Large effects of preparative techniques on lymphocyte cyclic AMP content

John P. MOORE, Gerry A. SMITH, T. Robin HESKETH and James C. METCALFE

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 8 May 1983/Accepted 11 July 1983)

1. When a cell suspension is formed by disruption of a pig lymph node into medium, large and transient increases in intracellular cyclic AMP occur. 2. Similar effects are observed when pig lymphocytes are centrifuged and the cell pellets resuspended, or when the cells are subjected to rapid temperature changes. 3. These observations define the conditions required to manipulate the cells while maintaining a stable cyclic AMP concentration. Under these conditions, neither concanavalin A nor ionophore A23187 at mitogenic concentrations have any early effect on cyclic AMP in pig lymphocytes, but small increases in cyclic AMP (<2-fold) were observed at supramitogenic concentrations of concanavalin A (50 µg/ml) or A23187 (500 nM). 4. Mouse thymocytes show qualitatively similar but much smaller changes in cyclic AMP concentration in response to experimental manipulations, and no response to mitogenic or supramitogenic concentrations of concanavalin A below the cytotoxic value.

Although cyclic AMP has been implicated as an early mitogenic signal for cultured Schwann cells (Raff *et al.*, 1978) and for fibroblasts (Rozengurt *et al.*, 1981), the evidence for its involvement in the early stages of mitogenic lymphocyte stimulation is limited. Neither cyclic AMP nor cyclic AMP-elevating agents are mitogens (Watson, 1976), and the prolonged maintenance of raised intracellular cyclic AMP concentration inhibits the mitogenic stimulation of lymphocytes (Wang *et al.*, 1978). The effect of mitogens on cyclic AMP concentrations in lymphocytes has been the subject of many conflicting reports (reviewed by Hesketh, 1978), but the clearest pattern of consistent data is that there are no significant early changes in the cyclic AMP concentration in lymphocytes stimulated by optimal concentrations of mitogens. Changes in cyclic AMP concentration later in the cell cycle may, however, be important in regulating the initiation of DNA synthesis (Wang *et al.*, 1978).

Here we have measured cyclic AMP in pig lymphocytes and mouse thymocytes stimulated by concanavalin A and by ionophore A23187. We can detect no early changes in the cyclic AMP content of either type of lymphocyte in response to optimally mitogenic concentrations of either agent, but find that large changes in cyclic AMP can occur during cell preparation and that the 'basal' cyclic AMP concentration of the cells is very responsive to physical manipulation of the cell suspensions. We conclude that early increases in the cyclic AMP

concentration are not obligatory for the mitogenic stimulation of lymphocytes or for the early increases in the free cytoplasmic Ca^{2+} concentration caused by mitogens (Tsien *et al.*, 1982; Hesketh *et al.*, 1983).

Materials and methods

Cell preparations

Suspensions of pig mesenteric-lymph-node lymphocytes and BALB/c mouse thymocytes were prepared by gently squashing the tissue with a plastic plunger into medium at 37°C. The media used were RPMI 1640 (Flow Laboratories) supplemented with glutamine (2 mM) and gentamycin (Essex Chemie A.G.; 0.2 µg/ml) and buffered to pH 7.3 with either 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (RPMI/Hepes medium) or with 20 mM-Tris/24 mM- NaHCO_3 (RPMI/Tris/bicarbonate medium). Where indicated in the text, pig lymphocytes were purified by centrifugation (20 min, 525 g) on to a cushion of Ficoll [Pharmacia; 6.0% (w/v) in Triosil/water (1:5, v/v)] at room temperature. The interfacial layer of cells was suspended in medium and washed twice by centrifugation (5 min, 275 g, room temperature). Cell viability measured by eosin exclusion was over 95% for mouse thymocytes and purified pig lymphocytes and over 70% for unpurified pig lymphocytes. Cells prepared in RPMI/Tris/bicarbonate medium were incubated in a humidified atmosphere of

air/CO₂ (19:1) at 37°C, and cells prepared in RPMI/Hepes were incubated in the laboratory atmosphere at 37°C.

