the restriction in the supply of exogenous FFA by opposing the increase in the arteriovenous concentration difference elicited by isoproterenol [4–6].

In conclusion, propranolol impairs, to some extent, the rise in lactic concentration within myocardial tissue due to the combined action of moderate ischemia and a β-adrenergic drug, but it exerts a lesser influence on glycogenolysis and no influence on the reduction of free fatty acid content. The mechanism of the prevention by propranolol of angina pectoris attacks probably lies in limiting the increase in lactic acid production and decrease in pH within myocardial tissue, due to the combined action of moderate ischemia and a release of catecholamines [20–22].

Claude Bernard University, Department of Medical Pharmacology, Rockefeller Avenue, 8, F 69373 Lyon Cedex 2, France.

JEAN L. ANDRIEU
MICHEL LIÈVRE
QUADIRI TIMOUR CHAH
GEORGES FAUCON

REFERENCES

1. J. W. Black, W. A. M. Duncan and R. G. Shanks, *Br. J. Pharmac.* **25**, 577 (1965).
2. R. G. Shanks, *Am. J. Cardiol.* **18**, 308 (1966).
3. G. Deltour, *Thérapie* **25**, 293 (1970).
4. M. Laubie, H. Schmitt, M. Drouillat and M. Roblin, *Thérapie* **24**, 997 (1969).
5. D. G. Satchell, S. E. Freeman and S. V. Edwards, *Biochem. Pharmac.* **17**, 45 (1968).
6. T. N. Masters and W. Glaviano, *J. Pharmac. exp. Ther.* **167**, 187 (1969).
7. K. Ichihara and Y. Abiko, *Am. J. Physiol. Heart circ. Physiol.* **1**, H 349 (1977).
8. D. R. Christian, G. S. Kilsheimer, G. Pettett, R. Paradise and J. Ashmore, *Adv. Enzyme Regul.* **7**, 71 (1969).
9. S. L. Gartner and G. V. Vahouny, *Am. J. Physiol.* **222**, 1121 (1972).
10. J. J. Lech, G. J. Jesmok and D. N. Calvert, *Fedn. Proc.* **36**, 2000 (1977).
11. J. L. Andrieu, C. Vial, B. Font, D. Goldschmidt, M. Lièvre and G. Faucon, *Archs int. Pharmacodyn. Thér.* **237**, 330 (1979).
12. K. Schwartz, P. Rey, M. H. Bui and M. A. De Mendoca, *J. molec. cell. Cardiol.* **5**, 235 (1973).
13. H. U. Bergmeyer, *Methods of Enzymatic Analysis*, p. 266. Academic Press, New York (1965).
14. H. U. Bergmeyer, *Methods of Enzymatic Analysis*, p. 59. Academic Press, New York (1965).
15. H. U. Bergmeyer, *Methods of Enzymatic Analysis*, p. 1825. Academic Press, New York (1974).
16. W. G. Duncombe, *Clinica chim. Acta* **9**, 122 (1964).
17. L. H. Opie and P. Owen, *Am. J. Cardiol.* **38**, 310 (1976).
18. L. H. Opie, *Jap. Circulation J.* **42**, 1223 (1978).
19. T. N. Masters and V. V. Glaviano, *J. Pharmac. exp. Ther.* **182**, 246 (1972).
20. P. Keelan, *Br. Med. J.* **1**, 897 (1965).
21. D. H. McKenna, R. J. Corliss, S. Sialer, W. C. Zarnstorff, C. W. Crumpton and G. G. Rowe, *Circulation Res.* **19**, 520 (1966).
22. E. M. Dwyer, L. Wiener and J. W. Cox, *Circulation* **38**, 250 (1968).

Biochemical Pharmacology, Vol. 29, pp. 2690–2692.

0006-2952/80/1001-2690 $02.00/0

Relationship between glycolysis and proliferation of L 1210 cells *in vitro*: effect of a new pharmacological effector: RA-233

(*Received* 1 *February* 1980; *accepted* 12 *May* 1980)

In most *in vitro* cultures, cells are highly dependent on glucose consumption for generation of metabolic energy, although under certain conditions, glutamine can also provide such a source [1]. The part glucose plays is especially clear in the case of cancer cells, which are characterized by intense glycolytic activity [2] that can be considered a factor in tumoral growth [3].

