INVERSELY RELATED OSCILLATIONS IN THE CONTENTS OF CYCLIC GMP AND THE TOTAL ADENINE NUCLEOTIDES IN STEADY-STATE PERFUSED RAT HEARTS

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1. Introduction

While investigating amino acid incorporation into protein in Langendorf perfused hearts we made the surprising observation that the tissue contents of cyclic AMP and cyclic GMP showed very significant variations when measured at time points 20 min apart during a 2 h perfusion period [1]. Despite these large temporal variations in the cyclic nucleotides, the heart preparation is apparently in a steady-state for up to 2 h as judged by oxygen uptake, glucose utilization, nicotinamide nucleotide ratio, glycogen turnover, lactate turnover, alanine output [2,3] protein-synthesis rate [1,4], beat rate and contraction amplitude [5]. It had been suggested that cellular feedback-regulation systems might give rise to stable oscillations on which metabolic steady-states could rely [6] and moreover oscillations which appeared to be related to glycolytic oscillations had been observed in the concentrations of the adenine nucleotides and IMP in particle-free rat skeletal-muscle extracts [7]. The possibility that such oscillatory phenomena were responsible for the cyclic nucleotide variations was investigated by measuring in both hearts and perfusate all purine nucleotides, nucleosides and their degradation products at selected time points over 80 min of perfusion. Highly significant differences were found in the contents of ATP, ADP, GTP, cyclic AMP, cyclic GMP in the ATP/ADP ratio and in the sum of the adenine nucleotides at time points 10 or 20 min apart [5]. The ATP/ADP ratio and the contents of GTP and cyclic AMP showed an identical pattern of variation which was distinct from that of the total adenine nucleotide content. The very large nett increases and decreases in the total adenine nucleotides extracted could neither be explained as operation of Lowenstein's purine nucleotide cycle [7] nor as metabolism of any known nucleotide precursor, derivative or polymer and it was suggested that an unsuspected substantial storage form of purine nucleotide might exist in mammalian heart [5].

This paper reports that when hearts are sampled more frequently very regular statistically significant temporal variations in total adenine nucleotide content are discerned. Furthermore the changing adenine nucleotide content is mirrored by inverse alterations in the cyclic GMP content. Since the cyclic GMP content also appears to respond to the non-synchronous variations in total guanine nucleotide, it is argued that guanylate cyclase activity may be regulated by the level of free adenine nucleotide in the cell.

2. Methods

Langendorf perfusion of hearts from male rats bred from an original Sprague-Dawley strain was as in [2] and the extraction and assay methods used were those in [5]. A single value at any time point was derived from a single heart freeze-clamped after perfusion for that time. Great care was taken to ensure that hearts used for the same time point were perfused and extracted on different days. All the values were related to the wet tissue weight obtained prior to perfusion by a standardized procedure [2]. The values quoted are means ± SEM using Bessel's correction for small numbers. Significance testing except for the special analysis of variance described in the text was by Student's *t*-test.

3. Results and discussion

Fifty-two hearts were perfused in 2 approximately equal batches ~3 months apart. The perfusion and

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processing of each batch took ~6 weeks. Not all the time-points were present in each batch in that the first omitted 10, 15 and 50 min samples while the second had no 80 min values. The mean values at zero-time (that is after 10 min washout preperfusion [2]), were different in some cases and the individual time point values were expressed as percentages of the mean zero-time value before combining the results from the 2 batches.

3.1. *The adenine nucleotide content*

Fig.1 gives the values obtained for the sum of the ATP, ADP and AMP contents (cyclic AMP is negligible) of hearts perfused for the time shown. When these contents at several consecutive time points were tested against each other using Student's *t*-test and found to be significantly different, this was taken as indicating that the content of nucleotide in the heart is exhibiting real variations with time and not merely reflecting the variance of the parent population. An alternative statistical approach to assess the validity of this conclusion is to determine whether the variation between the sample means is different from the population variance as indicated by variation within the individual samples. Thus if the 'between sample variation' should prove to be significantly greater than the 'within sample variation', then it may be concluded that the samples were not drawn from the same population but from populations whose mean values differed. In practice these may be compared using Snedecor's *F*-test which is valid provided:

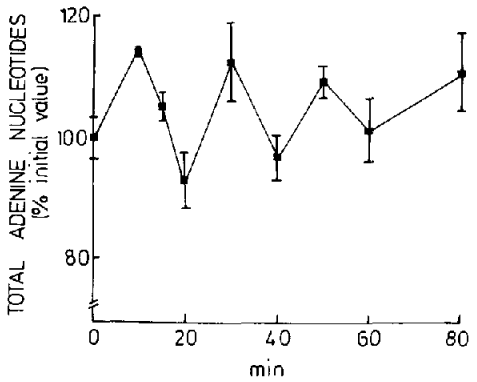

Fig.1. The variation of total adenine nucleotides during perfusion. The value for each heart was obtained by summing the ATP, ADP, and AMP contents. The mean zero-time point represents 4020 ± 376 (8) nmol/g (the values for each batch were 3687 ± 170 and 4353 ± 335). The total number of hearts was 51.