Assays

Mitogenic stimulation was assayed by the uptake of [6-³H]thymidine (Moore *et al.*, 1982), lactate output from lymphocytes was determined as described by Hesketh *et al.* (1983) and ATP was measured by the procedure of Pozzan *et al.* (1980). For cyclic AMP assays, lymphocytes (100 µl, containing 1×10^6 – 5×10^6 cells) were killed by the addition of 10 µl of 3M-HClO₄ and the sample was neutralized with 20 µl of 2M-triethanolamine/KOH buffer, pH 14.0. After incubation for 1 h at 4°C, insoluble material was precipitated by centrifugation (30 s, 14 000 g) and the cyclic AMP content of 125 µl of the supernatant solution was assayed by the binding-protein method of Brown *et al.* (1971). Solutions of cyclic AMP (0–50 pmol/100 µl) in medium were processed in the same manner to provide a standard curve for each experiment. When cell extracts were incubated with cyclic AMP phosphodiesterase (Boehringer, from ox heart), no material assaying as cyclic AMP remained.

Concanavalin A (3× recrystallized) was supplied by Miles–Yeda and ionophore A23187 by Calbiochem. A23187 was stored as a 500 µM solution in dimethyl sulphoxide at –25°C and diluted 20–100-fold in medium immediately before addition to cells. Prostaglandin E₁ (Sigma) was stored at –25°C as a 3 mM solution in ethanol. Ro-20/1724 [(±)-4-(3-butoxy-4-methoxybenzyl)-2-imidazolinone] was given by Mr. T. Collins of Roche Products, Welwyn Garden City, Herts., U.K., and was stored at –25°C as a 5 mM solution in ethanol. In all experiments the equivalent ethanol concentration (0.1–1%, v/v) was without effect on the parameter being measured.

Results and discussion

Changes in the cyclic AMP concentration in pig mesenteric-lymph-node lymphocytes during preparation of a cell suspension

In preliminary experiments we found that the 'basal' cyclic AMP concentration in pig lymphocytes was very variable and declined on incubation of the cells after the isolation and purification procedure described by Hesketh *et al.* (1977). Since these basal variations (0.5–30 pmol of cyclic AMP/10⁶ cells) were greater than previously reported mitogen-stimulated changes (2–10-fold; see Hesketh, 1978), we have characterized some of the experimental conditions that cause the basal cyclic AMP concentration to vary. Variable basal cyclic AMP values have previously been reported by, among others, Coffey *et al.* (1977), Lönnroth &

Lönnroth (1977) and Watson (1976). The last author concluded that such variation "undoubtedly ... affects the magnitude and kinetics of changes detected in the levels of cyclic AMP following mitogenic stimulation".

Mesenteric lymph nodes were routinely transported, packed in crushed ice, to the laboratory from a local abattoir. The nodes were, however, usually warm for up to 1 h after death of the animal because they are insulated by the fatty tissue of the intestinal fascia. The internal pH of a lymph node was typically in the range 6.2–6.5, as measured by insertion of a glass electrode into the tissue: disruption of the tissue by gentle squashing into 10 vol. of RPMI/Tris/bicarbonate medium immediately acidified the medium by approx. 0.3 pH unit. The acidification could be accounted for quantitatively by release of lactic acid accumulated in the tissue as a result of hypoxia *post mortem*. Despite this period of hypoxia, more than 70% of the cells in a freshly prepared lymphocyte suspension are viable, as judged by eosin exclusion.

