EFFECT OF SODIUM ION ON LEVELS OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE IN GUINEA PIG CEREBRAL SLICES

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Abstract—Accumulation of cyclic AMP was studied in guinea pig cerebral slices when Na^+ levels in the bathing medium were varied or agents which affect tissue Na^+ content were added. When NaCl was gradually replaced with Tris-HCl or choline chloride, cyclic AMP formation was progressively enhanced. When Na^+ was below 30 mM, cyclic AMP formation reached the maximum (approximately 30 fold), but this increment was not blocked by tetrodotoxin. The stimulatory effect of high K^+ was nearly linear over 120 mM and became much more prominent when Na^+ was decreased to 30–40 mM in the bathing medium. The effect of high K^+ also was not blocked by tetrodotoxin. Ouabain (10^{-4} M), electrical pulses and glutamate (5×10^{-3} M), each stimulated cyclic AMP formation about 17-, 7- and 5-fold, respectively. Tetrodotoxin (2×10^{-6} M) completely blocked the effects of electrical pulses and partially blocked the effects of glutamate and ouabain. It is suggested that the increase of cyclic AMP in cerebral cortical slices may be related to the decrease in Na^+ gradient across the cell membrane.

In slices of guinea pig cerebral cortex, the accumulation of adenosine 3',5'-monophosphate (cyclic AMP) appears to be stimulated by several factors including: (i) stimulation by biogenic amines (1), (ii) membrane depolarizing agents (2), (iii) adenine ribonucleotides (3) and some amino acids (4). We report herein the relationship between sodium ion and cyclic AMP formation in guinea pig cerebral slices and discuss the possibility that the formation of cyclic AMP is determined by the concentration gradient of sodium ion across the cell membrane.

MATERIALS AND METHODS

Hartley guinea pigs weighing 250–300 gms were decapitated and the cerebri removed and immersed in ice cold incubation medium. The cortex (0.2–0.3 mm thick) was separated and chopped tissue from 2–3 animals was transferred into a beaker containing 50 ml of incubation medium. Preincubation was carried out for 30 min at 37°C. This preincubation was sufficient to reduce the level of cyclic AMP to a stable minimum. After preincubation, the tissue was divided into 8–12 portions, estimated to contain between 3–6 mg of protein and each portion was transferred into 10 ml of fresh medium in a set of glass centrifuge tubes.

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Incubation was then carried out for ten min at 37°C in the presence or absence of various agents. After incubation, the medium was decanted and 1–2 ml of ice cold 10% trichloroacetic acid was added and the tube was immersed in dry-ice acetone. The incubation medium (pH 7.5) consisted of 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM KH₂PO₄, 2 mM MgSO₄, 40 mM Tris-HCl buffer and 10 mM glucose. The solution was gassed during all incubation procedures with oxygen. The control values of cyclic AMP contents observed in slices incubated in Tris-HCl buffer were more stable and lower than that in bicarbonate buffer. The frozen tissue was homogenized in a glass homogenizer after the addition of 1.0 p mole of cyclic [³H] AMP (about 12,000 cpm) for the estimation of recovery. The homogenate was centrifuged at room temperature for 20 min at 2,000 rpm. One ml of 1 N NaOH was added to the precipitate and protein was estimated by the method of Lowry *et al* (5). The supernatant was extracted 5 times with 5 volumes of ether which had been saturated with water. The residual ether was evaporated at 55°C with a stream of air. Each extract was applied to a column packed with one gram of basic aluminum oxide (6) and eluted with 5 ml of distilled water. The eluate fraction was dried with a rotary evaporator at 55°C and the flask was extracted with 300 μ l of water. Ten μ l of sample was used for assay of cyclic AMP and 50 μ l of sample was used for estimating recovery. The average recovery of cyclic AMP was about 60%.

Cyclic AMP was estimated by a modification of the method of Gilman (7). Binding protein was purified from rabbit muscle by the method of Reiman *et al* (8).

Electrical pulses were administrated to the cerebral cortex slices according to the method of Kakiuchi *et al* (9) with a slight modification. Twelve volt condenser pulses were applied for 10 min with a time constant of 0.4 msec and a frequency of 100 Hertz.

Cyclic AMP (Na salt) was purchased from Kohjin Co., tetrodotoxin from the Sankyo Co., and basic aluminum oxide from Merck Chemical Co. Cyclic [³H] AMP (24.1 Ci/mole) was purchased from New England Nuclear.

RESULTS

Effect of sodium ion on cyclic AMP formation

When NaCl in the Krebs Ringer solution was completely replaced by Tris-HCl (pH 7.5) or choline chloride, cyclic AMP accumulation in the slices was greatly increased (Fig. 1). In Tris-Ringer (Na⁺-free), cyclic AMP levels were twice as high as in choline-Ringer (Na⁺-free). When NaCl was gradually added to the bathing medium (Tris-Ringer), cyclic AMP formation began to decrease above 10 mM NaCl and reached the control level when NaCl exceeded 30 mM. In choline-Ringer (Na⁺-free), the level of cyclic AMP formation decreased to the control level when NaCl exceeded 20 mM.