(i) The data have been collected at random;
(ii) The sample variance is independent of the sample mean;
(iii) The individual estimates of the sample are equally distributed about the sample mean.

The last 2 of these requirements often do not hold for biological experiments but the difficulty may be surmounted by using logarithms of the measured values rather than the absolute quantities. Table 1 gives the result of this statistical analysis confirming that the total extractable adenine nucleotide pool is showing significant time-dependent oscillations at least for the first 40 min of perfusion. The data do not of course give the shape of this oscillation although the points could be fitted by a damped sinusoidal oscillation of the form:

$$y = (100 + mt) + Ce^{-\gamma t} \cdot \cos \frac{2\pi}{l} (t - t_0)$$

where m is the slope of the increasing baseline, C is the maximum amplitude at some time t_0 after zero time and l is the wavelength. The findings do however suggest that some oscillatory process removing and regenerating free adenine nucleotides is present which appears to be synchronised by the isolation or perfusion of these hearts and which tends to diminish with perfusion time.

Table 1 also shows that the 'between sample' variation of ATP, the major component in cell adenine nucleotides, like that of AMP is not significantly greater than the 'within sample' variation. AMP indeed shows relatively little variation (see also [5]) and the findings provide no support for the contention that adenylate kinase may be at equilibrium in the heart and that small changes in ATP concentration would be reflected by relatively large changes in AMP level [8,9]. The telling component for both the total adenine nucleotide content and the ATP/ADP ratio appears to be ADP and since a substantial proportion of muscle ADP is believed to be tightly bound to actin [10,11], myosin [12] or possibly even to sarcoplasmic reticulum [13], the fractional variations in free ADP could be considerably magnified in the cell.

3.2. *Cyclic GMP and the adenine nucleotide content*

Table 1 shows that the cyclic GMP content of these hearts was also showing significant oscillatory behaviour. When the time-course was examined (fig.2) it appeared to vary ~180° out of phase with the total adenine nucleotides although in a somewhat less regular fashion. When the adenine nucleotide and cyclic GMP

Table 1

	Perfusion time (min)	F	$k-1$	$N-k$	P
Total adenine nucleotides	40	2.64	5	30	$P < 0.05$
Total adenine nucleotides	80	1.90	8	42	$P < 0.1$
ATP	80	1.54	8	42	$P > 0.1$
ADP	80	3.32	8	43	$P < 0.01$
AMP	80	1.21	8	37	$P > 0.1$
ATP/ADP ratio	80	2.98	8	42	$P < 0.01$
Cyclic GMP	80	2.38	7	39	$P < 0.05$
GTP	80	2.95	7	37	$P < 0.05$
GDP	80	0.33	7	26	$P > 0.1$

The 'between sample variance' (V_B) and the 'within sample variance' (V_W) were calculated from the expressions:

$$V_B = \frac{1}{k-1}\left(\sum_{i=1}^{k}\frac{\left[\sum_{j=1}^{n} x_{i,j}\right]^2}{n_i} - \frac{\left(\sum_{i=1}^{k}\left[\sum_{j=1}^{n} x_{i,j}\right]\right)^2}{N}\right)$$

$$V_W = \frac{1}{N-k}\left(\sum_{i=1}^{k}\left[\sum_{j=1}^{n} x_{i,j}^2\right] - \frac{\left(\sum_{i=1}^{k}\left[\sum_{j=1}^{n} x_{i,j}\right]\right)^2}{N}\right)$$

where x = logarithm of each measured value, n = no. of items within each sample, k = no. of samples, N = total number of items, $k-1$ = no. of degrees of freedom in the estimate of V_B, $N-k$ = no. of degrees of freedom in the estimate of V_B

data are plotted against one another a negative correlation is observed ($r = -0.66$; $0.1 > p > 0.05$) which becomes highly significant ($r = -0.98$; $p < 0.001$) provided the 20 and 40 min time points are ignored although these too seem to show some correlated behaviour (fig.3). This suggests that cyclic GMP might

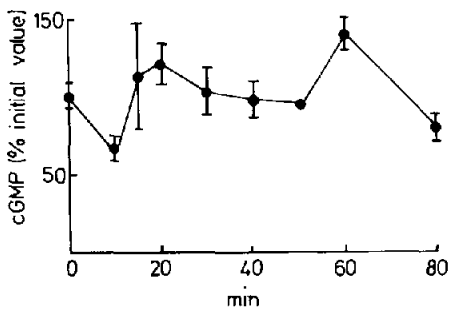

Fig.2. The variation of cyclic GMP content during perfusion. The hearts are the same as those in fig.1 except that only 2 hearts were measured at 50 min. The zero time value was 27.3 ± 3.7 pmol/g.