It is customary to express glucose consumption as a function of the number of cells counted at the end of incubation [4]. However, this cannot take account of cell growth kinetics during long-time culture which are often necessary for pharmacological purposes. We have thus attempted to describe glucose consumption by a mode of expression which takes greater account of cell proliferation, and is not dependent on time of incubation or number of cells seeded. This model has been applied to study the metabolic effects of a new anticancer drug on L 1210 cells.

Methods

Cells and media. Cell culture conditions have already been described [5] but will be recalled here briefly: L 1210 cells were incubated in Dulbecco's modified Eagle medium containing 10% foetal calf serum supplemented with antibiotics, bicarbonate and glutamine (GIBCO reagents). Final volume in microtitration plates was 0.2 ml, in a water-saturated atmosphere containing 5% CO_2. Initial cell concentrations were 2×10^5 cells/ml and 5000 cells/ml for 24- and 48-hr cultures, respectively. A few 48-hr cultures were also grown in 25 cm^2 flasks, the initial concentration being 25,000 cells/ml. The absence of mycoplasm contamination was verified by Flow Laboratory Kit (Ref. 5.070).

The pharmacological effector used was RA 233 (Boehringer-Ingelheim Laboratories) or 2,6-bis-(diethanol amino)-4 piperidino-pyrimido (5,4-d)-pyrimidine, some of whose effects on cancer cells have already been described [6]. Experimental solutions were prepared from a 2×10^{-2} mol/l solution in N/50 hydrochloric acid, diluted as required in the culture medium. Control cultures without effector were grown concomitantly. All experiments were carried out in triplicate.

Cell counts. Cells were counted in a Malassez hematimeter and the lethality was evaluated by the loss of cell refringence observed with a phase contrast microscope.

The number of cell doublings (n) between times t_1 and t_2 was calculated according to the following formula:

$$n = \frac{\log N_2 - \log N_1}{\log 2},$$

where N_1 and N_2 were the number of cells measured at times t_1 and t_2, respectively.

Table 1. Results of glucose consumption (*Kglc*) under various culture conditions

	Experiments		
Time of incubation	24 hr	24 hr	48 hr
Initial cell concentration/ml	200,000	100,000	5000
Number of experiments in triplicate	16	3	7
Kglc (nmoles/10^6 cells/hr): mean ± S.E.	407 ± 54	443 ± 77	395 ± 57

Measurement of anaerobic glycolysis. The glucose and lactate in the medium were determined according to Trinder [7] and Gutman and Wacklefeld [8], respectively, using Boehringer-Mannheim reagents.

Expression of results. The problem was to express the glucose consumption measured between the two times t_1 and t_2 in a way that took account of cell growth evolution during that period and was not based on an isolated count at the end of the experiment. When incubation periods were short (0.5–2 hr), cell growth evolution was negligible [4, 9]. However, the same does not apply when prolonged contact is sought between the cells and the pharmacological effector in order to come closer to therapeutic conditions. After determining the experimental conditions under which the growth of cells is exponential, glucose consumption was calculated and then related to the cell population whose growth was integrated during the period between t_1 and t_2. Cell growth evolution for this time interval was such that $N_2 = N_1 \times e^{kt}$, with $k = \frac{\log 2}{T}$ (T = one doubling time) and N_2 and N_1 being number of cells.

During the same period, the metabolically active cellular mass was integrated, i.e.

$$\int_{t_1}^{t_2} N(t)\,dt = N_1 \times \frac{e^{kt}}{k}\Big|_{t_1}^{t_2}$$

$$\text{rendered by } N_1 \frac{e^{kt_2} - e^{kt_1}}{k}.$$

This allowed definition of a glucose consumption coefficient (*Kglc*) for the time period under consideration, written thus:

$$Kglc = \frac{M \times k}{N_1 (e^{kt_2} - e^{kt_1})} \text{ (moles.}10^6\text{cells}^{-1}\text{. hr}^{-1}\text{)},$$

in which M is the amount of glucose that disappeared from the culture medium during the period considered. If $t_1 = 0$, the denominator becomes $N_1 (e^{kt_2} - 1)$.

The significance of the results obtained was checked by the use of correlation coefficients and by the Student t-test or paired series method.

