Antidiuretic Effects of Dibutyryl-Cyclic AMP Microinjected into the Hypothalamic Paraventricular Nucleus in a Water-Loaded and Ethanol-Anesthetized Rat

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Abstract—Effects of dibutyryl-cyclic AMP (db-cAMP) and cyclic AMP (cAMP) when microinjected into the hypothalamic paraventricular nucleus (PVN) in a water-loaded and ethanol-anesthetized rat on the rate of urine outflow, urine osmotic pressure and other visceral functions were investigated. The microinjection of db-cAMP decreased the rate of urine outflow with concomitant increase in the urine osmotic pressure, but did not change mean blood pressure, heart rate, respiration rate and rectal temperature. The antidiuretic effect of db-cAMP was more potent than the effect of cAMP, the median effective doses (ED₅₀) being approx. 40 nmol for db-cAMP and more than 300 nmol for cAMP, respectively. The time-courses for the antidiuretic effects and for the increase in the urine osmotic pressure showed a similar pattern, with the maximal effect at approx. 30 to 40 min and the duration of approx. one hour or longer. The effect of db-cAMP was potentiated by pretreatment with methylxanthines and inhibited by pretreatment with atropine. A second microinjection of db-cAMP induced a less potent antidiuretic effect than the first microinjection (tachyphylaxis). The results indicated the antidiuretic effects of microinjection of db-cAMP and cAMP into the PVN, and a possible mechanism for this was discussed.

The paraventricular nucleus (PVN) in the hypothalamus is known to contain magnocellular neurons which synthesize antidiuretic hormone (ADH). The hormone synthesized in the PVN is transported through the axonal tracts to the neurohypophysis. When the neurons are stimulated, the hormone is released from the neurohypophysis into the general circulation and enhances water reabsorption from the distal tubule and collecting duct of the kidney, resulting in an antidiuretic effect (1–4).

The presence of an acetylcholine (ACh) system in the nucleus (5, 6) and the finding that iontophoretic application of ACh excites the neurons of the PVN (7) have suggested a role of cholinergic innervation in the nucleus. Microinjection of muscarinic agonists such as oxotremorine and ACh into the nucleus causes potent antidiuretic

effects, which are blocked completely by preinjection of a muscarinic antagonist, atropine, indicating that the stimulation of muscarinic receptors induced the antidiuretic effect (8).

On the other hand, histofluorescence of catecholamine-containing varicosities and immunohistochemical localization of nerve terminals containing catecholamine-synthesizing enzymes on the magnocellular neurons in the PVN strongly suggest adrenergic regulation of ADH release (9–12). The β -adrenoceptor agonist isoproterenol when microinjected into the PVN induces a strong antidiuretic effect, which is stereospecific and blocked completely by β -adrenoceptor antagonists. The antidiuretic effect induced by the β -adrenoceptor agonist is inhibited partially by the muscarinic antagonist (13), suggesting a portion of the

effect may be due to the release of ACh from presynaptic area. Since adenosine-3',5'-cyclic monophosphate (cAMP) as a second messenger of β -adrenoceptor stimulation is known to enhance the release of ACh in the neuromuscular junction (14), the intestine (15, 16) and the brain (17–19), we tried to test the effects of microinjection of cAMP and its analog into the PVN in the present study by determining the following: antidiuretic effects, inhibitory effects by the muscarinic antagonist, and effects on other visceral functions which might affect the urine outflow. Urine osmotic pressure was also measured in order to know whether the antidiuretic effect was due to enhanced reabsorption of water by antidiuretic hormone.

Materials and Methods

Animals and drugs: Male Wistar rats, weighing 280–320 g, were used. Adenosine-3',5'-cyclic monophosphate (cAMP) sodium salt and N⁶,-O^{2'}-dibutyl-adenosine-3',5'-cyclic monophosphate (db-cAMP) sodium salt (Sigma Chemical Co., St. Louis, MO), atropine sulfate (Iwaki Co., Tokyo), phenoxybenzamine hydrochloride (Nakarai Chemicals, Kyoto), theophylline (Wako Pure Chemical Industries, Ltd., Osaka), and 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical Co., St. Louis, MO) were purchased. The other chemicals used were the analytical grade available.

Measurement of urine outflow and urine osmotic pressure: Urine outflow was measured by the method of Dicker, with some modifications (8, 20). The rats were starved overnight for approx. 17 hr, having free access to water. The animals were loaded orally through a catheter with a volume of water equivalent to 5% of the body weight and then the same volume of 12% ethanol. The cannulae were inserted into the trachea, bladder, and external jugular vein, respectively. The rat was then fixed in a stereotaxic instrument for rats (Takahashi Co., Tokyo). The number of drops of urine outflow from the cannula inserted into the bladder was counted using a photoelectric drop counter (DCT 102, Unique Medical Inc. Tokyo) and recorded as single pulses. Three percent ethanol in Locke solution was infused at a

constant rate of 0.10 ml/min through the cannula inserted into the jugular vein in order to maintain a constant level of anesthesia and a constant rate of urine outflow. Osmotic pressure of urine was measured by the freezing point depression method (The Fiske Osmometer, Fiske Associates, Inc., Uxbridge, MA).

