

Adenyl cyclase system and cerebral energy state

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SYNOPSIS The energy charge potential of the cerebral adenylate system, the cerebral lactate: pyruvate system, and the cerebral glycogen level were used to characterize the cerebral energy state of the brain. Cerebral adenyl cyclase activity and cyclic AMP concentrations were investigated to evaluate their possible influence on the regulatory processes in brain metabolism. These biochemical parameters were evaluated on the cortical motor area of the brain of the beagle dog in hypovolaemic hypotension during acute hypoxia (obtained by altering the composition of the inhalation mixture) and during the post-hypoxic recovery (three minutes after the restoration of normal ventilation). This experimental model of acute hypoxia was also used for investigating the action of some substances acting at β -adrenergic receptor level or at vascular level. The substances were perfused into the carotid artery at the rate of 0.5 ml/min for three to six minutes. During the first stage of hypoxia, the adenyl cyclase system is probably an important biochemical regulatory factor. This system becomes less important when a critical threshold is crossed (oxygen arterial partial pressure <25-20 mmHg) below which other factors become rate-limiting. A β -receptor stimulating agent enhanced the mechanisms of physiological post-hypoxic recovery; inhibition of this action was obtained with a β -receptor blocking agent. Under these experimental conditions, no correlation existed between the vascular effects of the agents and brain metabolism.

Previous studies (Villa, 1975) have indicated that the biochemical changes observed in the brain under various experimental conditions could probably also be related to the cyclic AMP system. The importance of this cyclic nucleotide as a regulatory factor of the glycolytic pathways is particularly relevant in the mature brain, glucose being the main substrate. Glycolysis (defined as either glycogen→lactate, or glucose→lactate) is of only minor importance in the normoxic adult brain.

The levels of cyclic AMP in the cells depend upon the opposite activities of at least two enzymes: adenyl cyclase, and a specific phosphodiesterase. The adenyl cyclase system seems to be composed of two types of subunits and it has been suggested that a part of the regulatory subunit may be an adrenergic receptor (Robison *et al.*, 1967). In a variety of tissues, β -adrenergic blocking agents are able to inhibit the formation

of cyclic AMP in response to catecholamines (Stock and Westermann, 1966; Butcher and Sutherland, 1967). Adenyl cyclase from the brain, localized in a particulate fraction (De Robertis *et al.*, 1967), has been extracted through lyophilization and lipid extraction with the retention of catecholamine sensitivity and has been partially solubilized (Sutherland *et al.*, 1962). Also, the formation of cyclic AMP was stimulated in the cortical and cerebellar tissues not only by catecholamines (Klainer *et al.*, 1962), but also by histamine (Kakiuchi and Rall, 1968).

The phosphodiesterase from the brain (Cheung, 1967) was inhibited by ATP and pyrophosphate, and also by citrate. This action is counteracted by competitive inhibition by methylxanthines (Butcher and Sutherland, 1962; Cheung, 1967). Cyclic AMP may play a role in the release of acetylcholine from nerve endings (Breckenridge *et al.*, 1967) and this effect, modulated by catecholamines, can be blocked by β -adrenergic blocking agents (Biver and Goffart, 1965;

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Breckenridge *et al.*, 1967). Nevertheless, a high concentration of α -adrenergic blocking agents also exerts a similar effect (Biver and Goffart, 1965; Bowman and Raper, 1967). This effect is probably not exerted at the receptor level, but rather results from an interference with the action of cyclic AMP (Northrop and Parks, 1964; Aulich *et al.*, 1967). There is also evidence that, in some cases, the increase in the concentration of cyclic AMP to levels which seem higher than necessary may be an illustration of the concept of spare receptors (Ariens, 1966; Butcher and Sutherland, 1967; Grahame-Smith *et al.*, 1967).