Time course of cyclic AMP formation in Na⁺-free medium

Cyclic AMP formation reached almost the maximal level 2 min after changing to a Na⁺-free medium. Cyclic AMP content in these slices began to decrease after 25 min (Fig. 2).

Effect of sodium and potassium ions on cyclic AMP formation

When KCl was gradually added to the Krebs Ringer solution (up to concentrations of 120 mM), cyclic AMP formation linearly increased (Fig. 3). However, when NaCl in high K^+ (46 mM) Ringer was gradually replaced with Tris-HCl, accumulation of cyclic AMP

FIG. 1. Effects of sodium and potassium ions on cyclic AMP accumulation. Chopped slices of cerebral cortex were preincubated in normal Ringer's solution. The preincubated slices were transferred into the fresh medium with a varying composition of ions and then incubated for an additional 10 min. In Tris-Ringer (Na^+ -free) or choline-Ringer (Na^+ -free), NaCl was completely replaced by Tris or cholinium ions. In the dose response curve, NaCl was gradually added to Na^+ -free Ringer up to 60 mM and the normal Ringer was used at a concentration of 120 mM NaCl. High K^+ (46 mM) Ringer was made by adding KCl to the normal Ringer. ○ ○ Tris-Ringer (Na^+ -free); ● ● choline-Ringer (Na^+ -free); ■ ■ high K^+ (46 mM) Ringer

FIG. 2. Time course of cyclic AMP accumulation in slices incubated in Tris-Ringer (Na^+ -free). After preincubation of chopped slices in normal Ringer's for 30 min, the slices were transferred to Tris-Ringer (Na^+ -free) and incubated as shown.

FIG. 3. Effect of potassium ion on cyclic AMP accumulation. Preincubation and incubation were identical to those in Fig. 1. In the dose response curve, KCl was gradually added to normal Ringer's up to 120 mM. KCl and KH_2PO_4 were completely replaced by Tris and NaH_2PO_4 at zero KCl.

in slices began to increase even more than was observed with the addition of KCl to the normal Ringers and reached the maximum at Na^+ concentrations of approximately 30 mM (Fig. 1). This maximal value was less than that found in slices incubated in Tris-Ringer (Na^+ -free) solution. However, the complete omission of NaCl from high K^+ Ringer somewhat reduced the levels of cyclic AMP (Fig. 1).

Effect of tetrodotoxin on cyclic AMP formation

When electrical stimulation was applied to a layer of cerebral cortex slice, cyclic AMP levels increased about 5–7 times above the level found in the control (Table 1). The effect of electrical stimulation was completely blocked by 2×10^{-6} M tetrodotoxin. However, increases in cyclic AMP levels associated with Na^+ -free Ringer or high K^+ Ringer were not prevented by tetrodotoxin. The effects of ouabain and glutamate were partially blocked by tetrodotoxin (Table 1).

TABLE 1. Effect of tetrodotoxin on cyclic AMP content

	Cyclic AMP (p moles/mg protein)
Control	14.0 ± 2
Control + tetrodotoxin (2×10^{-6} M)	10.0 ± 2
Electrical pulses (E.P.)	95.5 ± 10
E.P. + tetrodotoxin	13.5 ± 3
Tris-Ringer (Na^+ -free)	290.5 ± 25
Tris-Ringer (Na^+ -free) + tetrodotoxin	302.5 ± 28
High K^+ (86 mM)	144.4 ± 15
High K^+ + tetrodotoxin	141.2 ± 14
Ouabain (10^{-4} M)	245.0 ± 20
Ouabain + tetrodotoxin	120.5 ± 11
Glutamate (5×10^{-3} M)	68.6 ± 7
Glutamate + tetrodotoxin	47.0 ± 5

A layer of cerebral cortex slice was stimulated by 12 volt condenser pulses, at a time constant of 0.4 msec and a frequency of 100 Hertz. All samples were incubated for 10 min. Each value represents the mean $\pm$ S.D. obtained from four separate experiments.

TABLE 2. Effect of lithium ion on cyclic AMP content

	NaCl (120 mM)	LiCl (120 mM)
	Cyclic AMP (p moles/mg protein)	
Control	11.5 ± 2	14.0 ± 2
Electrical pulses	56.0 ± 3	12.5 ± 2
Adenosine (2×10^{-4} M)	202.0 ± 11	9.5 ± 2
High K^+ (86 mM)	135.0 ± 14	9.0 ± 2
Histamine (5×10^{-4} M)	53.0 ± 3	7.5 ± 1

LiCl completely replaced NaCl in Li^+ -Ringer solution. Samples were incubated for 10 min. Each value represents the mean $\pm$ S.D. obtained from four separate experiments.

Effect of lithium chloride on cyclic AMP accumulation

Lithium can replace Na^+ in the formation of the action potential (10). The accumulation of cyclic AMP was not enhanced by electrical stimulation for 10 min in a Li^+ -Ringer solution (Table 2). In addition, the effects of adenosine, high K^+ and histamine were completely prevented by Li^+ -Ringer.

