23 Control analysis of signal transduction

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Introduction

Control analysis allows quantitation of how metabolic pathways are internally controlled and how they are regulated by external effectors. It has been used extensively to measure the control and regulation of energy metabolism in mitochondria and cells by many workers (see [1,2]) and by us [3–10]. It has not been used experimentally in other areas of cell biology, such as signal transduction, although the principles have been addressed [11–13]. Regulation during signal transduction is incompletely understood, and current work in the field concentrates on identifying pathways and on the mechanisms of interactions.

We present an experimental control analysis of signal transduction using mitogenic activation of thymocyte energy metabolism as a simple model system (see also [14]). Con A is a T cell mitogen that acts as polyclonal antigen and stimulates thymocyte respiration by up to 40% [6]. This stimulation is stable for 25 min [8], so the system changes from one pseudo steady state to another, allowing simpler experimental and theoretical analysis. Several signalling pathways are involved in the activation of T cells [15,16]. A quantitative experimental description of regulation should be able to indicate the relative importance of each of the different signalling routes that triggers a change in respiration. Each signalling pathway may have effects on several of the reactions that affect respiration, so a quantitative analysis should also be able to indicate for each signalling route the relative importance of activation of different reactions in stimulating respiration. Finally, it should be possible to integrate all of the measurements into an overall quantitative picture of signal transduction.

Methods

The thymus from a female rat (4–8 weeks old) was removed, rinsed, and pressed through nylon mesh into 3 ml RPMI 1640 medium/1% FCS. Small thymocytes were retrieved by density gradient centrifugation using RPMI 1640 medium with glutamine and glucose [17] and suspended at 5×10^7 cells/ml in RPMI 1640 with glutamine, without glucose, containing 25 mM $NaHCO_3$, 10 mM HEPES and 20 μg gentamycin/ml.

Thymocytes were incubated at 37 °C under 5% CO_2/95% air with 20 μg Con A per 5×10^7 cells. They were pre-treated with inhibitors as appropriate: cypermethrin (2 μM, 30 min) to inhibit calcineurin [18], bisindolylmaleimide I (1 μM, 30 min) to inhibit protein kinase C [19], PD 98059 (20 μM, 60 min) to inhibit MAPKK [20], myxothiazol (8 nM) to partially inhibit electron transport or oligomycin (2.5 ng/ml) to partially inhibit ATP synthesis [8]. Oxygen consumption was measured in Clark electrodes and mitochondrial membrane potential, $\Delta\psi_m$, was determined using [^{3}H]TPMP$^+$ [8]. Experimental statements in this paper were significant, based on Monte Carlo analysis [14,21].

Results and discussion

We analysed signal transduction at two levels. At the first level we analysed the relative importance of different signalling pathways. The system consisted of a cellular variable, thymocyte respiration, which was responsive to changes in the input level of the activatory signal (Con A). Con A affects steady state respiration through a number of routes, probably TCR-linked [22]. Here, we consider four signalling pathways: protein kinase C, the MAPK pathway, calcineurin and a single block of unrelated, unidentified pathways. The four pathways were each defined by inhibitor specificity; thus we defined the calcineurin pathway as the part of the signal inhibitable by cypermethrin. At the second level we analysed the way each signalling pathway acted on different parts of oxidative phosphorylation to give the overall stimulation. The system had fewer signalling intermediates (MAPK was included under PKC), but was extended to include the two blocks of metabolic reactions that produce and consume $\Delta\psi_m$.