The purpose of the present work was: (1) to correlate the activity of the adenylyl cyclase system with the cerebral energy state (alterations of the cerebral energy charge potential were obtained through a controlled change of arterial oxygen partial pressure during hypovolaemic hypotension in the beagle dog); (2) to test the effect on the adenylyl cyclase system of agents exhibiting an affinity for β -adrenergic receptors, and exerting either a mimetic—for example, bamethan—or lytic—for example DCI—action. *In situ* β -mimetic compounds often show a vasodilating power which can modify the metabolic state of the tissue. For the sake of comparison, the vasodilating substance papaverine, which exerts a direct action on smooth muscle fibres, was also used. The activity of the adenylyl cyclase system of the brain was evaluated through both the enzyme activity and the cyclic AMP concentration. The energy state of the brain was evaluated on the basis of the balance of the adenylate pool (ATP, ADP, AMP), of the balance of the lactate:pyruvate system, and of cerebral glycogen concentrations.

METHODS

ANIMALS AND ANAESTHESIA The experiments were carried out on male beagle dogs aged 240–360 days and weighing from 9.3 to 12.9 kg. Before the experiments the dogs were maintained under standard environmental conditions (temperature = $22 \pm 1^\circ\text{C}$; relative humidity = $60 \pm 5\%$) and were fed only a standard diet as pellets with water *ad libitum*.

The surgical procedure was performed on animals preanaesthetized with urethane (0.4 g/kg intraperitoneally). Electrical activity of the brain was used to determine the degree of anaesthesia, which was induced and maintained during the surgical

procedure by chloralose (20–40 mg/kg intravenously). The dogs were artificially ventilated with air or a 4% or 6% nitrogen-oxygen mixture with an intratracheal Warne tube. During the operative procedure and the experiment, the animals were paralysed by intravenous injection of gallamine triethiodide (2–3 mg/kg).

OPERATIVE PROCEDURE The procedure (Benzi *et al.*, 1973) consisted mainly of isolating both common carotid arteries, with ligation of all their branches except the internal carotid and the superior thyroid arteries. The zygomatic, maxillary, auricular, and supraorbital vessels were all occluded by ligation or compression. Each of the isolated superior thyroid arteries was cannulated with a polystyrene tube and connected by a two-way cannula to a perfusion apparatus (to infuse the saline solution or the drug solution) and to a glass syringe (to draw anaerobic arterial blood samples). The head was fixed to a head-holder with the confluence of the cerebral venous sinuses approximately 10 cm higher than the heart. Monopolar electrodes were inserted in the left and right frontal, parietal and occipital areas. After a longitudinal incision, a 2–2.5 cm diameter hole was made in the frontoparietal area. A plastic funnel was fitted into the hole and the skin was sutured tightly around the funnel; subsequently, the plastic funnel was sealed with a rubber stopper and thermally insulated.

Both femoral arteries were cannulated: one connected to a physiological pressure transducer and the other to a heparinized Wolfe bottle to withdraw arterial blood from the systemic circulation. The Wolfe bottle was connected to a femoral vein to infuse the drained blood into the systemic circulation. Connection was made by a large silicone-coated paraffin cannula joined to a peristaltic pump.

The arterial oxygen partial pressure (PaO_2) and the carbon dioxide partial pressure (PaCO_2) were measured using anaerobic samples of blood from the superior thyroid arteries. The rectal temperature was monitored with a telethermistor, and intermittent heating was applied to keep the dog's temperature as close to 37°C as possible. Electroencephalographic tracings and systemic blood pressure were recorded using a Schwarzer 12-channel recorder.

EXPERIMENTAL PLAN When the PaO_2 was reduced below 35 mmHg, the phosphocreatine content was reduced, but there were no changes in the adenylate system until the PaO_2 was reduced below 25 mmHg. In any case, PaO_2 below 25 mmHg causes changes in the adenosine phosphates to be exaggerated by even small decreases in the blood pressure (Siesjö and Nilsson, 1971). Therefore, the hypoxia experiments

were carried out on beagle dogs in hypovolaemic hypotension lasting 22 to 30 minutes. In fact, it is possible that regulatory mechanisms allow near normalization of the energy state when hypotension is prolonged (Holmin *et al.*, 1974).

After the operative procedure a period of 30 minutes was allowed before hypotension was induced. The dogs were slowly bled from the arterial femoral cannula into a heparinized Wolfe bottle. A mean blood pressure of 60 mmHg was attained within eight to 20 minutes and then cautious arterial withdrawals or venous infusions by a peristaltic pump were made so that the mean blood pressure was held as constant as possible for 25 minutes. Normally, the blood pressure could be held at the specified level to within ± 4.0 mmHg. If large variations occurred or if a substantial part of the blood had to be reinfused to keep the blood pressure constant, the dog was discarded.

