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Adenylyl Cyclase mRNA Expression does not Reflect the Predominant Ca^{2+}/Calmodulin-Stimulated Activity in the Hypothalamus

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Abstract

Only three (Types I, II, V) of the six currently-described subtypes of adenylyl cyclase are prominently expressed in the rat brain. These species are differently sensitive to Ca^{2+}, $\beta\gamma$ subunits of G-proteins and protein kinase C. A knowledge of the susceptibility of the cAMP-signalling system in particular brain regions to these diverse modes of regulation can shed light on the mechanism of action of the neurotransmitters that modify neuronal activity in such regions. Cyclic AMP is extensively involved in the physiological functions of the hypothalamus. We have used in situ hybridization histochemistry with synthetic oligonucleotides to examine the expression in the rat hypothalamus of the three major brain subtypes of adenylyl cyclase-Ca^{2+}/calmodulin-stimulable (Type I), Ca^{2+}-insensitive (Type II) and Ca^{2+}-inhibitable (Type V). The hypothalamus expresses high levels only of Type II mRNA, particularly in the supraoptic and paraventricular nuclei. Curiously, the strong expression of the Ca^{2+}-insensitive Type II mRNA and the lack of expression of the major brain specific Type I mRNA does not correlate with the adenylyl cyclase activity, which is largely Ca^{2+}/calmodulin stimulable in plasma membranes prepared from the hypothalamus.

The recent realization that adenylyl cyclases are a superfamily of at least six discrete molecular species (1–12) with distinct responses to Ca^{2+}, $\beta\gamma$ subunits of G-proteins and phorbol esters (2, 6, 12–16) has raised the possibility that the discrete expression of individual species in particular brain areas may provide discrimination of a variety of neuronal signals or intracellular messages (17). Consequently, knowledge of the cyclase expressed in particular brain regions can outline the potential modes of regulation to which the cAMP signalling pathway is subject. For instance, type I adenylyl cyclase, which can be stimulated by Ca^{2+}/calmodulin, has long been implicated in learning and memory processes in invertebrates (18–20). In vertebrates, recent biochemical studies, coupled with high expression of type I in the hippocampal formation (21–23) has strongly implicated this species in the hippocampal model of learning and memory-NMDA-mediated long-term potentiation. By contrast, type V adenylyl cyclase mRNA, a Ca^{2+}-inhibitable species, is highly localized to caudate putamen, nucleus accumbens and olfactory tubercle in the rat (9, 24), which suggests a selective susceptibility to dopaminergic and muscarinic cholinergic receptor regulation. However, the Ca^{2+}/calmodulin-insensitive, but uniquely phorbol ester-sensitive, adenylyl cyclase-type II, is apparently rather diffusely distributed throughout the rat brain, with the strongest signals in pyramidal cells of CA1-CA3 layers of the hippocampus and granular cells of the cerebellum, along with moderate levels in other areas, including the thalamus and hypothalamus (23, 25). The foregoing three subtypes (I, II, V) are the only representatives of the currently described species that are abundantly expressed in the rat brain. Since previous data had demonstrated an extensive implication of cAMP in the physiological functions of the hypothalamus (26–29), we decided to investigate the expression of the major brain adenylyl cyclases in this region. The hypothalamus has the advantage over more complex brain regions that the physiological role played by many of its nuclei are rather well understood in terms of the regulation to which they are subject and their hormonal output. Therefore, this seemed like an area that could benefit from a close analysis of the adenylyl cyclase species expressed therein, as a means of anticipating the potential modes of regulation to which the cAMP signalling pathway is subject.

To obtain more precise information about the dominant cAMP-generating pathway in the hypothalamus, we examined the distribution of the three predominant types I, II and V mRNA by in situ hybridization histochemical analysis with rat-specific, synthetic oligonucleotides as probes. A strong expression of type II mRNA was found in magnocellular neurons of both supraoptic (SO) and paraventricular (PVN) nuclei; the other subtypes I and

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V were virtually absent. The effects of Ca^{2+} on hypothalamic adenylyl cyclase activity were also explored. Surprisingly, a very prominent stimulation of activity by Ca^{2+}/calmodulin was encountered. These results indicate that although the Ca^{2+}-insensitive type II adenylyl cyclase, may be the most prominent mRNA, the most prominent functional activity is Ca^{2+}/calmodulin stimulable.