A large and transient increase in cyclic AMP occurred when a lymph node was disrupted into medium or when 1–3 mm-thick slices of lymph node were incubated in medium or in the laboratory atmosphere (Fig. 1). All of the cyclic AMP measured was intracellular; no cyclic AMP could be detected in the medium after removal of the lymphocytes or lymph-node slice by centrifugation (3 s, 14 000 g). The increase in cyclic AMP was only 1.5-fold (from 2.2 to 3.2 amol/cell) when a cell suspension was prepared from lymph-node slices previously incubated for 90 min at 37°C in RPMI/Tris/bicarbonate medium under the conditions described in the legend to Fig. 1(b). It is therefore unlikely that the cyclic AMP increases described in Fig. 1(a) are a direct consequence of the alteration in intercellular interactions caused by dispersion of the tissue into a cell suspension.

The initial rates of cyclic AMP production were very similar when nodes were dispersed into medium at 23°C or 37°C, indicating that perturbation in the temperature of the lymph node did not cause the cyclic AMP increases: the rate of decline from peak values was, however, much slower when the cells were subsequently incubated at 23°C than at 37°C. The composition of the medium into which the nodes were disrupted did not affect the rate of cyclic AMP production or the peak value reached: neither parameter was altered by omission of glucose, Ca²⁺, Mg²⁺, Tris or bicarbonate from the medium, nor by the inclusion of Hepes, amino acids or the vitamin components of Eagle's Minimal Essential Medium (Gibco). The cyclic AMP increases were similar in media at pH 6.5 and 7.3. The decline in cyclic AMP from the peak value was slower in the presence of the cyclic AMP phosphodiesterase inhibitor Ro-

Fig. 1. Alterations in the cyclic AMP content of pig lymphocytes on preparation of a cell suspension and in lymph node slices

(a) A mesenteric lymph node was excised from the surrounding tissue, cut in half and rapidly disrupted into a cell suspension in either RPMI/Tris/bicarbonate medium (●), or a balanced salts solution (150 mM-NaCl, 3.7 mM-KCl, 3 mM- Na_2HPO_4 , 3.5 mM- KH_2PO_4 , 0.5 mM- MgCl_2 , 0.43 mM- CaCl_2 , pH 7.3) buffered with the same Tris and NaHCO_3 concentrations (○), at 37°C. The cell suspension (5×10^7 cells/ml) was incubated at the same temperature and the cyclic AMP content determined at the times indicated. Similar data were obtained from experiments in which either medium was buffered with 25 mM-Hepes. (b) Slices (1–3 mm thick, weighing 50–150 mg) were removed from the interior of mesenteric lymph nodes and incubated at 37°C either in 0.5 ml of RPMI/Tris/bicarbonate medium (▲) or without medium in the laboratory atmosphere (△). Sectioning of the nodes and transfer of the slices to their experimental environment took 1.5–2 min. At the times indicated, the cells were killed by transfer of the slices to a tube containing 0.6 ml of RPMI 1640/3M- HClO_4 (5:1, v/v) and samples of the solution were removed for assay of cyclic AMP. The weights of the slices were determined after addition to the acid solution. All cyclic AMP measurements presented in this and subsequent Figures are the averages of triplicate determinations; if not indicated by error bars, the standard deviations were within 15% of the mean value.

20/1724 (10 μM): 90 min after preparation of the cell suspension the cyclic AMP concentration was approx. 2-fold higher than in cells prepared without the inhibitor.

The cause(s) of the cyclic AMP increases described above has not been unequivocally demonstrated. One possibility is that they are a consequence, through an unknown mechanism, of the re-admission of oxygen to hypoxic cells. Although the cyclic AMP increases described in Fig. 1(a) still occurred when lymph nodes were disrupted into degassed medium in an atmosphere of nitrogen, the peak cyclic AMP concentration was decreased in four experiments to between 50% and 90% of the peak value in cells from the same node disrupted in the laboratory atmosphere. We note that 50–100-fold increases in the cyclic AMP content of ischaemic guinea-pig cerebral cortex occur on restoration of the blood supply and that this has been correlated with a rise in the tissue ATP content and the onset of aerobic metabolism (Kobayashi *et al.*, 1977). This effect may also account, at least in part, for cyclic AMP increases in the pig lymphocyte; the ATP concentration in these cells, assuming a cell volume of 175 fl (Felber & Brand, 1982), increased from 1 mM to 4 mM within 15 min of preparation of a cell suspension, whereas the rate of lactic acid production was 3-fold lower after 1 h incubation at 37°C than in a freshly prepared suspension.

