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THE EFFECT OF INTRACEREBROVENTRICULARLY ADMINISTERED DIBUTYRYL ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE ON CEREBROSPINAL FLUID AND SERUM DOPAMINE-β-HYDROXYLASE IN RABBITS

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Abstract—Intracerebrospinal injected or infused dibutyryl adenosine 3′,5′-cyclic monophosphate provoked (a) an initial small decrease of cerebrospinal fluid dopamine-β-hydroxylase activity which lasted for 2 hours, followed by a significant rise of a 4–6 hours duration, together with a rise in total cerebrospinal fluid protein concentration, (b) a significant increase in serum dopamine-β-hydroxylase activity. Pretreatment of rabbits with two intracisternal injections of 6-hydroxydopamine completely abolished the dibutyryl adenosine 3′,5′-cyclic monophosphate induced release of dopamine-β-hydroxylase in the cerebrospinal fluid but only partially the release of dopamine-β-hydroxylase in the serum.

Although it was not earlier than 1970 that Rasmussen [1] formulated his hypothesis on the cAMP‡, involvement in the excitation–secretion process in general, a vast amount of experimental data has been produced since then [2]. As far as the release of the noradrenergic transmitter is concerned, the results have led to conclusions in favour [3–6] as well as against [7, 8] this hypothesis.

Recently we studied the effect of various stimulants on the central noradrenergic system of rabbits *in vivo* [9, 10] using DβH in the CSF as a measure for release of the neurotransmitter in the brain [9, 11]. The reliability of this model in the study of the excitation-secretion coupling system has been debated before [12]. We found that the intravenous injection of a convulsive dose of bicuculline causes a significant increase in CSF-DβH being preceded by a transient but significant increase in CSF-cAMP [13]. An analogous observation has been reported recently for the adrenal medulla showing cAMP release preceding the release of catecholamines after acetylcholine stimulation [14]. Both observations prompted us to investigate the effect of cAMP itself on the release of the noradrenergic neurotransmitter from the brain.

The present study gives an account of the effects of intracerebroventricularly administered dBcAMP, a stable analogue of cAMP, on the levels of DβH in the CSF and serum of rabbits. The use of phosphodiesterase-inhibitors was intentionally avoided since apart from an increase in cAMP levels, other pharmacological activities might complicate the results (see [15]).

MATERIALS AND METHODS

Male rabbits (2–2.5 kg), anaesthetized with an intravenous injection of a 25 per cent urethane (w/v in 0.9 per cent saline) solution (5 ml/kg) were used in all experiments.

The general outline of the experiments was as follows: after anaesthesia and preparation for sampling of CSF and serum and intraventricular administration [10], the animals were infused with dBcAMP in one of the lateral ventricles. Various periods of infusion time and various concentrations of dBcAMP were tested (see Results). Most results are obtained with a continuous infusion of dBcAMP during the whole experiment at 0.25 μmole per hour dissolved in 254 μl of saline or artificial CSF [16]. The samples taken before the start of the experiment were considered as control value and taken as 100 per cent.

Cerebrospinal fluid samples (300 μl) were collected from a catheter, inserted into the cisterna magna, by means of a peristaltic pump, at 100 μl/min. Another cannula was placed in a femoral artery for blood collection and continuous monitoring of blood pressure by means of a Statham (P23 AA) transducer and a Dynograph recorder (Type R Beckman). Both CSF and blood samples were collected on ice, the blood samples were centrifuged at low speed and the serum recovered for DβH assay.

In some animals 6-OHDA pretreatment was performed by injection into the lateral ventricles of 100 μl of saline containing 0.1 mg of ascorbic acid and 500 μg of 6-OHDA. A second injection into the contralateral ventricle was given one week later. Animals were used for experiments 1–2 weeks after

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‡ Abbreviations: cAMP: adenosine 3′,5′-cyclic monophosphate; dBcAMP: dibutyryl adenosine 3′,5′-cyclic monophosphate; CSF: cerebrospinal fluid; DβH: dopamine-β-hydroxylase; CNS: central nervous system; 6-OHDA: 6-hydroxydopamine.

the second injection. Control rabbits received the same volume of the vehicle by the same route at similar intervals.