Results

Expression of Types I, II and V adenylyl cyclases mRNAs

Comparisons between expressions of subtypes I, II and V adenylyl cyclase mRNAs were established at two levels of the hypothalamus (Fig. 1 and 2) using identical probe concentrations and similar *in situ* hybridization procedures. As seen in Fig. 1 and 2, hybridization signals of type I, II and V adenylyl cyclases mRNA were clearly different in the hypothalamus. At anterior levels, type II adenylyl cyclase probe yields strong to moderate signals in several hypothalamic nuclei, such as SO, PVN and the anterior hypothalamic nucleus (Fig. 1A). By contrast, type I and V probes yielded no signals in these regions (Fig. 1B, C). At the posterior level of the hypothalamus, moderate signals are observed for type II in the ventromedial hypothalamic and arcuate nuclei (Fig. 2A, B). No hybridization signals were found at this level with type I and V probes (Fig. 2C, D). To control for the ability of each of these probes to detect adenylyl cyclase mRNAs, intensities of signals detected in the hypothalamus were compared with those found in thalamus and other parts of rat brain (Figs. 1 and 2). Outside the hypothalamus, strong expression of type I occurs in the hippocampal formation, such as the pyramidal cells of CA2 and granular layer of the dentate gyrus, and additional moderate signals are detected in cortical areas, piriform cortex and several thalamic nuclei, such as anterodorsal, ventrolateral, ventroposterior, ventromedial and reticular thalamic nuclei (Fig. 2C). The other major brain adenylyl cyclase, type V is expressed at a high level in the caudate putamen (Fig. 2D). Thus, the lack of I and V mRNA from hypothalamus is credible given their prominent expression elsewhere by the same analysis.

To further examine type II mRNA expression in hypothalamus, autoradiograms were obtained in a series of sagittal (Fig. 3) and coronal sections (Fig. 4). The sagittal section indicates that type II adenylyl cyclase gives its strongest signals in areas such as the hippocampal formation and supraoptic nuclei (Fig. 3). A strong signal is also detected in granular layers of cerebellum (data not shown). As previously observed (12, 14), type II probe gives a signal similar in intensity in both pyramidal cells of CA1-CA3 layers and granular cells of the dentate gyrus (Fig. 2A, B; 3). Sagittal sections also reveal moderate amounts of type II mRNA in several areas, including cerebral cortex, olfactory tubercle, and substantia nigra. Ventrally, the strongest signal of type II mRNA is found in SO (Fig. 3). Indeed, this signal is similar in intensity to that observed in the hippocampal formation.

Coronal sections show the expression of type II mRNA at two different levels of SO (Fig. 4A, B). Type II mRNA expression is highest in magnocellular neurosecretory neurons that are present in both SO and PVN. This signal is higher in intensity than that detected in the amygdaloid complex and piriform cortex. Scattered and strongly labeled accessory magnocellular neurons are also detected in preoptic periventricular and anterior hypothalamic nuclei (Fig. 4A). Above the optic chiasm, the parvocellular neurons of suprachiasmatic nucleus (SCh) exhibit a moderate expression of type II adenylyl cyclase (Fig. 4A). Lower amounts of type II mRNA are seen in other hypothalamic areas, including anterior and preoptic periventricular-, ventromedial- and anterior hypothalamic nuclei. More caudally to SO, the major hypothalamic magnocellular cell groups found in both SO and PVN are strongly labeled with type II probe (Fig. 4B). Strong hybridization signals corresponding to the accessory magnocellular neurons are also detectable at this level. As seen in Fig. 4A and B, hybridization signals found in other hypothalamic nuclei are homogeneous and similar in intensity to those observed dorsally, at the level of thalamic nuclei.

Fig. 5 presents a schematic series of coronal sections corresponding to the expression of type II mRNA at different levels of the hypothalamus. Separate neuronal populations expressing high to moderate levels of type II mRNA are indicated. The strongest signals occur in SO and moderate signals are seen in SCh, whereas other dorsal and anterior hypothalamic nuclei exhibit lower levels of type II mRNA (Fig. 5A). More caudally to SO, moderate expression of type II mRNA are found ventrally at the level of ventromedial hypothalamic and arcuate nuclei (Fig. 5B, C). Homogeneous and low signals occur in other hypothalamic nuclei, including dorsomedial and lateral hypothalamic nuclei. Dorsally to the hypothalamus, the highest expression of type II mRNA is seen in zona incerta, extending to the reticular thalamic nuclei.