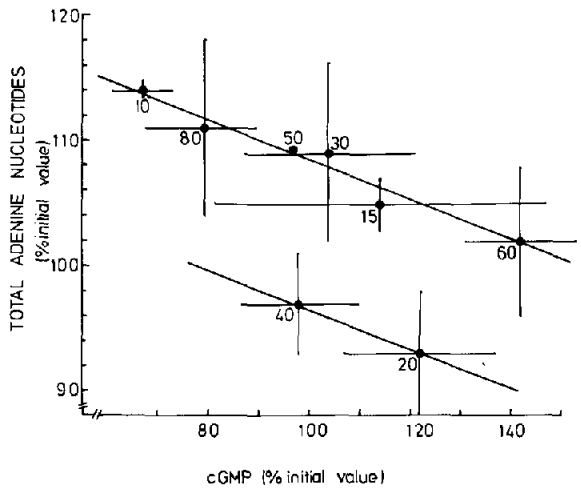

Fig.3. The correlation of cyclic GMP and the total adenine nucleotide content. The points are the data (and SEM) shown on fig.1,2. The numbers refer to the time point at which that particular set was measured.

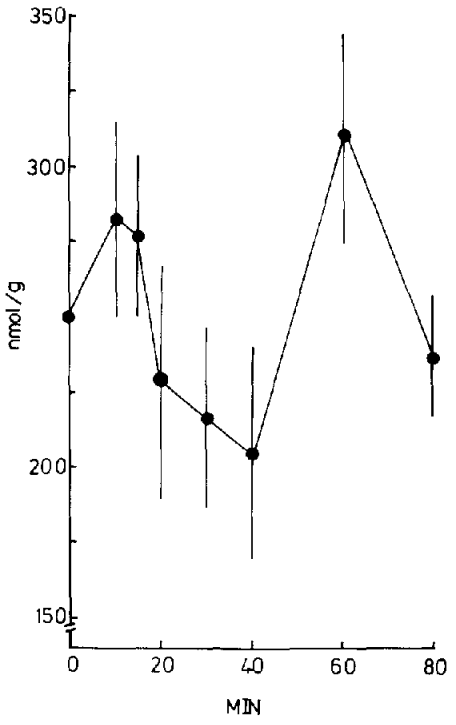

Fig.4. The variation of total guanine nucleotides during perfusion. The data are derived from the sum of the GTP and GDP contents of only 34 hearts since in some cases there was incomplete resolution of guanine nucleotide from a large neighbouring peak on HPCL. GMP and cyclic GMP were negligible. The mean GTP/GDP ratio was ~3.

in some way control, or be controlled by, the level of adenine nucleotides in the cell. A possible explanation for the different behaviour at the 20 and 40 min time points may be found by considering the sum of the guanine nucleotides (fig.4) whose variation, largely due to changes in GTP content (table 1), is different from that of the adenine nucleotides. In particular at 20 and 40 min the guanine nucleotide (and GTP) content is low. Hence if it were assumed that the adenine nucleotides control cyclic GMP levels and not vice versa, a low adenine nucleotide content could lead to activation of guanylate cyclase. The resulting increase in cyclic GMP content would, however, be constrained by insufficient GTP compared to the circumstances at other time points when the cyclase was activated or, as at 30 min, when the relative inactivity of the cyclase is satisfied by lower levels of substrate. ATP at physiological concentrations is a very powerful inhibitor of cardiac guanylate cyclase in vitro [14–16]: lowering the adenine nucleotide levels may relieve such inhibition in vivo.

The function of the observed 'sequestration' of adenine nucleotides is open to conjecture. It has been suggested that coarse control for the basal metabolic rate is needed to minimise build up of intermediates introduced by slight imbalances in fine coordinate control [17]. One possibility is that regulation of the nucleotide coenzyme content could determine overall metabolic activity without selectively altering cellular processes. The findings reported here raise the further possibility that cyclic GMP is related to this regulation of available adenine nucleotide. In this context it is noteworthy that effectors such as acetylcholine which reduce cardiac work and hence would be expected to lead to lowered adenine nucleotide content are observed to raise heart cyclic GMP content just as stimulants like isoproternol conversely decrease it [18].

4. Conclusions

Isolation and Langendorf perfusion of rat hearts results in sustained damped oscillations of the total free adenine nucleotide, guanine nucleotide and cyclic GMP contents. The cyclic GMP level is inversely proportional to the total extractable adenine nucleotides and the nature of their variations suggests that the cyclic GMP content may be partly determined by the total adenine nucleotide concentration and hence related to the controlled sequestration of adenine nucleotides in the cell.

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