DβH was assayed with the method of Molinoff *et al.* [17] as adapted for the determination of DβH in CSF-samples [9]. Two hundred microliters of the 1/5 diluted CSF-samples or of the 1/10 diluted serum samples were used for the assay and incubated at 37° for 2 hr in a reaction mixture containing 20 μl of 1 M sodium acetate, pH 5.0, 25 μl of 0.5 M sodium fumarate, pH 5.0, 10 μl of 12 mM pargyline in H_2O, 1500 units of catalase in 10 μl of H_2O, 10 μl 0.03 M tyramine, pH 5.0, 25 μl of $CuSO_4$ in the appropriate concentration to give optimal activity; each sample was assayed in the presence of six different concentrations of $CuSO_4$, ranging from 0.16 to 9.6 μM. Duplicate samples kept at 0° in the first step were used as blanks. After 2 hr of incubation the pH of the mixture was raised to 8.6 by adding to each tube 100 μl of a mixture of 40 μl of 1 M Tris–HCl buffer, pH 8.6, 10 μl of bovine adrenal phenyl-ethanolamine-*N*-methyltransferase (PNMT), purified as described by Molinoff *et al.* [17] and 50 μl of a solution containing 50 nCi of *S*-adenosyl-L-methionine-methyl-^{14}C (sp. act. 55 mCi/mmole). Forty five minutes later, the PNMT reaction was stopped by the addition of 0.5 ml of 0.5 M borate buffer, pH 10. The labeled synephrine was extracted into 6 ml Toluene Scintillator (Packard) isoamylalchol mixture (6/4). Four ml of the organic phase were transferred to a counting vial for liquid scintillation counting. Standard assays, consisting of 250 and 500 pmoles of octopamine added to the entire reaction mixture, were run in all experiments. In this assay, one unit (U) of activity represents the formation of 1 nmole of octopamine from tyramine/hr at 37°.

Noradrenaline was determined by the radiochemical method of Henry *et al.* [18]. Proteins in the CSF were determined by the protein–dye binding method of Bradford [19], using bovine serum albumin as standard. Contamination of the CSF with serum proteins was monitored as described by Chubb *et al.* [20] using the difference between the isoenzyme pattern of CSF and serum acetylcholinesterase. For statistical analysis of data Student's *t*-test was used.

RESULTS

Intracerebroventricular infusion of dBcAMP (0.25 μmole/hr, continuous infusion during the experiment, approx. 5–6 hr) produces a significant increase in CSF-DβH of 4–6 hr duration, starting after an initial delay period of 2 hr (Fig. 1(A)). When the infusion time was reduced to 90 min (3 experiments) or when the concentration of dBcAMP was reduced to 5 nmoles/hr in a continuous infusion experiments (3 experiments), the same delay period followed by an increase in CSF-DβH was seen, indistinguishable from the above mentioned observations. In control experiments, only a slight increase was seen towards the end of the experiment.

When dBcAMP was added to the DβH incubation mixture, no alteration in enzymatic activity was observed (less than 5 per cent deviation from control at 0.25 μmole dBcAMP/ml incubation mixture).