Adenylyl cyclase activity

The predominant expression of type II mRNA in the hypothalamus raises the possibility that the adenylyl cyclase activity in hypothalamic membranes may be largely Ca^{2+}-insensitive. In Fig. 6, the effects of increasing concentrations of Ca^{2+} in the presence or absence of calmodulin on adenylyl cyclase activity were determined. When calmodulin (1 μM) is present, Ca^{2+} stimulated activity in a concentration-dependent manner, with a maximal stimulation measured in the presence of approximatively 0.7 μM free Ca^{2+}. At concentrations of Ca^{2+} greater than 0.9 μM, activity declined (Fig. 6A). Ca^{2+} stimulates hypothalamic activity by at least 3-fold. In the absence of added calmodulin, a small increase in Ca^{2+}-dependent activity was observed. This increase is likely due to endogenous calmodulin in the membrane preparation or assay components, such as creatine phosphokinase or bovine serum albumin (Fig. 6B). Thus, our data showed that Ca^{2+} predominantly stimulates enzyme activity in hypothalamic membranes by a calmodulin-dependent mechanism.[1]

Discussion

Ca^{2+}/calmodulin-stimulated adenylyl cyclase was the first member of this signal transduction family to be purified and cloned from bovine brain (1). Indeed, the susceptibility of brain cyclase to this regulation rendered it distinct from peripheral adenylyl cyclase activity. Further cloning studies have now identified 6

[1]It is not possible to determine whether hypothalamic adenylyl cyclase can be stimulated by PKC in these experiments, since the experiments implicating type II with PKC were only successful with phorbol ester treatment of intact transfected HEK 293 cells (14–16).