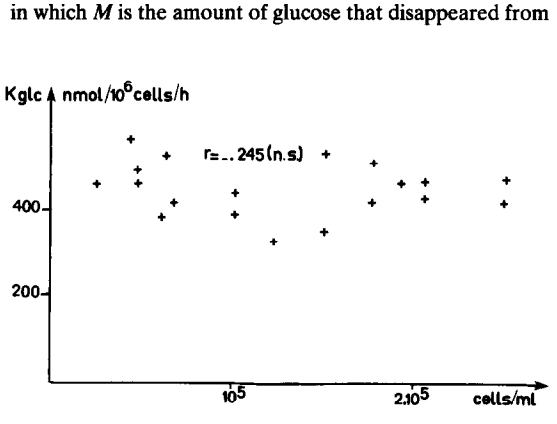

Fig. 1. Variations in the coefficient for cellular consumption of glucose (*Kglc*) as a function of the number of cells seeded. Duration of experiments was 24 hr.

Results

L 1210 cell growth. Under the culture conditions described, L 1210 cell numbers grew exponentially as a function of time, and the number of doublings per 24 hr was 2.23 ± 0.21 for 24-hr incubations (mean ± S.E. of eight experiments in triplicate). Similar results were obtained using any concentration of cells or time of incubation (see Methods). Cell death was around 5 per cent.

Glucose consumption cell coefficients (Kglc). Glycolysis is the chief source of energy for cells, under the conditions described above. It is primarily anaerobic, with a constant lactate/glucose ratio of about 1.8.

The coefficients *Kglc* calculated under different *in vitro* cell growth conditions, using the technique defined earlier, are indicated in Table 1. It shows that *Kglc* is reproducible under various culture conditions. Moreover, *Kglc* was measured in 24-hr cultures with initial cell concentrations ranging from 25,000 to 250,000 cells/ml. On the basis of 19 experiments, no significant correlation was established (Fig. 1), and the same applied to three series of 48-hr cultures with initial concentrations of 5000, 10,000 or 20,000 cells/ml.

Non-significant results were similarly obtained when expressing the *Kglc* as a function of the number of cells harvested at the end of 24- and 48-hr experiments. Similarly, the *Kglc* showed no correlation with the number of doublings, and if assayed every 4 hr, it remained constant for 48-hr cultures.

Effects of RA 233. According to its concentrations, RA 233 had inhibitory or lethal effect on L 1210 cells. After 24 hr of culture, LC_{50} and IC_{50} were, respectively, 5×10^{-4} moles/l and 2×10^{-4} moles/l. After 48 hr of cultures, IC_{50} was 1×10^{-4} mole/l. With highly toxic concentrations (i.e. 10^{-3} mole/l) lethality became perceptible only after 4 hr of culture, then grew sigmoïdally to reach 50 per cent after 8 hr and more than 90 per cent after 12 hr. The inhibitory effect was reversible: after 24 hr of culture with IC_{50}, cells were washed and cultured again in fresh medium without RA 233, after which growth was similar to control cells.

The effect on glycolysis was studied using RA 233 in the culture medium for 48 hr at a concentration of 1×10^{-4} mole/l. Growth remained exponential throughout the experiments and the number of doublings per 24 hr was 1.98 ± 0.25 (mean ± S.E. of six experiments in triplicate). This result differed very significantly from control values when the two were compared by the paired t method ($P = 0.0002$).

The *Kglc* was 489 nmoles/10^6 cells/hr ± 54 (for three experiments in triplicate). Comparison of this result with control values by the paired t method showed a significant increase ($P = 0.002$). The effect of RA 233 on glycolysis

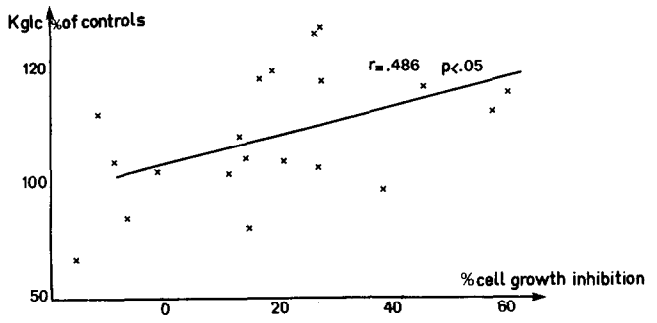

Fig. 2. Relationship between the coefficient for cellular consumption of glucose and inhibition of the growth of L 1210 cells incubated in the presence of RA 233. There was no increase in the percentage of dead cells.

is reversible; after washing of cells, *Kglc* falls to normal levels. When the RA concentrations were altered, the percentage increase in *Kglc* correlated with the percentage of cell inhibition and multiplication ($r = 0.486$; $P < 0.05$; Fig. 2).