An increase in CSF proteins, ranging between 2

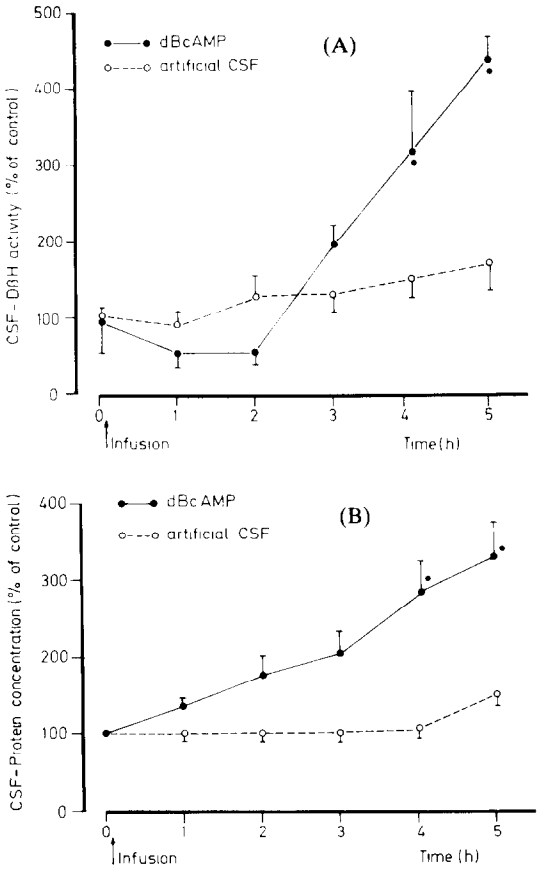

Fig. 1.(A) Change in DβH activity in the cerebrospinal fluid of rabbits, expressed as percentage of control value, during an intracerebroventricular infusion of 0.25 μmole 10^{-6} mole dBcAMP/hr at a speed of 254 μl/hr. Basal CSF-DβH level (= 100 per cent of control): 0.98 ± 0.09 U/ml (n = 6). *P < 0.05 as compared to controls. (B) Change in protein concentration in the cerebrospinal fluid of rabbits, expressed as percentage of control value, during an intracerebroventricular infusion of 0.25 μmole 10^{-6} mole dBcAMP/hr at a speed of 254 μl/hr. Basal CSF-protein level (= 100 per cent of control): 0.28 ± 0.04 mg/ml (n = 6). *P < 0.05 as compared to controls.

and 3 times the pre-infusion values was also observed at the end of a 5-hr infusion experiment (Fig. 1(B)). This increased protein level, which did not occur in control animals, was not due to contamination with serum proteins as the isoenzyme pattern of acetylcholinesterase in CSF samples of control animals and of dBcAMP treated animals was exactly the same, and different from the serum pattern. One single band was observed in the CSF samples, the serum pattern was more complex and contained more than two bands of acetylcholinesterase activity.

In the serum, DβH activity increases upon dBcAMP infusion, no delay period being noticed (Fig. 2). Control values obtained after intracerebroventricular infusion of vehicle are shown for comparative purposes.

The effect of centrally administered dBcAMP on serum noradrenaline is shown in Fig. 3. The increase in serum levels of noradrenaline during the experiment is statistically different from control values. In

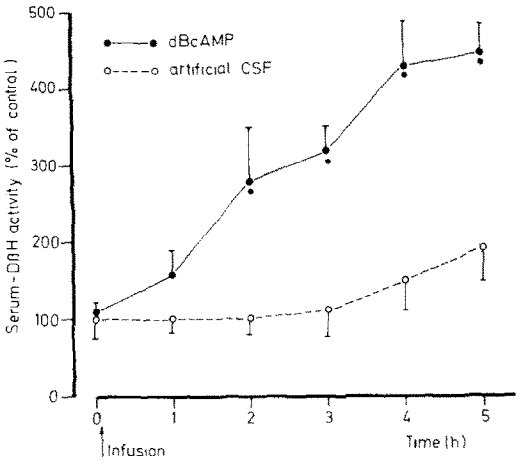

Fig. 2. Change in serum-DβH activity, expressed as percentage of control value, during intracerebroventricular infusion of 0.25 μmole 10^{-6} mole dBcAMP/hr at a speed of 254 μl/hr. Basal serum DβH level (= 100 per cent of control): 3.36 ± 0.55 U/ml (n = 6). *P < 0.05 as compared to controls.

all these experiments, blood pressure was continuously monitored through the femoral artery. The infusion of dBcAMP causes a non-significant decrease of 13 per cent (range 10–15) in mean arterial blood pressure as compared to the pre-infusion blood pressure.

When the rabbits were pretreated with two intracisternal injections of 6-OHDA at one week interval, basal CSF-DβH levels (0.98 ± 0.09 U/ml, n = 6) decreased to a significant degree (0.62 ± 0.09 U/ml, n = 4), compatible with previously observed values [10], while serum-DβH levels remained unaffected. Upon intracerebroventricular infusion of dBcAMP into lesioned rabbits, the dBcAMP induced effect on CSF-DβH was abolished (Fig. 4(A)). The serum response was only partially influenced in so far that 6-OHDA pretreatment resulted in a 25–50 per cent lowering of the dBcAMP evoked increase in DβH in the control serum (Fig. 4(B)).

