OPPOSITE EFFECTS OF DIBUTYRYL CYCLIC GMP AND DIBUTYRYL CYCLIC AMP ON GLUCOSE 1,6-DIPHOSPHATE LEVELS AND THE ACTIVITIES OF GLUCOSE 1,6-DIPHOSPHATE PHOSPHATASE AND PHOSPHOFRUCTOKINASE IN DIAPHRAGM MUSCLE

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1. Introduction

Glucose 1,6-diphosphate (Glc-1,6-P_2), the coenzyme of phosphoglucomutase [1–3], was postulated by us to play a highly important role in the regulation of glucose metabolism in muscle (reviewed [4]). Glc-1,6-P_2 participates in the control of key enzymes, and is one of the strongest activators (de-inhibitors) of phosphofructokinase [5–7], the rate-limiting enzyme in glycolysis. N^6-2′-*O*-dibutyryl cyclic AMP (dbcAMP) increases Glc-1,6-P_2 levels and activates phosphofructokinase in the isolated rat diaphragm, mimicking the action of epinephrine [8]. We have also found that the activity of the enzyme that degrades Glc-1,6-P_2, Glc-1,6-P_2 phosphatase, undergoes changes in muscle; the changes are reciprocal to the levels of Glc-1,6-P_2, under various conditions such as starvation, refeeding, and muscular dystrophy [9–11]. Here we have examined whether the changes in Glc-1,6-P_2 levels induced by dbcAMP in the isolated rat diaphragm, can be attributed to a change in the activity of Glc-1,6-P_2 phosphatase. In view of the 'Yin–Yang Hypothesis' [12] of biological regulation through the opposing influences of cyclic GMP and cyclic AMP, we also tested the influence of N^2-2′-*O*-dibutyryl cyclic GMP (dbcGMP) on this enzyme's activity and its effects on the Glc-1,6-P_2 levels and the activity of phosphofructokinase in the isolated rat diaphragm. The results show that the two cyclic nucleotides exert opposite effects on these parameters.

2. Methods

Male Charles River albino rats (80–120 g), fed ad libitum or fasted for 24 h, were anesthetized with sodium pentobarbital. Hemidiaphragms were prepared as in [13], and incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer containing glucose (1 mg/ml) and gassed with 95% O_2–5% CO_2. After incubation, the hemidiaphragms were rapidly frozen between a pair of aluminium tongs precooled in liquid N_2. Frozen diaphragms were powdered in a mortar cooled in liquid N_2, and the powder was used for extraction of Glc-1,6-P_2 and enzymes. Glc-1,6-P_2 was extracted as in [8] and measured by the fluorometric method [14]. Preparation of extracts and assay of phosphofructokinase were as in [15]. One milliunit of phosphofructokinase activity catalyzed the formation of 1 nmol fru-1,6-P_2/min at 25°C. Glc-1,6-P_2 phosphatase was extracted and assayed as in [9]. One milliunit of Glc-1,6-P_2 phosphatase activity catalyzed the degradation of 1 nmol Glc-1,6-P_2/15 min at 37°C. Protein was estimated by the method in [16].

3. Results

Table 1 reveals that the addition of dbcAMP to the incubation medium of hemidiaphragms from 24 h fasted rats caused a significant reduction in the activity of Glc-1,6-P_2 phosphatase. This offers an explanation for the rise in Glc-1,6-P_2 levels induced by dbcAMP under identical conditions [8]. The effect of dbcAMP was dose-dependent and was most pronounced in hemidiaphragms from starved rats in which the activity of Glc-1,6-P_2 phosphatase is markedly elevated. (We had found that fasting had a similar effect on this enzyme's activity in mouse leg muscle [11].) On the

Table 1
The influence of dibutyryl cyclic AMP and dibutyryl cyclic GMP on the activity of Glc-1,6-P_2 phosphatase in the isolated rat diaphragm

Addition to incubation medium	Glc-1,6-P_2 phosphatase activity (milliunits/mg protein)	Effect (%)	p value
A.			
None	0.95 ± 0.09 (7)		
dbcAMP (2 mM)	0.56 ± 0.08 (7)	−40	<0.0005
B.			
None	0.45 ± 0.04 (6)		
dbcGMP	0.77 ± 0.04 (6)	+81	<0.00005