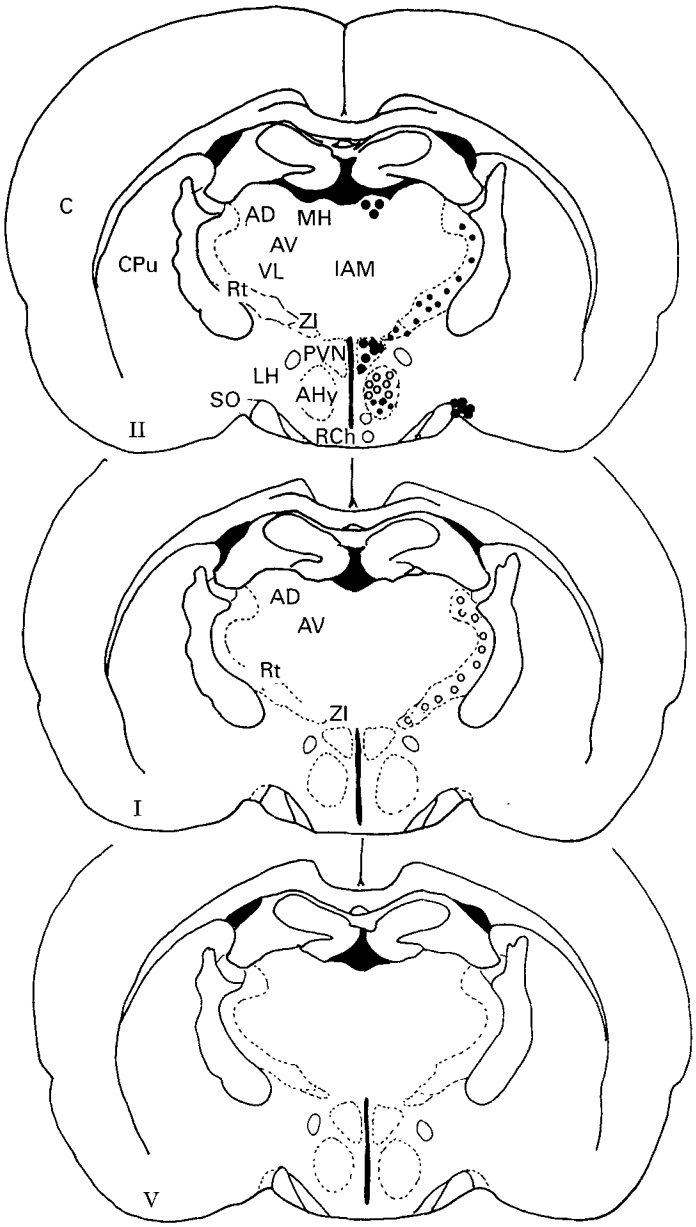

FIG. 1. Schematic series of coronal sections illustrating the distribution of Types II, I and V mRNA-containing neurons (dots) at the level of hypothalamus. Relative intensity of labeling was established as follows: ● strong, • moderate, ○ weak. Type II probe is strongly expressed in both hypothalamus and thalamus in contrast to type I and V probes. Abbreviations: AD: anteroventral thalamic n., AHy: anterior hypothalamic n., AV: anterodorsal thalamic n., C: cortex, CPu: caudate putamen, IAM: interanteromedial thalamic n., LH: lateral hypothalamic n, MH: medial habenular n., PVN: paraventricular n., RCh: retrochiasmatic n., Rt: reticular thalamic n., SO: supraoptic n., VL: ventrolateral thalamic n., ZI: zona incerta.

adenylyl cyclase species, which—rather than being redundant, repetitive isoforms—have distinct regulatory susceptibilities and tissue distributions (2–12). Thus, types I and III are stimulated by Ca^{2+}, acting through calmodulin (12, 30, 31); types II and IV are insensitive to Ca^{2+} (2, 12) and types V and VI are inhibited by Ca^{2+} (4, 6). Furthermore type I is inhibited by $\beta\gamma$ subunits of G-proteins, whereas type II is stimulated by $\beta\gamma$ subunits (12, 13). In addition, phorbol esters treatment of transfected HEK 293 cells exerts a profound stimulation of type II adenylyl cyclase, which is a unique quality among the known adenylyl cyclases (14–16). In many cases these observations, which initially emanated from in vitro studies, have been validated or strongly supported by studies performed in intact cells (24, 32–34). Therefore, it is now appropriate to assume that physiological regulators which either elevate $[Ca^{2+}]_i$, liberate $\beta\gamma$ subunits of G-proteins, or activate PKC, can significantly modify the functioning of the cAMP signalling pathway. These multiple regulatory modes for adenylyl cyclase prompts a detailed examination of distinct adenylyl cyclase species expression in the brain as an essential element of understanding how cAMP may modulate neuronal function. Previous studies had implicated cAMP signalling in many aspects of hypothalamic function, including the regulation of gene transcription, mRNA levels, propeptide processing and peptide secretion (26–29).