No large cyclic AMP increases were detected when the thymus from a BALB/c mouse was disrupted into medium to form a cell suspension within 2 min of the death of the animal. The cyclic AMP content in thymocytes was initially in the range 3–5 amol/cell, but subsequently declined at an approximately constant rate during incubation of the cell suspension at 37°C. After 60–90 min incubation the cyclic AMP content was constant in the range 0.5–1.5 amol/cell. Similar decreases in the cyclic AMP content of mouse splenocytes occurred when cell suspensions, prepared as described by Hesketh *et al.* (1977), were incubated at 37°C.

Effect of centrifugation on the cyclic AMP content of lymphocytes

A standard procedure for the purification of pig lymphocytes involves centrifugation of a lymphocyte suspension on to a cushion of Ficoll/Isopaque, followed by washes of the interfacial cells by centrifugation and resuspension (see, e.g., Hesketh *et al.*, 1977). Large increases in pig lymphocyte cyclic AMP were found when the packed cells were resuspended after either centrifugation step (Fig. 2), although the increase was much greater on resuspension of a cell pellet than when the layer of cells at the Ficoll/medium interface was resuspended. The changes in cyclic AMP concentration were not

Fig. 2. *Effect of centrifugation on the cyclic AMP content of pig lymphocytes*

A suspension of pig lymphocytes in RPMI/Tris/bicarbonate medium (7×10^7 cells/ml) was incubated for 1 h at 37°C before the start of the experiment (zero time). A portion (5 ml) of the cell suspension was incubated in a plastic conical centrifuge tube (11 cm $\times$ 1.7 cm; Nuncatom) at 23°C in the laboratory atmosphere ($\bullet$). Two portions (5 ml) of the cell suspension were centrifuged (500g, 20 min) at 23°C in identical tubes and the cell pellets were resuspended in 5 ml of either fresh medium at 23°C ($\blacksquare$) or the supernatant solution ($\square$). Both cell suspensions were incubated at 23°C in the laboratory atmosphere and their cyclic AMP content was assayed at the times indicated. A fourth portion (7 ml) of the suspension was layered on a cushion (2 ml) of Ficoll/Isopaque (see the Materials and methods section) and centrifuged as described above. The layer of cells (2 mm thick, containing 2.5×10^8 cells) at the interface was removed, resuspended in 5 ml of fresh medium at 23°C and incubated as described above before cyclic AMP assay ($\blacktriangle$).

caused by alteration of the temperature of the cell suspension during these procedures, since similar data to those shown in Fig. 2 were obtained from experiments in which all of the procedures were performed in a room maintained at a constant temperature of 37°C . However, cyclic AMP was maintained at a high value for longer if the cells were incubated at 23°C rather than at 37°C after resuspension of the cell pellet. The cyclic AMP changes shown in Fig. 2 were not dependent on the inclusion of Ca^{2+} , Mg^{2+} , glucose or amino acids in the medium, but the magnitude of the effect was found to depend on the duration of the centrifugation step and on the number of cells centrifuged. Thus, in a typical experiment, the peak cyclic AMP content recorded when pellets containing 5×10^8 cells were resuspended after a 5 or 20 min centrifugation (525g) were 2.2 and 8.0 amol/cell respec-

tively, and, after resuspension of 0.6×10^8 cells centrifuged for 5 min (525g), the peak value was 1.2 amol/cell. The cyclic AMP content in uncentrifuged cells was 0.2 amol/cell.

Transient cyclic AMP increases were also found when mouse thymocytes were centrifuged into a pellet and then resuspended in medium. The magnitudes of the increases (typically less than 2-fold when 1×10^8 cells were centrifuged for 20 min at 525g) were, however, considerably less than those that occurred when pig lymphocytes were subjected to comparable centrifugations.