The experiments were carried out by evaluating the cerebral biochemical parameters in dogs before, during, and after arterial hypoxaemia. The three periods in each experiment were usually: (1) steady-state period of hypovolaemic hypotension of 10 minutes, during air ventilation; (2) six to 12 minutes of acute hypoxia induced by altering the inspired oxygen concentration with a 6% or a 4% oxygen-nitrogen mixture in the respirator. Saline solution or drugs were perfused through the superior thyroid arteries during the last six minutes of hypoxia, at the rate of 0.5 ml/min, at the molar concentration indicated below; (3) three minutes of recovery of respiration with air, and perfusion through the superior thyroid arteries with saline solution or drugs.

ANALYTICAL TECHNIQUES At the set time, the motor area of the cortex was frozen *in situ* by pouring liquid nitrogen into the plastic funnel fitted into the cranial vault. Ventilation with air or with oxygen-nitrogen mixture was continued for at least two minutes after the beginning of the regional brain freezing. A portion of the frozen brain was chiselled out using a rotating cold hollow tube during continuous irrigation with liquid nitrogen. The frozen cerebral material was then immersed into liquid nitrogen for 10 to 15 minutes and was subsequently extracted with perchloric acid. The initial extraction was performed at from -10° to -15°C , using frozen 3 M perchloric acid, while the subsequent steps were carried out at $0-5^{\circ}\text{C}$ until a neutral perchlorate-free extract was obtained. The steps which followed were: extraction with 3 M perchloric acid at subzero temperatures, homogenization for 15 s, additional extraction with 3% perchloric acid, centrifugation, neutralization of the combined extracts with 5N KOH to pH 5.5 and separation of the potassium

perchlorate by centrifugation. The neutralized, potassium perchlorate-free extract was then used for immediate analyses of lactate, pyruvate, and adenosine mono-, di-, and triphosphate, and glycogen according to Le Baron (1955), Hohorst *et al.* (1959), Minard and Davis (1962), Schmidt (1961), Lowry *et al.* (1964), Schmahl *et al.* (1965). Samples for the cyclic AMP assays were prepared from the cerebral frozen tissue by extraction and purification procedures involving ion-exchange column chromatography with two fractionation systems, according to Butcher *et al.* (1965). The activities of the adenyl cyclase system were evaluated by determining the rate of cyclic AMP produced in the assay mixture, according to De Robertis *et al.* (1967). Each value was calculated from the mean of at least four determinations performed blind on the same sample.

SUBSTANCES USED The following substances were perfused into the carotid arteries, at the indicated molar concentration: (1) 1-(p-hydroxyphenyl)-2-butylaminoethanol: bamethan = 2.5×10^{-4} M; (2) 1-(3,4-dichlorophenyl)-2-isopropylamino-ethanol hydrochloride: DCI = 5×10^{-4} M; (3) 6,7-dimethoxy-1-veratrylisoquinoline hydrochloride: papaverine = 5×10^{-4} M.

CEREBRAL ENERGY CHARGE POTENTIAL According to Atkinson (1968), the energy charge potential (ECP) is defined in terms of actual concentrations of the adenine nucleotides as: $([\text{ATP}] + 0.5 [\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$. Therefore, the energy charge was estimated by assuming that the balance among concentrations of adenine nucleotides responds to the energy state of the cell rather than to the concentration of a single nucleotide.

This evaluation provides little satisfactory information as to: (1) the importance of barrier compartmentation as compared with kinetic compartmentation; (2) the interactions between feed-back modifiers and energy charge in the regulation of the production of energy storage compounds; and (3) the sequences involved in the control of the pyridine nucleotides. At any rate, the enzymes that are involved in the energy-yielding sequences, or that catalyse reactions at metabolic branch points, are affected by adenine nucleotides. (There are many exceptions to this general statement about the properties of branch point enzymes.) The energy-mediating systems act together in maintaining the adenylate pool system at as high a level of activity as possible.

STATISTICAL ANALYSIS Student's *t* test was applied to the differences in all instances.