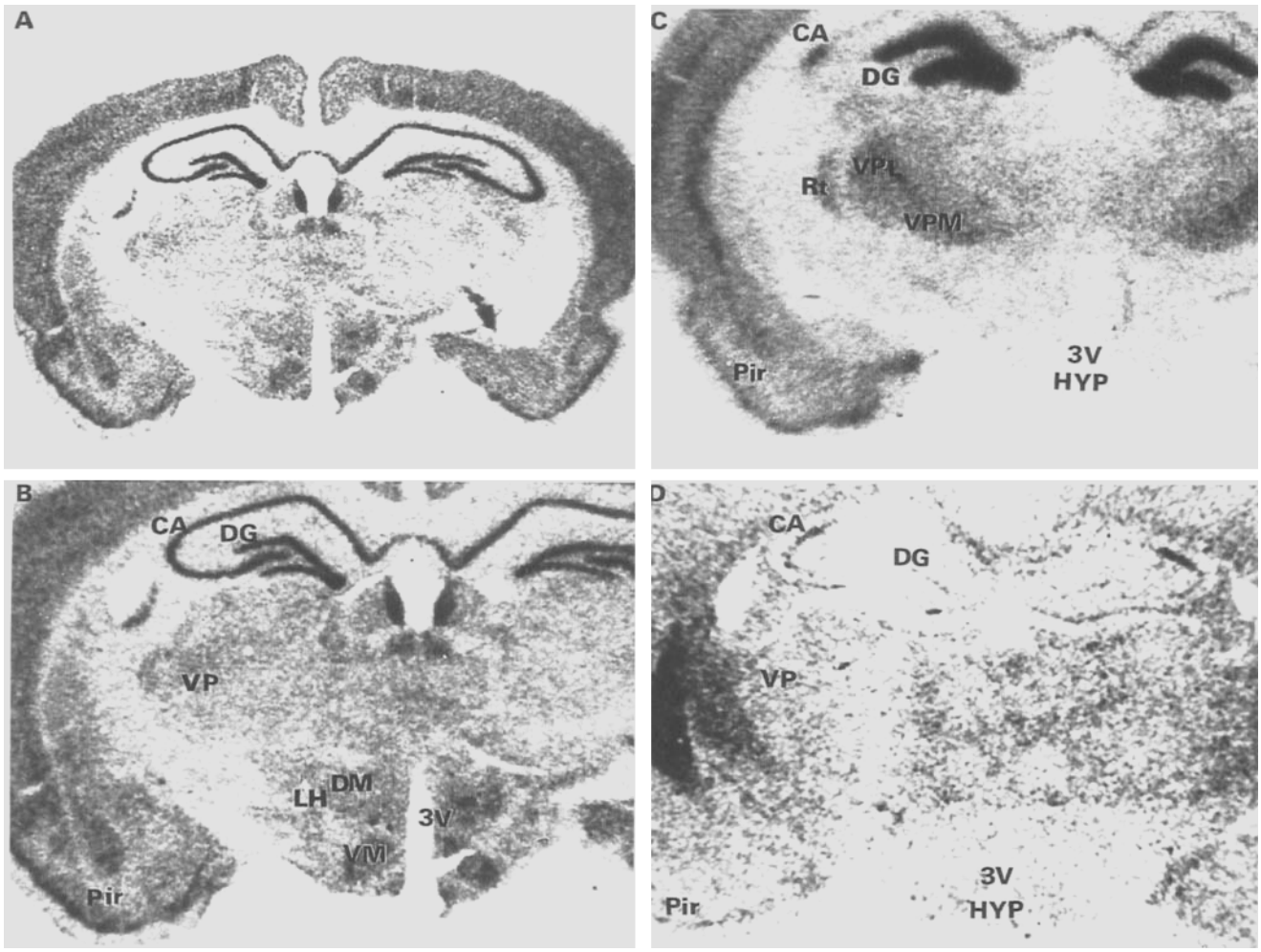

FIG. 2. Autoradiograms of coronal sections illustrating the expression of type II (A-B), type I (C) and type V (D) mRNA in the hypothalamus. Abbreviations: CA: fields CA1-3 of Ammon's Horn, DG: dentate gyrus, DM: dorsomedial hypothalamic n., Hyp: hypothalamus, LH: laterol hypothalamic n., Pir: piriform cortex; Rt: reticular thalamic n., VM: ventromedial hypothalamic n., VP: ventromedial hypothalamic n; 3V: 3rd ventricle.

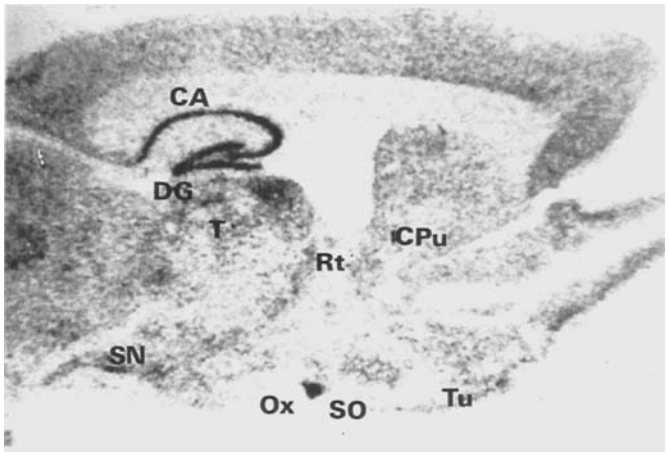

FIG. 3. Autoradiograms of a sagittal section showing the strong expression of type II mRNA in both hippocampal formation and supraoptic nucleus. Abbreviations: CA: fields CA1-3 of Ammon's Horn, CPu: caudate putamen, DG: dentate gyrus, Ox: optic chiasm, Rt: reticular thalamic n., SN: substantia nigra, SO: supraoptic n., T: thalamus, Tu: olfactory tubercle.