First level of analysis: the relative importance of different signalling pathways

By using inhibitors both alone and in combination and measuring their effects on the Con A-induced increase in respiration, we calculated how much of the signal passed through each inhibitable pathway, and which pathways overlapped (Fig. 23.1). Inhibition of PKC depressed the Con A stimulation by 54%. Inhibition of

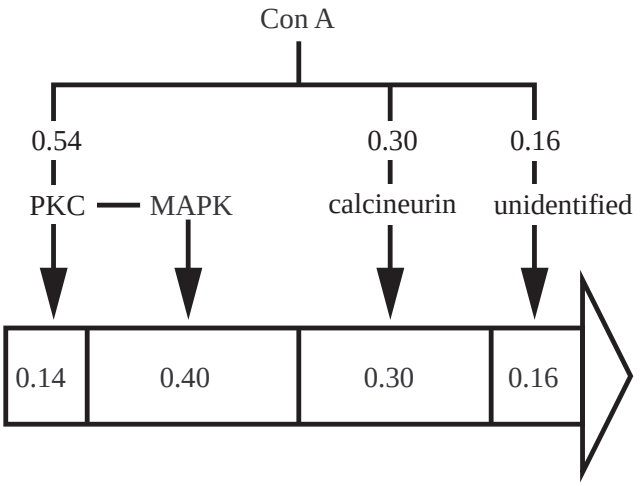

Fig. 23.1 Quantitation of signalling pathways during Con A stimulation of thymocyte respiration. Cells treated with inhibitors of signal transduction were stimulated with Con A. The decrease in Con A stimulated respiration was expressed as fraction of control without inhibitors (large open arrow); numbers indicate how strongly each signalling pathway contributed to the Con A-induced increase in respiration. Means from 5–7 experiments.

MAPKK depressed the stimulation by 40%, but the effects of inhibition of PKC and MAPKK were not additive, showing that the MAPK signal was entirely contained within the PKC pathway. Thus 54% of the Con A signal passed through PKC; this signal route then split, with 14% of the Con A signal passing to respiration directly, and 40% of the signal passing to respiration indirectly, through the MAPK pathway. Inhibition of calcineurin depressed the stimulation by Con A by 30%. This effect was additive with inhibition of either PKC or MAPKK, showing that calcineurin provided an independent signal transduction route from Con A to respiration. 80% signal reduction was achieved by combining the three inhibitors, so other, unidentified pathways carried 16–20% of the signal. Fig. 23.1 summarises these results.

Second level of analysis: regulation of processes producing and consuming $\Delta\psi_m$

Quiescent and stimulated cells had similar values of $\Delta\psi_m$, so there was equal activation of the supply and demand for $\Delta\psi_m$ by Con A. How strongly did each signalling pathway affect respiration through the two blocks of reactions that produce or consume $\Delta\psi_m$? We calculated the integrated elasticity (*IE*) [23] of each block to the large step change (Δq) in that part of the Con A signal passing through each signalling route as:

$$IE^{i}_{\Delta q} = \Delta J_i - \sum_{all\ x} \varepsilon^{i}_{x} \cdot \Delta x \tag{23.1}$$

where ΔJ_i is the change in the measured rate of block *i*, and ε^{i}_{x} is the elasticity of block *i* to intermediate *x* ($\Delta\psi_m$). We then multiplied *IE* by the control coefficient of block *i* over respiration rate to give the integrated response of respiration to Con A through each signalling route [23]:

$$IR^{a}_{\Delta q} = \sum_{all\ i} C^{a}_{i} \cdot IE^{i}_{\Delta q} \tag{23.2}$$

where *IR* is the integrated response of variable *a* (respiration rate) to Δq and *C* is the control coefficient of block *i* over respiration. Assumptions made in this calculation of partial responses are discussed in detail in [23]. Quiescent and stimulated cells had similar values of $\Delta\psi_m$, so it was reasonable to use elasticities to $\Delta\psi_m$ (based on infinitesimal changes in $\Delta\psi_m$) when calculating integrated responses to large changes in Con A.

Thus, each of the signalling routes quantified in Fig. 23.1 was analysed to calculate how the total response to Con A was partitioned into individual effects on the subsystems. Partitioning of the PKC route was calculated as follows; calculations for the other routes followed the same pattern. Starting from the Con A stimulated state, we measured the effect of the PKC inhibitor on respiration and $\Delta\psi_m$. Separately, we determined the elasticities of each of the blocks to $\Delta\psi_m$ from the effects of adding submaximal myxothiazol or oligomycin (see [8]). We calculated the integrated elasticity of each block to the PKC signal by correcting for the indirect effects of the PKC signal on each block through changes in $\Delta\psi_m$ using eq. 23.1. The control coefficients of the blocks over respiration were calculated from the elasticities to $\Delta\psi_m$ [24]. The response of respiration to Con A via PKC from Fig. 23.1 was broken down into partial integrated responses via each of the blocks using eq. 23.2.