DISCUSSION

The concentration of cyclic AMP in cerebral cortex slices was greatly increased when Na^+ in the bathing medium was replaced by Tris or cholinium ions. Tris-Ringer (Na^+ -free) was more effective than choline-Ringer (Na^+ -free) in this respect. Hypertonic choline- or Tris-Ringer (Na^+ -free) made up with the addition of 50 mM sucrose also showed similar stimulatory effects on cyclic AMP accumulation in cerebral slices. Shimizu and Daly (11, 12) also have reported that complete omission of Na^+ from the Krebs-Ringer medium and replacement with other cations, such as cholinium, tetramethylammonium and NH_4 or with sucrose causes an enhanced accumulation of cyclic AMP. This stimulatory effect is not seen with cholinium or tetramethylammonium ions in the presence of 25 mM NaCl (11). We also observed that the effect of Na^+ -free Ringer cannot be attributable to the presence of either Tris or cholinium ions in the bathing medium, since increased accumulation of cyclic AMP was abolished by the addition of NaCl above 30 mM in the presence of these cations. Shimizu *et al* (2) also suggested that the formation of cyclic AMP in the brain slices is greatly stimulated by depolarizing agents such as ouabain and veratridine and high K^+ medium. The effect of the Na^+ -free Ringer observed in this study, however, cannot be explained by a depolarizing mechanism, since it has been reported that Na^+ -free Ringer in which Na^+ is replaced by Tris or cholinium ion does not produce a depolarization in excitable tissues within a short time period, but rather results in a hyperpolarization (13, 14, 15). Furthermore, hypertonic Ringer (NaCl, 225 mM) had no stimulatory effect on cyclic AMP formation (data was not shown.), though it has been reported that excess NaCl causes a depolarization (13, 15). As shown in Fig. 2, the large increase in cyclic AMP content in slices occurred within 2 min following the reduction in Na^+ . The data further indicate that the effect of the Na^+ -free system may be direct, since the metabolic disturbances which take place secondarily in brain slices incubated in Na^+ -free Ringer are not observed for at least 10 min (10). It is also known that participation of Na^+ is necessary for the occurrence of the action potential and, that lithium can replace Na^+ in this process (10). The accumulation of cyclic AMP in slices was not stimulated by electrical pulses in Li^+ -Ringer in which Na^+ was replaced by lithium. Lithium ion itself would be an potent inhibitor of cyclic AMP formation in slices, since histamine and adenosine did not stimulate cyclic AMP accumulation in Li^+ -Ringer. Dousa and Hetcher (16) have shown that F^- sensitive brain adenylate cyclase is directly inhibited by lithium. Lithium ion at high concentrations (1 to 50 mM) also reduces norepinephrine-induced formation of cyclic AMP in rat cerebral cortex slices (17) and histamine-induced formation of cyclic AMP in slices from rabbit cerebral cortex (17) and rat hypothalamus (18).

There is also an alternative way to increase the cyclic AMP content in brain slices. Application of electrical pulses to slices or exposure of slices to glutamate resulted in an increase in the cyclic AMP content (Table 1). These treatments are known to increase the intracellular concentration of Na^+ (19). Interestingly, tetrodotoxin which is known to block the increase of sodium conductance (20, 21) inhibited the effect of electrical pulses completely but not the effect of the Na^+ -free system.

The increase in cyclic AMP content produced by high K^+ , which increases the intracellular Na^+ (22) was nearly linear up to 120 mM K^+ but not as prominent as the metabolic responses or release of biogenic amines produced by high K^+ (23, 24). When Na^+ was reduced in high K^+ Ringer, however, cyclic AMP formation was greatly stimulated. The complete omission of NaCl from high K^+ (46 mM) Ringer somewhat reduced the levels of cyclic AMP. Shimizu and Daly reported similar results with high K^+ (150 mM) Ringer (11). Thus the cyclic AMP level in cerebral tissue was shown to increase either when the concentration of Na^+ in the medium decreased or when the intracellular Na^+ concentration increased. These observations can be reasonably explained if it is assumed that the concentration of cyclic AMP in the cell is regulated by the sodium ion gradient across the cell membrane. The active transport of amino acids and sugars across the cell membrane exemplified such a mechanism. Here, the rate of transport is dependent upon the Na^+ gradient but independent of intracellular concentrations of Na^+ (25, 26).

Our conclusion, however, would be compatible with the depolarizing hypothesis proposed by Shimizu and Daly (11), if a depolarization of the cerebral cells can be induced by the decrease in Na^+ gradient across the cell membrane, according to an equation reported by Hodgkin and Katz (27).

It has been reported that the effects of both depolarizing agents and adenosine were suppressed by theophylline (1, 3). In addition, the effects of depolarizing agents have been attributed to the release of adenosine (12). Since the effect of Na^+ -free Ringer was also suppressed by theophylline, it is postulated that decrease in the sodium gradient across the cell membrane may induce a release of adenosine, which in turn stimulates the formation of cyclic AMP.

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