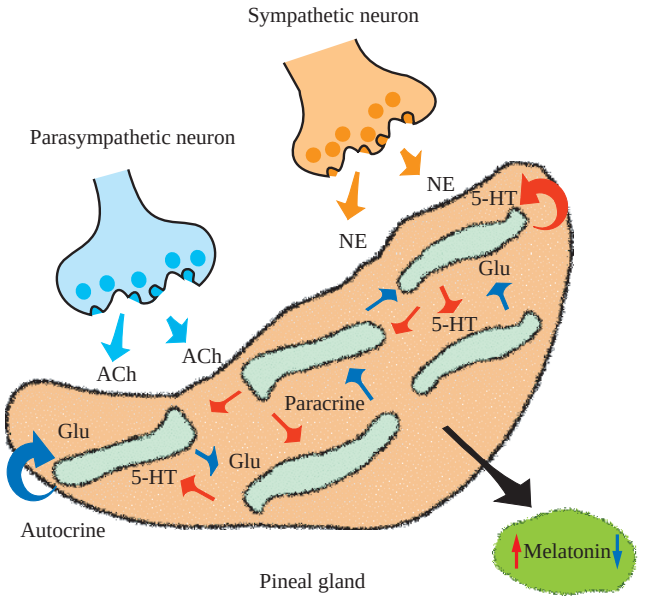

Fig. 5. Proposed paracrine network for the regulation of melatonin synthesis in the pineal gland. Upon receiving norepinephrine from sympathetic neurons, pinealocytes secrete serotonin by exocytosis. Subsequently, the secreted serotonin stimulates melatonin synthesis in a receptor-mediated manner. Acetylcholine from parasympathetic neurons triggers the exocytosis of glutamate, inhibiting melatonin synthesis. Thus, pinealocytes use serotonin and glutamate as intercellular chemical mediators to trigger opposite responses. V-ATPase plays a central role in the accumulation of these neurotransmitters. Glu, L-glutamate; 5-HT, serotonin; NE, norepinephrine; ACh, acetylcholine.

pinealocytes use glutamate and serotonin as paracrine chemical mediators that have opposite effects on melatonin synthesis. This paracrine signalling should be under neuronal control, with acetylcholine and norepinephrine as transmitters. Fig. 5 summarizes the proposed paracrine network for the regulation of melatonin synthesis in the pineal gland. We should emphasize that melatonin synthesis is primarily regulated through an on/off mechanism (switching) from the suprachiasmatic nucleus to sympathetic neurons (Axelrod, 1974; Klein, 1985; Reiter, 1991; Foulkes et al., 1997). This paracrine network may help the on/off mechanism to ensure precise regulation of daily changes in melatonin synthesis. It is noteworthy that retinal cells, another important class of cell that produces melatonin (Tosini and Menaker, 1996), use L-glutamate and dopamine as regulators: retinal cells secrete L-glutamate and receive glutamate signals from bipolar cells and dopamine signals from dopaminergic amacrine cells so as to exert a negative effect on melatonin synthesis (Morgan and Boelen, 1996). Further studies, especially characterization of the secretion of serotonin and of the interrelationship between serotonin and L-glutamate secretion, will be important in explaining the physiological basis for the putative paracrine network regulating melatonin synthesis.

Conclusions

Since finding that microvesicles are acidic organelles containing L-glutamate, we have detected the presence of a novel regulatory mechanism for melatonin synthesis. The regulatory mechanism consists, at least in part, of systems for the output, input and termination of glutamate signals and of serotonin signals. V-ATPase plays an important role in the novel signal-transduction system through the storage and secretion of neurotransmitters. Similarly, microvesicles in various endocrine cells participate in the storage and secretion of neurotransmitters. In pancreatic islets of Langerhans, receptors for glutamate, acetylcholine and γ-aminobutyrate that are functionally expressed regulate hormonal secretion in the endocrine cells (Satin and Kinard, 1998). Although more extensive studies are necessary, microvesicle-mediated paracrine-like signal transduction seems to be one of the common features of endocrine cells for the regulation of hormonal synthesis and secretion.

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