The cyclic AMP response to centrifugation and resuspension was refractory in that the magnitude of the response decreased with time of incubation at 37°C before centrifugation. Thus repeated centrifugation of a cell suspension at 60 min intervals caused progressively smaller cyclic AMP increases, and no increase was detectable after incubation for 3 h. In this respect, the centrifugation/resuspension response resembles the desensitization to hormonal stimuli of adenylate cyclase of lymphocytes and other cells that occurs on incubation of the cells at 37°C (Nordeen & Young, 1978; Kurokawa *et al.*, 1979; Zick *et al.*, 1983). It is uncertain whether the desensitization to centrifugation/resuspension is a consequence of the cyclic AMP changes that occur on preparation of the cell suspension.

The mechanism by which cyclic AMP increases are generated on resuspension of a packed cell pellet is not known. We have, however, found that centrifugation causes an increase in the rate of lactic acid output from lymphocytes, and it seems likely that this is a consequence of hypoxia within the pellet. If so, the cyclic AMP increases that occur on resuspension of a cell pellet and those that occur on disruption of a lymph node (Fig. 1) may both be due, at least in part, to the relief of hypoxia. This would be compatible with the findings that the magnitude of the cyclic AMP increase is proportional to the size of the cell pellet and to the duration of the centrifugation step.

Effect of temperature changes on lymphocyte cyclic AMP concentration

The cyclic AMP concentration in lymphocytes responded rapidly to changes in the incubation temperature (Fig. 3). Zick *et al.* (1978, 1982) have reported that transferring mouse thymocytes from 4°C to 37°C triggers a rise and then an exponential fall in intracellular cyclic AMP. The data presented in Fig. 3 demonstrate that pig lymphocyte cyclic AMP increases transiently on transferring the cells from 37°C to 23°C , and that this rise exceeds that caused by the addition of Ro-20/1724 ($10 \mu\text{M}$) at 37°C . The cyclic AMP pulse produced when the temperature of pig lymphocytes is lowered from 37°C to 23°C may result from differences in the

Fig. 3. Effect of temperature changes on the cyclic AMP content of pig lymphocytes

(a) A suspension of pig lymphocytes (4×10^7 cells/ml) in RPMI/Hepes medium was prepared as described in the Materials and methods section and preincubated for 1.5 h at 37°C in 100 μ l portions in polyethylene tubes (3.9 cm $\times$ 1 cm; Sarstedt). One set of tubes were then transferred to 23°C (zero time) and incubated at 23°C for 5 min (▲) before transfer back to 37°C. Other tubes of cells were incubated at 37°C throughout, with (■) or without (●) addition of 10 μ M-Ro-20/1724. In a similar experiment, the maximum cyclic AMP value recorded when 10 μ M-Ro-20/1724 was added simultaneously with transfer of the cells from 37°C to 23°C was 5.2 ± 0.8 amol/cell. (b) Pig lymphocytes were purified in RPMI/Tris/bicarbonate medium as described in the Materials and methods section. Immediately after the final centrifugation step, the cell suspension (4×10^7 cells/ml) was divided into 50 μ l portions and incubated in tubes (3.9 cm $\times$ 1 cm) at 37°C (●) or at 4°C (○). After 1 h some cell samples were transferred from 37°C to 4°C (□) and after a further 1 h some were transferred back to 37°C (Δ). Other cell samples that had been incubated at 4°C for 2 h were then transferred to 37°C (▲).

We conclude that it is essential to avoid any perturbations of the incubation temperature in studies of the effects of hormones or mitogens on lymphocyte cyclic AMP, or in studies of any process that may be modulated by the cyclic AMP concentration in the lymphocyte.

Effect of mitogens on lymphocyte cyclic AMP concentration

The preceding studies defined conditions under which lymphocytes can be manipulated while maintaining a basal cyclic AMP concentration that is sufficiently stable for the effect of mitogens on cyclic AMP concentrations to be determined.