Microinjection of drugs: A stainless cannula (outer diameter: 200 μ m) was inserted stereotaxically and unilaterally into the PVN according to the atlas of König and Klippel (21). Dibutyl-cAMP and cAMP were dissolved in saline (pH approx. 7), IBMX was dissolved in 3% ethanol in saline (pH approx. 7), and other compounds in an artificial cerebrospinal fluid (CSF: 128 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 0.65 mM NaH₂PO₄ and 4.8 mM NaHCO₃, pH 7.4). Drugs were injected using a microsyringe in a volume of 1 μ l through the cannula. Then 2 μ l of the artificial CSF was injected at a rate of approx. 0.3 μ l/min. The microinjection was performed when the urine outflow reached a constant rate of 0.05–0.16 ml/min within one hour after the animal was fixed in the stereotaxic instrument. Effects of drugs on urine outflow during every 10 min were expressed as a percent of the initial constant outflow.

In the experiment to test the effect of pretreatment with atropine or timolol, microinjection of db-cAMP was carried out at 30 to 60 min after premicroinjection of atropine when the urine outflow had recovered to the initial level or at 20 to 30 min after premicroinjection of timolol. In the experiments to see the effects of pretreatment with phenoxybenzamine and phosphodiesterase inhibitors, the time interval between the premicroinjection and microinjection of cAMP or db-cAMP was 20 min. The effect of preinjection of a drug was estimated as the change in antidiuretic effect caused by the injection of db-cAMP with and without the pretreatment in separate rats because of tachyphylaxis in the antidiuresis induced by the dibutyl analog.

Identification of the sites of inserted cannula: The position of the tip of the cannula inserted stereotaxically into the PVN was confirmed by the following methods: 1)

functionally, by the appearance of an anti-diuretic effect by microinjecting a depolarizing dose (400 nmol) of KCl through the cannula and 2) histochemically, by the localization of the tip of the cannula in a group of magnocellular cells in the PVN positively stained by the method of Gomori (22).

Measurement of blood pressure, heart rate, respiration rate and rectal temperature:

Mean blood pressure and heart rate were measured through a cannula inserted into the carotid artery with a pressure transducer (MPU-0.5-290-O-III, Nihon Kohden Kogyo, Co., Tokyo) and by an electrocardiograph (FD-14, Fukuda, Tokyo), respectively. Respiration rate was measured by a thermister probe (SR-115S, Nihon Kohden Kogyo, Co.) inserted into a tracheal catheter. These three indices were recorded simultaneously on a recticorder (RJG-3004-2, Nihon Kohden Kogyo, Co.). Rectal temperature was monitored by a thermister probe (MGA III-219, Nihon Kohden Kogyo, Co.) inserted into the rectum.

Statistical analysis: Significance of differences between mean values was determined by Student's *t*-test. The differences were considered significant at $P < 0.05$.

Results

Effects of microinjection of db-cAMP and cAMP on urine outflow: Figure 1a shows the

effects of various doses of db-cAMP on urine outflow as a function of time after their microinjections into the PVN. Dibutyl-yl-cAMP caused the antidiuretic effect in a dose-dependent and time-dependent manner. The urine outflow decreased within 20 min, with a minimal outflow at 30 to 40 min, and recovered to the initial level at approx. 1.5 hr after microinjection of 50 and 100 nmol of db-cAMP. Vehicle alone microinjected into the nucleus did not change the urine outflow. As shown in Fig. 1b, the antidiuretic effect of microinjection of cAMP was much less than that of db-cAMP.

Dose-effect curves for db-cAMP and cAMP: Figure 2 demonstrates the dose-effect curves for the antidiuretic effect induced by db-cAMP and cAMP microinjected into the PVN. The approx. median effective doses (ED₅₀) were estimated to be 40 nmol for db-cAMP and more than 300 nmol for cAMP.