Previous preliminary *in situ* hybridization studies of Type II adenylyl cyclase mRNA had indicated a rather widespread expression of this species in the rat brain (23, 25). The strongest signals were apparently localized in the hippocampus, olfactory tubercle, neocortex, piriform and cerebellar cortex. The present results, which include a detailed examination of the hypothalamus, indicate that Type II is the predominant adenylyl cyclase mRNA expressed in this region and yields strong signals in both SO and PVN, which are similar in intensity to those observed in the hippocampus. These nuclei represent a homogeneous population of magnocellular cells, which contain both oxytocin (OT) and vasopressin (AVP) (35–37). These cells also synthetize other peptides, notably opioid peptides (38). Osmotic stimuli elicit a coordinated release of OT and AVP in both nuclei, which is accompanied by an apparently cAMP-mediated increase in the amount of Gs and Gi (26, 39). Furthermore, continuous treatment of hypothalamic neurons with either PKC-activators or forskolin markedly elevates AVP secretion, suggesting both PKC- and PKA-modes for regulation of AVP gene transcription (40, 41).

Moderate levels of Type II mRNA were also detected in other hypothalamic nuclei, again in contrast with the absence of expression of types I and V mRNA. Such nuclei included the SCh,

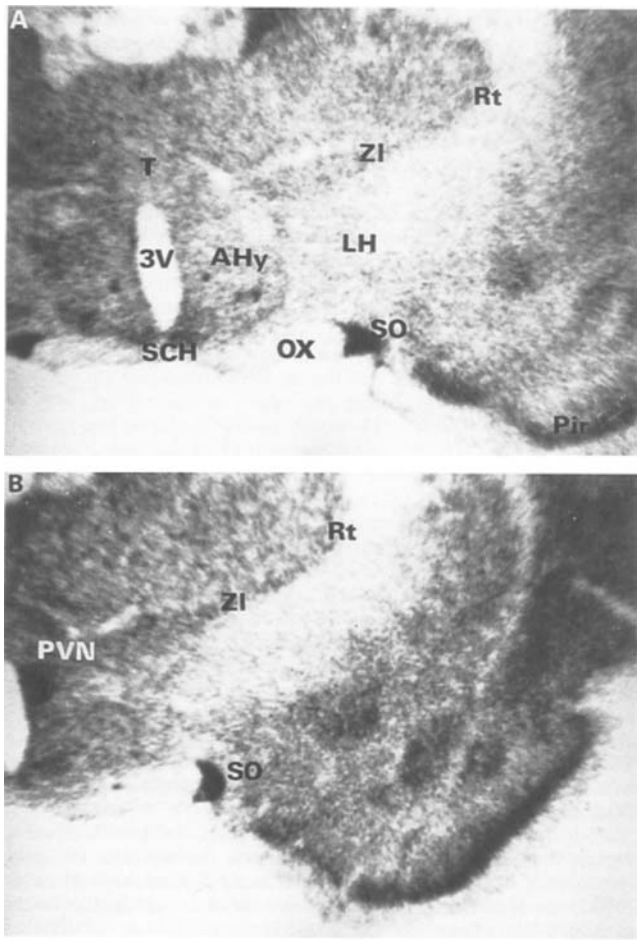

FIG. 4. Autoradiograms of coronal sections showing the expression of type II mRNa at two levels of supraoptic nucleus (SO). Abbreviations: AHy: anterior hypothalamic n., LH: lateral hypothalamic n., Ox: optic chiasm, Pir: piriform cortex, PVN: paraventricular n., Rt: reticular thalamic n., SCh: suprachiasmatic n., SO supraoptic n; T. thalamus, ZI: zona incerta, 3V: 3rd ventricle.