The cyclic AMP concentration in pig lymphocytes was stable provided that the cells were incubated for 60–90 min at 37°C after isolation, Ficoll/Isopaque centrifugation and washing. The effects of a range of concentrations of concanavalin A and A23187 on the initial cyclic AMP content of pig lymphocytes and on subsequent [3 H]thymidine uptake are shown in Fig. 4. No cyclic AMP increases were stimulated within 30 min of the addition of mitogenic concentrations of concanavalin A (0.5–5 μ g/ml) or the bivalent-cation ionophore A23187 (40–150 nM). Both prostaglandin E_1 (an activator of adenylate cyclase; Best *et al.*, 1977) and Ro-20/1724 (an inhibitor of cyclic AMP phosphodiesterase; Sheppard *et al.*, 1972) stimulated increases in the cyclic AMP content of replicate samples of the pig lymphocyte suspension. There were small (<200% of control) cyclic AMP increases in pig lymphocytes treated with concentrations of concanavalin A and A23187 that were supraoptimal for mitogenic stimulation (Fig. 4). The cyclic AMP increases were maximal after 5 min and were sustained for 30 min (see also Metcalfe *et al.*, 1980). Optimal and supraoptimal concentrations of concanavalin A did not affect the cyclic AMP increase stimulated by Ro-20/1724 (10 μ M).

Mitogenic and supramitogenic concentrations of concanavalin A and A23187 had no detectable effect on the cyclic AMP content of mouse thymocytes within 30 min (Fig. 4) under conditions in which large increases were stimulated by prostaglandin E_1 or Ro-20/1724 (Fig. 5). Longer incubations with high concentrations of concanavalin A or A23187 caused a decrease in cyclic AMP, owing to their cytotoxic effects (Hesketh *et al.*, 1977). Although prostaglandin E_1 and Ro-20/1724 were synergistic in their stimulation of thymocyte cyclic AMP, concanavalin A (50 μ g/ml) had no effect on the cyclic AMP increase when added with Ro-20/1724 (Fig. 5).

The data described above imply that concanavalin A neither activates adenylate cyclase nor inhibits the cyclic AMP phosphodiesterase of pig lymphocytes or mouse thymocytes. We conclude

thermal activation profiles of adenylate cyclase and the membrane-bound cyclic AMP phosphodiesterase (Houslay & Palmer, 1978).

Fig. 4. *Effect of concanavalin A and A23187 on lymphocyte cyclic AMP concentration and on $[^3\text{H}]$ thymidine uptake* (a, b) Mouse thymocytes (1.5×10^7 – 4.5×10^7 cell/ml) or (c, d) purified and washed pig lymphocytes (3×10^5 – 5×10^7 cells/ml) were incubated for 1–2 h in RPMI/Tris/bicarbonate or RPMI/Hepes medium before addition of concanavalin A (a, c) or A23187 (b, d) at the concentrations shown. The incubations were continued at 37°C in the laboratory atmosphere for (●) 2 min, (■) 5 min, (▲) 20 min or (○) 30 min before determination of the cyclic AMP content of the cells. The cyclic AMP contents in control cells were 1–2 amol/cell and 0.5–1 amol/cell for mouse thymocytes and pig lymphocytes respectively. The effects of concanavalin A and A23187 on $[^3\text{H}]$ thymidine uptake into the cells after 42–48 h of culture are shown (◇). A23187 is not mitogenic for mouse thymocytes (Hesketh *et al.*, 1977).