TABLE 1

CEREBRAL CORTEX OF MOTOR AREA OF BEAGLE DOG IN HYPOVOLAEMIC HYPOTENSION: BEHAVIOUR OF SOME BIOCHEMICAL PARAMETERS AS FUNCTION OF ARTERIAL OXYGEN PARTIAL PRESSURE (PaO₂)

Intracarotid perfusion													
Substance	Molar concentration	(n)	PaO ₂ (mmHg)	Glycogen	Lactate	Pyruvate	Lactate: pyruvate ratio	ATP	ADP	AMP	ECP	Cyclic AMP	Adenyl cyclase activity
				(μmol.g ⁻¹)				(μmol.g ⁻¹)				(nmol.g ⁻¹)	(mU.g ⁻¹)
Saline solution	—	5	96.4 ± 2.3	3.04 ± 0.19	2.28 ± 0.10	0.122 ± 0.009	18.85 ± 0.67	2.28 ± 0.06	0.45 ± 0.03	0.09 ± 0.01	0.89 ± 0.01	3.22 ± 0.23	25.2 ± 1.5
Saline solution	—	3	45.0* ± 4.4	2.65* ± 0.12	2.87 ± 0.15	0.140 ± 0.014	20.64 ± 0.98	2.46 ± 0.09	0.41 ± 0.05	0.09 ± 0.02	0.90 ± 0.05	4.04 ± 0.12	31.1 ± 2.0
Saline solution	—	3	27.3* ± 1.3	1.42* ± 0.08	7.60* ± 0.56	0.209* ± 0.017	36.20* ± 1.90	2.22 ± 0.06	0.44 ± 0.04	0.12 ± 0.01	0.87 ± 0.01	6.08* ± 0.33	46.3* ± 3.5
Saline solution	—	5	18.8* ± 0.5	0.55* ± 0.06	23.54* ± 1.64	0.264* ± 0.039	90.53* ± 7.34	1.68* ± 0.09	0.46 ± 0.05	0.65* ± 0.10	0.70* ± 0.03	1.47* ± 0.17	12.9* ± 1.4
Bamethan	2.5 × 10 ⁻⁴	3	17.7* ± 1.2	0.67* ± 0.03	23.39* ± 2.71	0.249* ± 0.024	93.80* ± 0.09	1.76* ± 0.03	0.49 ± 0.12	0.51* ± 0.08	0.73* ± 0.01	1.38* ± 0.25	18.4* ± 1.5
DCI	5.0 × 10 ⁻⁴	3	17.6* ± 0.9	0.51* ± 0.08	23.68* ± 2.43	0.252* ± 0.032	94.65* ± 3.06	1.48* ± 0.11	0.50 ± 0.02	0.72* ± 0.06	0.63* ± 0.03	1.20* ± 0.09	11.2* ± 1.5
Papaverine	5.0 × 10 ⁻⁴	3	19.0* ± 0.6	0.59* ± 0.07	22.63* ± 1.80	0.258* ± 0.030	88.56* ± 3.28	1.82* ± 0.12	0.49 ± 0.04	0.56* ± 0.02	0.72* ± 0.01	1.81* ± 0.15	16.4* ± 2.2

Mean values ± SE of *n* preparations (four assays per preparation). The energy charge potential (ECP) was calculated as: $([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP])$. The drugs were perfused through the superior thyroid arteries, at the rate 0.5 ml/min for six minutes. * Statistical difference ($P < 0.05$) versus the value of control condition at PaO₂ = 96.4 mmHg. † Statistical difference ($P < 0.05$) versus the value of hypoxaemia condition at PaO₂ = 18.8 mmHg.

TABLE 2

CEREBRAL CORTEX OF MOTOR AREA OF BEAGLE DOG IN HYPOVOLAEMIC HYPOTENSION: BEHAVIOUR OF SOME BIOCHEMICAL PARAMETERS DURING POST-HYPOXIC RECOVERY