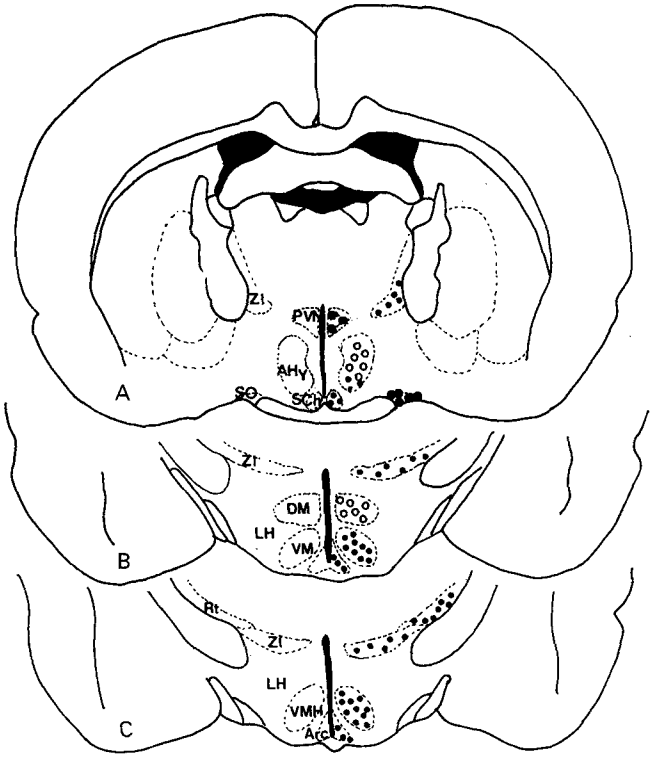

FIG. 5. Schematic series of coronal sections illustrating the expression of type II mRNA at different levels of hypothalamus. Relative intensity of labelling was established as follows: ● strong, ◉ moderate, ○ weak. Abbreviations: AHy: anterior hypothalamic n., Arc: arcuate n., DM: dorsomedial hypothalamic n., LH: lateral hypothalamic n., PVN: paraventricular n., Rt.: reticular thalamic n., SCh: suprachiasmatic n., SO: supraoptic n., VM: ventromedial hypothalamic n., ZI: zona incerta.

ventromedial and arcuate nuclei. Concerning the SCh, which is the site of circadian pacemaker that generates and regulates circadian rhythms (42–43), previous studies have described that cyclic changes in endogeneous cAMP are correlated with the mammalian circadian clock (44). The susceptibility of type II adenylyl cyclase to PKC activation and the presence of moderate levels of PKC in the SCh (45), support a potential regulation of this cyclase by PKC in this region. The ventromedial and arcuate nuclei also express high densities of Gsα (46, 47), which would be an essential element for the highly Gs responsive type II cyclase.

Comparison between the mRNA patterns and the adenylyl cyclase activity in the hypothalamus shows marked discrepancies; in particular, the predominance of Ca^{2+}-insensitive type II mRNA contrasts with an adenylyl cyclase activity that is largely Ca^{2+}/calmodulin stimulable. The stimulation of hypothalamic adenylyl cyclase by Ca^{2+} is quite comparable to the degree of stimulation seen in other brain regions, such as the cerebellum and hippocampus, which express high levels of type I mRNA. Two possibilities can explain this discrepancy: i) another, as yet unidentified Ca^{2+}/calmodulin-stimulable adenylyl cyclase mRNA may be present but undetected in the hypothalamus. In this

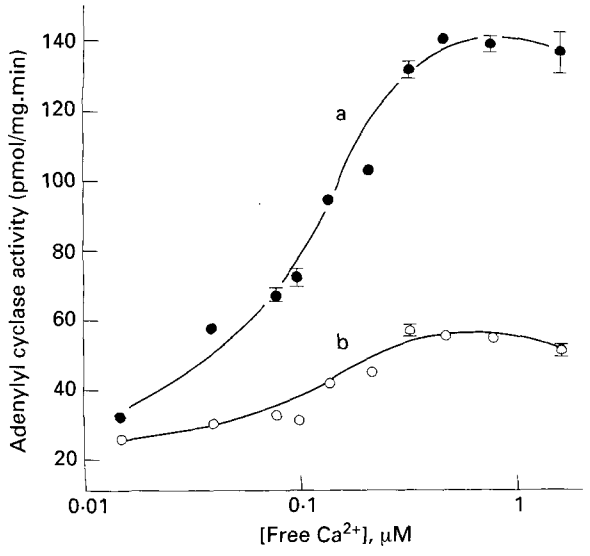

FIG. 6. Effects of Ca^{2+} and calmodulin on hypothalamic adenylyl cyclase activity. Hypothalamic plasma membranes were washed with EGTA and adenylyl cyclase activity was measured as described in the Materials and Methods. Adenylyl cyclase activity was measured in the presence of the indicated free Ca^{2+} concentrations in the presence (a) and absence (b) of calmodulin (1 μM). Data are from a representative of five similar experiments, performed in triplicate. SDs were $< 10\%$ of the mean.

context, another Ca^{2+}/calmodulin-stimulable adenylyl cyclase (type VIII) has been found at low levels in some hypothalamic nuclei, such as arcuate nuclei in recent in situ hybridization analyses (48, 49); ii) Cell bodies of other brain regions that innervate the hypothalamus may express mRNA for either types I, VIII or another Ca^{2+}/calmodulin stimulable adenylyl cyclase, which are transported as protein to hypothalamic terminal fields, resulting in the detection of functional activity but very little mRNA.