Paired hemidiaphragms from 24-h fasted rats (A) or from fed rats (B) were incubated for 30 min in the absence and presence of the specific cyclic nucleotide. Glc-1,6-P_2 phosphatase was prepared and assayed as in section 2. Values are means ± SEM, with no. expt. in parentheses

other hand, dbcGMP induced an effect opposite to that of dbcAMP when added to an incubation medium of hemidiaphragms from fed rats (table 1, exp. B); it caused an almost 2-fold increase in Glc-1,6-P_2 phosphatase activity. Fed rats were used, as the dbcGMP could not activate more the already activated enzyme in the fasted rats. As can be seen in table 2, in correlation to the activation of Glc-1,6-P_2 phosphatase by dbcGMP, the Glc-1,6-P_2 levels decreased under incubation with dbcGMP. Phosphofructokinase was strongly inhibited under these conditions. This inhibition is probably a result of both the increase in its direct allosteric inhibitor, cyclic GMP [7], and decrease in its potent de-inhibitor, Glc-1,6-P_2. As seen in table 3, Glc-1,6-P_2 had a very strong de-inhibitory effect on the inhibited phosphofructokinase in the diaphragms incubated with dbcGMP. Cyclic AMP also activated the enzyme (though concentrations ~5-times higher were needed). Both activators acted in a concentration-dependent manner. An interesting finding is that when Glc-1,6-P_2 (2.5 μM, which caused a 48% increase) and cyclic AMP (20 μM, which caused a 41% increase) were added to the reaction mixture together, they activated the enzyme synergistically (196% increase).

4. Discussion

The 'Yin–Yang Hypothesis' [12] suggests that cyclic AMP and cyclic GMP, through their opposing influences, may constitute a mechanism for cellular regulation in many systems. These results add support to this hypothesis. While dbcAMP inhibits the activity

Table 2
The effect of dibutyryl cyclic GMP on glucose 1,6-diphosphate levels and phosphofructokinase activity in the isolated rat diaphragm

	Glc-1,6-P_2 (μmol/kg wet wt)	Phosphofructokinase activity (milliunits/mg protein)
Control	5.54 ± 0.27 (11)	11.5 ± 0.6 (8)
dbcGMP	3.67 ± 0.25 (11)	5.4 ± 0.3 (8)
% effect	−33	−54
p value	<0.0005	<0.00005

Paired hemidiaphragms from fed rats were incubated for 30 min in the absence and presence of 10 μM dibutyryl cyclic GMP (dbcGMP). Glc-1,6-P_2 and phosphofructokinase were extracted and assayed as in section 2. ATP in the assay mixture for phosphofructokinase was 0.05 mM. Values are means ± SEM, with no. expt. in parentheses

Table 3
The activatory effect of glucose 1,6-diphosphate and cyclic AMP on phosphofructokinase from diaphragms incubated with dibutyryl cyclic GMP

Additions to incubation medium	Additions to assay mixture	Phosphofructokinase activity (milliunits/mg protein)	Effect (%)
None	None	6.96 ± 0.05	
dbcGMP	None	2.85 ± 0.03	−59
dbcGMP	Glc-1,6-P_2 (20 μM)	7.99 ± 0.19	+179[a]
dbcGMP	Glc-1,6-P_2 (10 μM)	6.21 ± 0.08	+117
dbcGMP	Glc-1,6-P_2 (5 μM)	4.92 ± 0.15	+71
dbcGMP	Glc-1,6-P_2 (2.5 μM)	4.25 ± 0.03	+48
dbcGMP	Cyclic AMP (100 μM)	9.95 ± 0.29	+243
dbcGMP	Cyclic AMP (50 μM)	6.90 ± 0.09	+138
dbcGMP	Cyclic AMP (20 μM)	4.08 ± 0.17	+41
dbcGMP	Cyclic AMP (10 μM	3.77 ± 0.06	+30
dbcGMP	Glc-1,6-P_2 (2.5 μM) + Cyclic AMP (20 μM)	8.58 ± 0.70	+196

[a] % activation by Glc-1,6-P_2 and/or cyclic AMP was calculated, in all the instances below, relative to phosphofructokinase activity in the dbcGMP-treated diaphragms (2.85 ± 0.03 milliunits/mg protein)