Experimental conditions													
	(n)	PaO ₂ (mmHg)	Glycogen	Lactate	Pyruvate	Lactate: pyruvate ratio	ATP	ADP	AMP	ECP	Cyclic AMP	Adenyl cyclase activity	
			(μmol.g ⁻¹)				(μmol.g ⁻¹)				(nmol.g ⁻¹)	(mU.g ⁻¹)	
Hypoxaemia	5	18.8* ± 0.5	0.55 ± 0.06	23.54* ± 1.64	0.264* ± 0.039	90.53 ± 7.34	1.68* ± 0.09	0.46 ± 0.05	0.65* ± 0.10	0.70* ± 0.03	1.47 ± 0.17	12.9* ± 1.4	
3 min after recovery of room air ventilation	5	97.8 ± 1.6	0.46 ± 0.06	13.4 ± 1.2	0.163 ± 0.015	87.54 ± 14.69	2.00 ± 0.05	0.56 ± 0.04	0.36 ± 0.03	0.78 ± 0.01	1.91 ± 0.16	15.3 ± 1.9	
Idem + bamethan (2.5 × 10 ⁻⁴ M)	3	97.7 ± 2.3	0.41 ± 0.03	13.8 ± 2.8	0.178 ± 0.029	79.43 ± 13.89	1.95 ± 0.08	0.45 ± 0.05	0.22 ± 0.01	0.83 ± 0.01	2.71* ± 0.27	20.4 ± 2.2	
Idem + DCI (5 × 10 ⁻⁴ M)	3	96.7 ± 3.4	0.62 ± 0.06	16.6 ± 0.3	0.192 ± 0.037	103.88 ± 17.72	1.91 ± 0.02	0.55 ± 0.04	0.37 ± 0.05	0.77 ± 0.01	1.30* ± 0.17	11.4 ± 1.8	
Idem + DCI (5 × 10 ⁻⁴ M) + bamethan (2.5 × 10 ⁻⁴ M)	3	97.3 ± 2.6	0.59 ± 0.10	13.6 ± 1.8	0.179 ± 0.010	77.63 ± 14.50	1.98 ± 0.06	0.55 ± 0.03	0.42 ± 0.02	0.76 ± 0.01	1.26* ± 0.16	11.0 ± 0.6	
Idem + papaverine (5 × 10 ⁻⁴ M)	3	98.0 ± 2.5	0.40 ± 0.01	14.3 ± 2.2	0.189 ± 0.009	74.80 ± 7.65	1.94 ± 0.04	0.54 ± 0.02	0.37 ± 0.03	0.78 ± 0.02	2.36 ± 0.54	14.5 ± 2.9	
Pre-hypoxia control condition	5	96.4 ± 2.3	3.04* ± 0.19	2.28* ± 0.10	0.122* ± 0.009	18.85* ± 0.67	2.28 ± 0.06	0.45 ± 0.03	0.09* ± 0.01	0.89* ± 0.01	3.22* ± 0.23	25.2* ± 1.5	

Mean values ± SE of *n* preparations (four assays per preparation). The energy charge potential (ECP) was calculated as: $([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP])$. The drugs were perfused through the superior thyroid arteries, at the rate of 0.5 ml/min, for three minutes. * Statistical difference ($P < 0.05$) versus the value of three minute recovery of room air ventilation, at PaO₂ = 97.8 mmHg.

RESULTS

Table 1 shows how the progressive decrease of arterial pO_2 induced biochemical changes in the cortex of the motor area of the brain. The decrease of the arterial oxygen pressure to about 25 mmHg induced a marked degradation of cerebral glycogen, and an increase of both adenyl cyclase activity and cyclic AMP tissue concentration. Consequently, the catabolism of cerebral glycogen is increased by the cyclic AMP activated system. Under this condition, the pattern of the increase of the cerebral lactate:pyruvate ratio was discharged from that of the energy charge potential, which was unchanged. This indicates that the increase in the lactate production from glycogenolysis helped to maintain the energy charge potential of the adenylate system at the steady state level.

When the arterial oxygen pressure fell to a value below 20 mmHg (Table 1), the acute hypoxaemia strongly modified overall brain metabolism. The further drop of the cerebral glycogen content was accompanied by a marked reduction of the adenyl cyclase activity and cyclic AMP level. Both the lactate concentration and the lactate:pyruvate ratio increased dramatically, thus indicating the activation of glycolysis.