The integrated elasticities showed that the signals through PKC and MAPKK stimulated the kinetics of the $\Delta\psi_m$-consuming block, but (if anything) slightly inhibited the kinetics of the $\Delta\psi_m$-producing reactions. The integrated responses after weighting by the flux control coefficients showed that the signals activated respiration directly through the $\Delta\psi_m$-consuming block and not through the $\Delta\psi_m$-producing block. We calculated by difference the properties of the unknown routes that are insensitive to inhibition. This calculation is error-prone, but showed that the unidentified pathways stimulated respiration via the $\Delta\psi_m$-producers much more strongly than via the consumers.

Fig. 23.2 summarises our conclusions and provides an approximate quantitative topology of the regulation of respiration in thymocytes by Con A. It shows the relative importance of different signalling pathways: PKC-related pathways carry about 54% of the Con A signal, calcineurin-related pathways carry about 30%, and

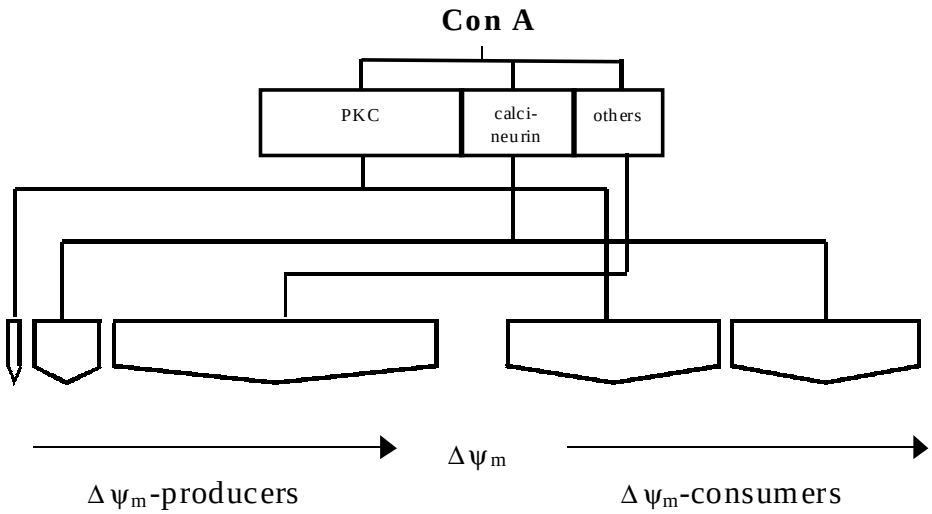

Fig. 23.2 Partition of the Con A stimulation of respiration in thymocytes. The large open pentagons represent the strength of the partial integrated responses to Con A through each indicated route. All effects are activations except for those of PKC and calcineurin on the $\Delta\psi_m$-producers, which are inhibitory.

unidentified signalling pathways carry the remainder. It also shows the interactions of these signalling mechanisms with metabolic reactions: the stimulatory effects of Con A on respiration acting through PKC and calcineurin mostly act through the consumers of $\Delta\psi_m$; the $\Delta\psi_m$-producers are much less important mediators. As $\Delta\psi_m$ does not change on Con A stimulation, these stimulatory effects of PKC and calcineurin must be balanced by an approximately equal activation of the $\Delta\psi_m$-producers by the unidentified pathways.

Our results show how control analysis can be used experimentally to quantify signal transduction pathways. We gathered large parts of metabolism or signal transduction into black-box groups of reactions [3–5] and simplified the application of control analysis to a system comprising both metabolic and signalling components, but, if required, resolution could be enhanced by using more inhibitors and measuring more intermediates.

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