Hemidiaphragms from fed rats were incubated for 30 min with 10 μM dibutyryl cyclic GMP (dbcGMP). Phosphofructokinase was prepared and assayed as in section 2, using 0.15 mM ATP in the assay mixture. Variable Glc-1,6-P_2 and cyclic AMP concentrations were added to the assay mixture. Values are means ± SEM for 6 expt. For all expt. $p < 0.0001$

of Glc-1,6-P_2 phosphatase, dbcGMP activates it. The opposite influences on the Glc-1,6-P_2 phosphatase lead to inverse changes in the Glc-1,6-P_2 levels in the diaphragm; dbcGMP brings about a decrease in Glc-1,6-P_2 levels, while dbcAMP has been shown [8] to cause a rise in Glc-1,6-P_2. Phosphofructokinase, the key enzyme of glycolysis, changes in full agreement to the levels of the cyclic nucleotides and the level of its potent activator Glc-1,6-P_2. On incubation with dbcGMP, the phosphofructokinase is inhibited, probably as a result of a combined action of the rise in concentration of its direct allosteric inhibitor, cyclic GMP, and the fall in levels of its activator (de-inhibitor), Glc-1,6-P_2. Adding very small concentrations of Glc-1,6-P_2 can overcome the inhibition of the enzyme (table 3).

We had reported that in muscular dystrophy the levels of Glc-1,6-P_2 in muscle are markedly reduced [17,18], while the cyclic GMP levels rise [17]. Concomitant to these changes, phosphofructokinase activity and glycolysis in the dystrophic muscle are severely depressed [17]. In light of these results, it is possible that the rise in cyclic GMP in the dystrophic muscle leads to the decrease in the Glc-1,6-P_2 levels, through its activatory effect on the Glc-1,6-P_2 phosphatase. The activity of Glc-1,6-P_2 phosphatase is markedly increased in the dystrophic muscle [9,11]. The strong deinhibitory effect of Glc-1,6-P_2 and cyclic AMP, which act synergistically on phosphofructokinase activity in muscle incubated with dbcGMP (table 3), leads us to believe that an increase in Glc-1,6-P_2 and cyclic AMP levels will overcome the inhibition of phosphofructokinase by cyclic GMP. We are doing experiments in this direction on dystrophic muscle.

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References

[1] Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A. C. and Caputto, R. (1948) Arch. Biochem. 19, 339–340.

[2] Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E. and Paladini, A. C. (1948) Arch. Biochem. 18, 201–203.
[3] Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F. and Trucco, R. E. (1949) Arch. Biochem. 22, 87–99.
[4] Beitner, R. (1979) Trends Biochem. Sci. 4, 228–230.
[5] Hofer, H. W. and Pette, D. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1378–1392.
[6] Krzanowski, J. and Matschinsky, F. M. (1969) Biochem. Biophys. Res. Commun. 34, 816–823.
[7] Beitner, R., Haberman, S. and Cycowitz, T. (1977) Biochim. Biophys. Acta 482, 330–340.
[8] Beitner, R., Haberman, S. and Nordenberg, J. (1978) Mol. Cell. Endocrinol. 10, 135–147.
[9] Beitner, R. and Cohen, T. J. (1979) IRCS Med. Sci. 7, 24.
[10] Beitner, R. and Nordenberg, J. (1979) IRCS Med. Sci. 7, 136.
[11] Beitner, R., Nordenberg, J. and Cohen, T. J. (1979) FEBS Lett. 104, 244–248.
[12] Goldberg, N. D., Haddox, M. K., Nicol, S. E., Glass, D. B., Sanford, C. H., Kuehl, F. A. jr and Estensen, R. (1975) in: Advances in Cyclic Nucleotide Research (Drummond, G. I. et al. eds) vol. 5, pp. 307–330, Raven, New York.
[13] Beitner, R. and Kalant, N. (1971) J. Biol. Chem. 246 500–503.
[14] Passonneau, J. V., Lowry, O. H., Schulz, D. W. and Brown, J. G. (1969) J. Biol. Chem. 244, 902–909.
[15] Beitner, R., Cohen, T. J., Nordenberg, J. and Haberman, S. (1979) Biochim. Biophys. Acta 586, 266–277.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Beitner, R., Haberman, S., Nordenberg, J. and Cohen, T. J. (1978) Biochim. Biophys. Acta 542, 537–541.
[18] Beitner, R. and Nordenberg, J. (1979) FEBS Lett. 98, 199–202.