The present observations, demonstrate that at least two distinct functional subtypes of adenylyl cyclases may participate in the modulation of cAMP signalling process in the hypothalamus. They also make the interesting point that mRNA expression alone does not necessarily reveal the predominant functional subtype, and remind us that presynaptic projections from other areas can considerably influence the gross regulatory picture. It is not inconceivable that type II adenylyl cyclase is the major cyclase within the cells of the identified hypothalamic nuclei and that all of the Ca^{2+}/calmodulin stimulated activity is presynaptic and originates outside of the hypothalamus. It will be very interesting to determine the origin and the nature of the Ca^{2+}/calmodulin stimulable adenylyl cyclases that has been detected in these studies.

Materials and Methods

Animals

Male Sprague Dawley rats weighing about 150–200 g were anesthetized with pentobarbital (50 mg/kg i.p.) and perfused through the aorta with 100 ml of 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). The brains were quickly removed, cut into 5 mm blocks and postfixed in the same fixation buffer for 3 h at 4 °C. Tissues were then immersed in 0.1 M PBS containing 30% sucrose (16 h, 4 °C), then frozen on powdered dry ice, and stored at −80 °C until use. Sagittal and coronal sections (30 μm) were cut, mounted on glass slides and stored at −80 °C.

Oligonucleotide probes

Oligonucleotides probes were synthetized using an applied Biosystem 394 DNA synthesizer. The types I, II and V penentemer ('50-mer') antisense oligonucleotides were as described previously (23, 24). The probes were labeled at the 3′ end with [^{35}S] dATP (1000 Ci/mmol, 37 TBq/mmol, NEN) and terminal deoxynucleotide transferase (Tdt, Stratagene) in a 20 μl mixture containing 100 μM potassium cacodylate (pH 7.2), 2 mM $CoCl_2$, Tdt (34 U), [^{35}S]-dATP (3 pmol/μl) for 1 h at 37 °C. Reactions were stopped by addition of ammonium acetate (2.5 M). Radiolabeled probes were separated from uncorporated dATP by ethanol precipitation.

In situ *hybridization studies*

In situ hybridization procedures were performed as described previously (23, 24). Free floating sections (30 μm) were acetylated in 0.1 M triethanolamide pH 8.0/0.25% acetic anhydride. Subsequently, they were rinsed with 2 × SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0), and prehybridized with a buffer containing 2 × SSC and 2 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) at 42 °C for 1 h. The sections were then hybridized overnight, in 50 μl of a mixture containing 50% formamide, 2 × SSC, 10% dextran sulfate, 1 × Denhardt's solution, 50 mM dithiothreitol, 0.5 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, and [^{35}S]-dATP-labeled probes (10^7 cpm/ml, 100 μl/tube). After hybridization, sections were rinsed sequentially in 2 × SSC for 15 min (room temperature, RT), 1 × SSC for 15 min (RT), 1 × SSC for 1 h at 37 °C, 0.5 × SSC (42 °C). They were mounted onto gelatin-coated slides, dried at RT, before being exposed to hyperfilm-βmax (Amersham) for 2 days at RT. Films were developed with Kodak D-19 solution, fixed with rapid fixer (Kodak) and washed with water.

Measurement of adenylyl cyclase activity

Hypothalami were removed from male Sprague-Dawley rats (250–300 g) and plasma membranes were prepared and adenylyl cyclase activity was measured as described previously using 10 μg membrane protein, 100 μM ATP, 1 mM $MgCl_2$, 200 μM EGTA and the indicated free Ca^{2+} concentrations (50, 51). The calculation of free Ca^{2+} was performed using an iterative computer program as described (52). Since the program considers the major determinants of free Ca^{2+} concentrations in the assay (i.e. Ca^{2+}, Mg^{2+}, Na^+, EGTA, ATP, and H^+ concentrations), the reported values are well-developed estimates of free Ca^{2+} concentrations. Background [Ca^{2+}] is assumed to be 10 μM.

Nomenclature

The terminology follows that of the atlas of Paxinos & Watson (53).

Acknowledgements

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