The increase in CSF-protein concentration after dBcAMP infusion (shown above), is reduced to a great extent in so far that no distinction can be made from the dBcAMP controls.

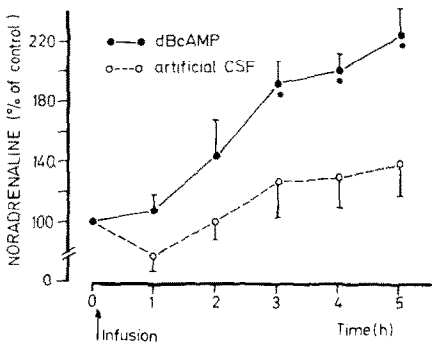

Fig. 3. Noradrenaline content in serum of rabbits as percentage of control value after intracerebroventricular dBcAMP infusion (0.25 μmole 10^{-6} mole/hr). Basal serum NA level (= 100 per cent of control): 1.52 ± 0.13 ng/ml (n = 3). *P < 0.05 as compared to controls.

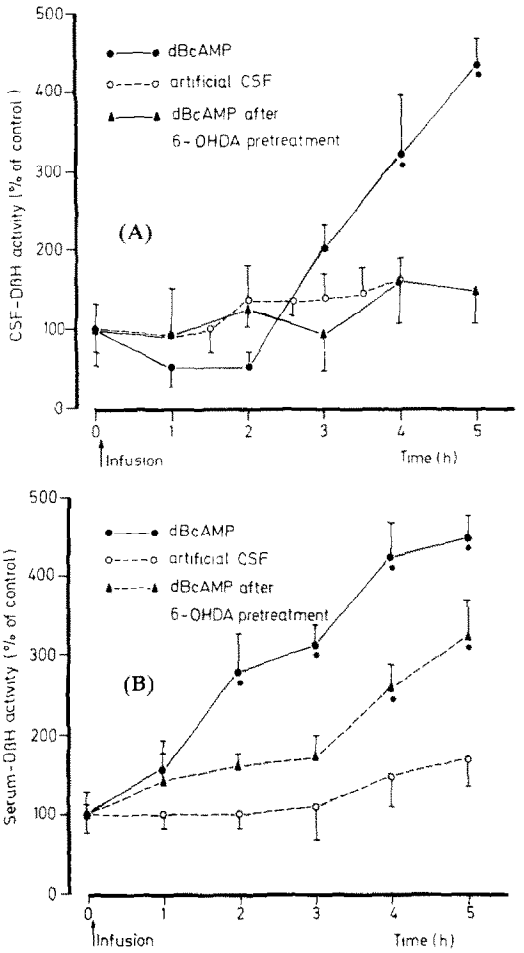

Fig. 4. (A) Comparison of the change in CSF-DβH activity, expressed in percentage of control value, in rabbits with or without 6-OHDA pretreatment followed by an infusion of dBcAMP (n = 3). *P < 0.05; the statistical significant difference is calculated vs the control, without 6-OHDA and with artificial CSF instead of dBcAMP. (B) Comparison of the change in serum-DβH activity, expressed in percentage of control value, in rabbits with or without 6-OHDA pretreatment followed by an infusion of dBcAMP (n = 3). *P < 0.05; the statistical significant difference is calculated vs the control, without 6-OHDA and with artificial CSF instead of dBcAMP.