Potentialiation by pretreatment with phosphodiesterase inhibitors and inhibition by pretreatment with muscarinic antagonist: As illustrated in Fig. 3a, a premicroinjection of 50 nmol theophylline potentiated the antidiuretic effect of microinjection of 50 nmol db-cAMP, but was not able to enhance the effect of microinjection of 100 nmol cAMP (Fig. 3b). A premicroinjection of 10 nmol IBMX potentiated the effect of cAMP (Fig. 3b) and tended to enhance the antidiuretic

Fig. 1. Time-courses for antidiuresis by microinjection of cyclic nucleotides (a: db-cAMP; b: cAMP) into the PVN. Abscissa indicates time in min after microinjection of 1 μ l of drug solution, and ordinate shows urine outflow presented as percent of the initial urine outflow (a, 0.107 ± 0.015 ; b, 0.092 ± 0.016 ml/min). Symbols which represent the urine outflow during the preceding 10 min period are means $\pm$ S.E. from 4–6 experiments.

effect of db-cAMP (Fig. 3a).

As shown in Fig. 4, the antidiuretic effect of 100 nmol db-cAMP was completely blocked by premicroinjection of 300 nmol atropine, a muscarinic antagonist, which inhibits completely the antidiuretic effect of ACh (8). The antidiuretic effect of db-cAMP was not altered by premicroinjection of 80

nmol phenoxybenzamine, an α -adrenoceptor antagonist, which blocked the antidiuretic effect of microinjection of 10 nmol nor-epinephrine (13). Timolol at 100 nmol was not

Fig. 2. Dose-effect curves for antidiuretic effects of microinjection of db-cAMP and cAMP into the PVN. Abscissa indicates the dose of cyclic nucleotides microinjected in 1 μ l of drug solution, and ordinate shows the minimal urine outflow during the preceding 10 min at 30 min or 40 min after microinjection presented as percentage of the initial urine outflow (for db-cAMP: 0.107 ± 0.015 ; cAMP: 0.092 ± 0.016 ml/min). Symbols are means $\pm$ S.E. from 4–6 experiments.

Fig. 4. Effects of pretreatment with atropine and phenoxybenzamine on antidiuresis induced by microinjection of 100 nmol db-cAMP into the PVN. $\circ$: control, microinjection of db-cAMP alone; $\bullet$: pretreatment with microinjection of atropine (300 nmol) at 30–60 min before the injection of db-cAMP; $\blacktriangle$: pretreatment with microinjection of phenoxybenzamine (80 nmol) at 20 min before the injection of db-cAMP. Abscissa and ordinate are as in Fig. 3. The initial urine outflow was 0.112 ± 0.023 ml/min. Symbols are means $\pm$ S.E. from 3–4 experiments. Significance was as in Fig. 3: * $P < 0.05$.

Fig. 3. Effects of pretreatment with theophylline and IBMX on antidiuresis induced by microinjection of db-cAMP and cAMP into the PVN. a: db-cAMP (50 nmol), b: cAMP (100 nmol). Premicroinjection of $\bullet$: theophylline (50 nmol) and $\blacktriangle$: IBMX (10 nmol) at 20 min before the injection of db-cAMP or cAMP. Abscissa indicates time in min after injection, and ordinate shows urine outflow presented as percent of the initial urine outflow (a, 0.108 ± 0.012 ; b, 0.116 ± 0.015 ml/min). Symbols are means $\pm$ S.E. from 4–6 experiments. * $P < 0.05$: Significance compared with the effect of injection of db-cAMP or cAMP alone at the same time point.

significantly able to inhibit the effect of db-cAMP.

Tachyphylaxis in the antidiuretic effect by microinjection of db-cAMP: The antidiuretic effect induced by the second microinjection of 100 nmol db-cAMP into the PVN was much less than the effect by the first injection (Fig. 5), while antidiuretic effects caused by the second microinjection of KCl, cholinergic agonists, and adrenoceptor agonists are

approx. equal to the effects by the first microinjection (8, 13).

Effect of microinjection of db-cAMP on urine osmotic pressure: Figure 6 shows the effects of microinjection of db-cAMP into the PVN on urine osmotic pressure as a function of time after microinjection, compared with the effects on urine outflow. After microinjection of 100 nmol db-cAMP, the urine osmotic pressure appeared to increase at

Fig. 5. Antidiuretic effects induced by repeated microinjection of db-cAMP (100 nmol) into the PVN. Drug was injected at the time indicated by the arrows. The time interval between the two arrows was approx. two hours. Abscissa and ordinate are as in Fig. 3. The initial urine outflow was 0.102 ± 0.023 ml/min at the time of the first injection and 0.081 ± 0.017 ml/min at the time of the second injection. Symbols are means $\pm$ S.E. from 4–5 experiments.