At pO_2 value below 20 mmHg (Table 1), the intracarotid perfusion of the β -adrenergic stimulating agent bamethan (Table 1) caused an increase in the adenyl cyclase activity, but not a concomitant increase in the cyclic AMP concentration; the cerebral energy charge potential remained highly depressed. The intracarotid perfusion of the β -adrenergic blocking agent *DCI* exerted no effect on the biochemical changes induced by hypoxaemia. The vasodilator agent papaverine did not affect the drop of the energy charge potential induced by hypoxaemia.

Table 2 shows the data evaluated three minutes after the post-hypoxia recovery. The biochemical pattern was shifted and a partial recovery of the cerebral energy charge potential occurred. The redox state of the brain, as measured by the lactate:pyruvate ratio, remained high, thus indicating the persistence of anaerobic mechanism. The perfusion of the β -adrenergic stimulating agent bamethan induced an increase of the adenyl cyclase activity and the cyclic AMP

level. On the contrary, the perfusions of the β -adrenergic blocking agent *DCI* and of *DCI*+bamethan blocked the recovery of the activity of the adenyl cyclase system. This indicates the importance of a β -adrenergic activation on the energy-coupling processes *in vivo*. The intracarotid perfusion of the vasodilator agent papaverine exhibited no effect on the biochemical changes induced by the recovery of air ventilation.

DISCUSSION

Before discussing the present results, some limitations should be pointed out. In the first place, the model used was an acute one: consequently, chronic studies might lead to different biochemical adjustments. Secondly, these data refer to the motor area of the cerebral cortex: therefore, other important areas of the brain might undergo different biochemical adjustments, because of the anatomical and functional heterogeneity of the brain.

In a first stage induced by hypoxaemia, at values of arterial O_2 partial pressure higher than 25 mmHg, the activation of the cerebral adenyl cyclase system seems to be a very important biochemical way for increasing glycogen metabolism, its utilization being strongly modulated by both the enzyme activity and the cyclic AMP level. The increase of the lactate:pyruvate ratio in the cerebral tissue is a function of the decrease of arterial oxygen pressure.

In a second stage induced by hypoxaemia, at values of arterial O_2 partial pressure lower than 20 mmHg, a critical threshold was probably exceeded, new biochemical conditions were established, and other biochemical mechanisms of regulation were involved. In fact, the adenyl cyclase system was cut off, while carbohydrate catabolism was high, as indicated by the very high lactate production, by the very high lactate:pyruvate ratio, and by the further glycogen reduction. In any case the low ATP levels reduced the substrate for cyclic AMP formation, while they enhanced the rate of transformation from inactive to active phosphofructokinase. On the other hand, the phosphorylase activation can be supported by tissue factors—for example, the kinase activating factor. In any case, during this condition, the β -adrenergic stimulation, the β -

adrenergic block or the vasodilatation exhibited no action on the cerebral energy charge. This indicates that the physiological autoregulation and feed-back phenomena overcome exogenous interventions. The β -adrenergic stimulation can induce an increase in the adenylyl cyclase activity but not an increase in the cyclic AMP level, probably because of the impairment of ATP.

During the post-hypoxic restoration, the cerebral energy charge potential exhibited a gradual recovery. The high lactate:pyruvate ratio indicated that the mechanism involved in the recharging of the adenylyl pool was still partially anaerobic. Perfusion with a β -receptor stimulating agent potentiated the adenylyl cyclase system and increased the cerebral level of cyclic AMP. Perfusion of a β -receptor blocking agent inhibited the activation by a β -stimulating agent. This fact is probably related to the block of the adenylyl cyclase receptor, the adenylyl cyclase protein being regarded as a β -adrenergic receptor (Robison *et al.*, 1967). No correlations exist between the vasodilator effect and the recovery of the cerebral energy-coupling systems. Though a strong vasodilator, papaverine proved unable to affect favourably the restoration of the cerebral energy charge potential.

In conclusion, the data obtained indicate that the adenylyl cyclase system is an important cerebral biochemical regulatory factor, but that this system becomes less important beyond a critical threshold of arterial oxygen partial pressure (lower than 25–20 mmHg). Under these conditions, other enzymatic systems—for example, phosphofructokinase—intervene as rate-limiting factors in glycolysis. Consequently, during hypoxaemia the cerebral adenylyl cyclase system seems to play a role as an activating factor, but is unable to sustain the activating step for long periods of time beyond a pO_2 critical threshold.

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