DISCUSSION

Intracerebroventricular infusion of dBcAMP increases both CSF- and serum-DβH activity. The concentration of dBcAMP used routinely in these experiments should not be considered as unphysiological for the animal. Indeed, it has been demonstrated that tissue-cAMP concentrations can reach values up to the mM range, which is much higher than the CSF or brain tissue concentrations ever reached in the present experiments [21, 22]. Central administration of dBcAMP appears to result in a steep increase in CSF-DβH, during the time of the experiment, delayed for approximately 2 hours. An easy explanation of the transient delay in CSF-DβH increase is not available. The hypothesis of a protein-synthesis inducing effect of dBcAMP, a well documented property of the nucleotide [15], seems to fit most of the results. Indeed, this latency period

could be due to the time required for induction of the biosynthetic mechanism and the time needed for a large molecule like DβH to migrate from its site of biosynthesis to its site of release into the CSF. Further evidence in favour of this explanation is found in CSF-protein determinations which show an increase in CSF-protein concentration after intracerebroventricular infusion of dBcAMP. The stimulatory effect of dBcAMP on the biosynthesis of DβH in other nervous tissue has been reported before [23]. No influence of dBcAMP on the protein–dye reaction was observed; hereby, a false positive result could be excluded.

When rabbits were pretreated with 6-OHDA, a significant decrease of CSF-DβH level was observed, as described before [10]. Furthermore, a complete disappearance of increase in CSF-DβH upon dBcAMP infusion was seen, most probably due to the degeneration of central catecholaminergic neurons by the intracerebroventricularly administered 6-OHDA. The fact that the increase in protein concentration was less pronounced in 6-OHDA treated rabbits suggests that the noradrenergic neurons at least provide part of the proteins in the CSF upon dBcAMP interaction. No conclusions are possible, however, as to the site of interaction of the nucleotide, either directly on the noradrenergic neurons or indirectly via other neuronal systems.

The possibility of contamination of CSF samples with serum needed serious consideration as only a very minor contamination might artificially increase the CSF–DβH activity [11]. Additionally, it has been described [24] that the blood–brain barrier is sensitive to cAMP and forms pinocytotic vesicles which are supposed to reflect an enhanced permeability of the barrier. In our experiments no difference was found between the isoenzyme pattern of acetylcholinesterase upon gel electrophoresis of CSF samples of control and dBcAMP treated rabbits. Despite the increase in protein concentration, only one band of enzyme activity was seen. If CSF samples would have been contaminated with serum-proteins, the acetylcholinesterase isoenzymes present in serum would have contaminated the CSF, resulting in a comparable pattern for both CSF and serum.

An additional argument for the hypothesis that serum-proteins do not contaminate the CSF is found in the 6-OHDA results, where the CSF-DβH level upon dBcAMP infusion remains within control values, while serum-DβH increases to significant higher values.

Intracerebroventricular infusion of dBcAMP gives rise to a significant, time-dependent increase in serum-DβH and noradrenaline. This could be due to the peripheral stimulation induced by central pathways not destroyed by 6-OHDA but affected by dBcAMP. It is known that dBcAMP in certain species induces convulsions [21], which in turn are shown to cause an increase in DβH and noradrenaline in serum [10]. It has to be mentioned, however, that our experimental set-up does not allow overt convulsions to occur due to the anaesthesia. It seems likely that these central pathways are neither dopaminergic nor noradrenergic as chemical sympathectomy does not prevent this effect. The possible spillover of centrally administered dBcAMP in the periphery cannot evoke the phenomenon as an intravenous injection of dBcAMP does not influence serum-DβH in a significant manner (unpublished results).

Both noradrenaline and DβH are believed to be stored in the same vesicle, and released simultaneously by an exocytotic process [25]. Their simultaneous appearance in the serum supports the hypothesis that the effect of centrally administered dBcAMP on the peripheral noradrenergic system is due to its interference with the release mechanism.

Pretreatment with 6-OHDA results in a diminished response to dBcAMP when compared to non-pretreated animals. It can be argued that serum-DβH is only partially affected by central noradrenergic and dopaminergic nuclei as both are largely destroyed by 6-OHDA pretreatment.

Although the present results suggest that cAMP is involved in the release mechanism, several fundamental questions remain unanswered such as: At what exact location in the brain does dBcAMP interfere? Is this interaction directly on the noradrenergic neurons or via other pathways involving other neuronal systems?

Even more important is the question of whether cAMP and the noradrenergic function are related to each other in such a way that an increase in cAMP above a certain threshold level is always accompanied by an activation of the noradrenergic secretory system, measured by an increase in DβH activity in CSF and serum.

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