Discussion and conclusions

The mode of expression is always a debatable issue in reports describing results obtained with cell cultures. Depending on the authors, the parameters studied are related either to the number of cells or to the amount of proteins or cellular DNA measured at the end of the experiments.

In our view, it is important to stress the following points. Lengthy incubation makes it necessary to take account of the kinetic evolution of the cell population. The method suggested here enables a metabolic parameter to be correlated with cellular growth over the same period, thus providing a much more homogeneous mode of expression. *Kglc* is independent of variables like the number of cells seeded and harvested, the number of doublings or the time of incubation. Expression in terms of *Kglc* can thus become a true constant of the cells studied and provide a mean of characterizing a line of cells. Furthermore, any other metabolic flux can be analysed in the same way, for instance macromolecular incorporation of radiolabelled precursors.

The effect of RA 233 illustrates the complex relations which may arise between glycolysis and cell growth. In L 1210 cells, RA 233 inhibits this growth while stimulating the *Kglc*. This effect seems rather specific since it correlates closely with the intensity of cell inhibition and is not observed when cell growth is altered by serum starvation for 12 hr: *Kglc* is 436 nmoles/10^6 cells/hr instead of 478 for control cultures (nonsignificant).

In chemotherapy, a close relationship is noted between antineoplastic activity in man and *in vitro* results with L 1210 cells [10]. The fact that with RA 233, cytotoxic activity is associated with a perturbation of a metabolic pathway characteristic of transformed cells (i.e. glycolysis) seems a promising further development.

In conclusion, we have described a mode of expression which relates a metabolic flux to cell growth and is independent of culture conditions. The effect on glycolysis of a pharmacological effector which inhibits cell growth while increasing glucose consumption was then described and discussed.

Acknowledgement—This work was supported by a grant from the Fédération Nationale des Centres de Lutte contre le Cancer de France (French National Federation of Centres for combating cancer).

*Laboratoires de Biochimie,
Institut Jean Godinot,
1, rue du Général Koenig,
51100 Reims, France.
†U.E.R. de Pharmacie,
51, rue Cognacq-Jay,
51100 Reims, France.

Y. CARPENTIER
J. KOUAMOUO
B. DESOIZE
J.-C. JARDILLIER‡

REFERENCES

1. H. R. Zielke, P. T. Ozand, J. T. Tildon, D. A. Sevdalian and M. Cornblath, *Proc. natn. Acad. Sci. U.S.A.* **73**, 4110 (1976).
2. C. E. Wenner, in *Cancer* (Ed. F. F. Becker), Vol. 3, p. 389. Plenum Press, New York (1975).
3. E. D. Savlov, J. L. Wittliff and R. Hilf, *Cancer* **39**, 539 (1977).
4. S. H. Gregory and S. K. Bose, *Expl Cell Res.* **110**, 387 (1977).
5. B. Desoize, Y. Carpentier and J.-C. Jardillier, *Biomedicine* **29**, 58 (1978).
6. H. Gastpar, *Hemat. Rev.* **3**, 1 (1972).
7. P. Trinder, *Ann. clin. Biochem.* **6**, 24 (1969).
8. I. Gutmann and A. W. Wahlefeld, in *Methods of Enzymatic Analysis* (Ed. H. V. Bergmeyer), p. 1464. Academic Press, New York (1974).
9. E. Kaminskas and A.C. Nussey, *Cancer Res.* **38**, 2989 (1978).
10. W. J. Darkin, V. J. Ghanta, C. M. Balch, D. W. Davis and R. N. Hiramoto, *Cancer Res.* **39**, 402 (1979).

‡ Author to whom all correspondence should be sent.