Fig. 6. Time-courses for change in urine osmolality after microinjection of db-cAMP into the PVN. $\circ$: urine outflow, $\bullet$: urine osmolality. Abscissa and the left ordinate are as in Fig. 3. The right ordinate shows osmolality of the urine outflow during the preceding 10 min. The initial urine outflow was 0.137 ± 0.018 ml/min. Urine osmolality presented as percent of the initial urine osmotic pressure (265 ± 18.9 mOsm). Symbols are means $\pm$ S.E. from 4 experiments. Significance was as in Fig. 3: * $P < 0.05$.

approx. 20 min, with a maximal osmotic pressure of approx. 200% of the initial control level at 40 min, and recovered to the initial level at approx. 1.5 hr. The pattern of time-course curves for urine outflow and for urine osmotic pressure showed an approx. mirror image with minor differences in their configurations.

Effect of microinjection of db-cAMP into the PVN on visceral functions: Some visceral indices which might be expected to be responsive to the microinjection of db-cAMP into the PVN and which might affect the urine outflow were monitored during the experiments. At 20 and 30 min after microinjection of 300 nmol db-cAMP, when it induced a marked decrease in the urine outflow, there were no changes in mean blood pressure, heart rate, respiration rate and rectal temperature. Heart rate increased by approx. 10% at 60 min, when the urine outflow was recovered to approx. 60% of the initial control outflow.

Discussion

This is the first paper that demonstrated antidiuretic effects of cAMP and the dibutyl analog microinjected into the PVN which contains magnocellular neurons synthesizing and releasing antidiuretic hormone, vasopressin. The dibutyl analog induced more potent antidiuresis than cAMP in a dose-dependent and time-dependent manner, the median effective doses (ED₅₀) for db-cAMP being approx. 40 nmol versus more than 300 nmol for cAMP. The time-courses of the effects were relatively slow, with a minimal urine outflow at approx. 30 to 40 min and the duration of approx. 1.5 hr after microinjection, suggesting that the effects are hormonal rather than through neuronal pathways as found in the case of antidiuretic effects induced by microinjection of cholinergic and adrenoceptor agonists (8, 13). The antidiuretic effect of db-cAMP closely correlated with an increase in urine osmotic pressure, indicating that reabsorption of water was enhanced during the antidiuresis.

Since the ED₅₀ value of KCl for depolarizing the neurons of the PVN was 320±78 nmol (8), db-cAMP whose ED₅₀ was approx. 40 nmol was approx. eight times more potent than KCl. The median

effective concentration for db-cAMP can be roughly estimated to be approx. 4 mM, a little higher concentration than the effective concentration usually used (18), provided that central neurons are depolarized by KCl at approx. 30 mM concentration. As rats were loaded with water and ethanol in order to keep the urine outflow constant and at a measurable flow rate in the present experiments, the true effective concentration for db-cAMP in unanesthetized rats without water- and ethanol-loading could be lower than the value estimated above.

The stronger potency to induce the antidiuretic effect of db-cAMP which is more permeable through the plasma membrane into the intracellular cytoplasm (23) than that of cAMP and the potentiation of their effects by pretreatment with the inhibitors of phosphodiesterase suggest that their effects may be related to an increased intracellular level of cAMP. However, it remains to be elucidated why theophylline and IBMX could not significantly potentiate the effects of cAMP and db-cAMP, respectively. The results might reflect differences of the two methylxanthines in the inhibitory effect of phosphodiesterase (24, 25) or in effects other than the enzyme inhibition.

Premicroinjection of a muscarinic antagonist, atropine, blocked completely the antidiuretic effect of db-cAMP, which suggests that the effect of db-cAMP was mediated through muscarinic receptor. One possible explanation for this observation is that db-cAMP may release a muscarinic substance, probably ACh from presynaptic cholinergic terminals on the neurons in the nucleus (5, 6) as has been demonstrated in the brain (17–19), neuromuscular junction (14), and small intestine (15, 16). However, our previous results that antidiuretic effects induced by norepinephrine and isoproterenol were inhibited only partially by pretreatment with atropine (13) suggest that the presynaptic effect of the catecholamines mediated through cAMP if present may be a part of the antidiuretic effects. Tachyphylaxis in the effect of db-cAMP could be due to the depletion of a relatively small pool of ACh in the presynaptic area as tachyphylaxis found in the indirect effects of ephedrine and

tyramine which release norepinephrine from the presynaptic terminals (26).

During antidiuresis induced by microinjection of db-cAMP (300 nmol), no significant changes were observed in various visceral functions such as mean blood pressure, respiration rate and rectal temperature which might affect the urine outflow, with a slight increase in heart rate which was not affected at the time of the maximal antidiuretic effect, at 30 to 40 min after microinjection. Therefore, the antidiuretic effect of db-cAMP is not likely due to the changes in these visceral functions.

In summary, the present data indicate for the first time antidiuretic effects of db-cAMP and cAMP when microinjected into the PVN which contains neurons synthesizing and releasing antidiuretic hormone. A possible mechanism of the effect of db-cAMP may be due to a release of ACh, which stimulates muscarinic receptors in the PVN (8).

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