Fig. 5. *Effect of concanavalin A, A23187, prostaglandin E_1 and Ro-20/1724 on the cyclic AMP content of mouse thymocytes*

Mouse thymocytes were washed once by centrifugation (275 g, 5 min, 23°C) and incubated at 4×10^7 cells/ml in RPMI/Tris/bicarbonate medium at 37°C for 1.5 h before addition of reagents: ○, no addition; ●, concanavalin A (50 $\mu\text{g}/\text{ml}$); ◆, 500 nM-A23187; ■, 10 μM -prostaglandin E_1 ; ▲, 10 μM -Ro-20/1724; □, 10 μM -prostaglandin E_1 + 10 μM -Ro-20/1724; △, concanavalin A (50 $\mu\text{g}/\text{ml}$) + 10 μM -Ro-20/1724. The cells were incubated at 37°C , and cyclic AMP was assayed at the times indicated.

that: (i) an early increase in cyclic AMP is not obligatory for the mitogenic stimulation of lymphocytes; (ii) the increase in the free cytoplasmic Ca^{2+} concentration caused by mitogenic concentrations of concanavalin A or A23187 (Tsien *et al.*, 1982; Hesketh *et al.*, 1983) neither depends on, nor induces, an immediate change in the cyclic AMP content of the cells.

J. P. M. held an M.R.C. Research Studentship. This work was supported by a grant from the S.E.R.C. to J. C. M. We thank Dr. A. N. Corps for ATP measurements and Mr. R. W. Plumb for technical assistance.

References

- Best, L. C., Martin, T. J., Russell, R. G. G. & Preston, F. E. (1977) *Nature (London)* **267**, 850–852
- Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. & Tampion, W. (1971) *Biochem. J.* **121**, 561–562
- Coffey, R. G., Hadden, E. M. & Hadden, J. W. (1977) *J. Immunol.* **119**, 1387–1394
- Felber, S. M. & Brand, M. D. (1982) *Biochem. J.* **204**, 577–585

- Hesketh, T. R. (1978) *Int. Rev. Biochem.: Biochemistry of Cell Walls and Membranes II* **19**, 63–91
- Hesketh, T. R., Smith, G. A., Houslay, M. D., Warren, G. B. & Metcalfe, J. C. (1977) *Nature (London)* **267**, 490–494
- Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J. C. (1983) *J. Biol. Chem.* **258**, 4876–4882
- Houslay, M. D. & Palmer, R. W. (1978) *Biochem. J.* **174**, 909–919
- Kobayashi, M., Lust, W. D. & Passonneau, J. V. (1977) *J. Neurochem.* **29**, 53–59
- Kurokawa, T., Kurokawa, M. & Ishibashi, S. (1979) *Biochim. Biophys. Acta* **583**, 467–473
- Lönnroth, I. & Lönnroth, C. (1977) *Exp. Cell Res.* **104**, 15–24
- Metcalfe, J. C., Pozzan, T., Smith, G. A. & Hesketh, T. R. (1980) *Biochem. Soc. Symp.* **45**, 1–26
- Moore, J. P., Smith, G. A., Hesketh, T. R. & Metcalfe, J. C. (1982) *J. Biol. Chem.* **257**, 8183–8189
- Nordeen, S. K. & Young, D. A. (1978) *J. Biol. Chem.* **253**, 1234–1239
- Pozzan, T., Corps, A. N., Montecucco, C., Hesketh, T. R. & Metcalfe, J. C. (1980) *Biochim. Biophys. Acta* **602**, 558–566
- Raff, M. C., Hornby-Smith, A. & Brockes, J. P. (1978) *Nature (London)* **273**, 672–673
- Rozengurt, E., Legg, A., Strang, G. & Courtenay-Luck, N. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4392–4396
- Sheppard, H., Wiggan, G. & Tsien, W. H. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 103–112
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *Nature (London)* **295**, 68–71
- Wang, T., Sheppard, J. R. & Foker, J. E. (1978) *Science* **201**, 155–157
- Watson, J. D. (1976) *J. Immunol.* **117**, 1656–1663
- Zick, Y., Cesla, R. & Shaltiel, S. (1978) *FEBS Lett.* **90**, 239–242
- Zick, Y., Cesla, R. & Shaltiel, S. (1982) *J. Biol. Chem.* **257**, 4253–4259
- Zick, Y., Cesla, R. & Shaltiel, S. (1983) *Biochim. Biophys. Acta* **762**, 355–365