Biochimica et Biophysica Acta 861 (1986) 187–193
Elsevier

BBA 73291

Leucine transport in relation to the activities of Na^+-H^+ antiporter and Na^+/K^+ pump stimulated by serum and a tumor promoter

Yasuhide Mitsumoto and Tetsuro Mohri

Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11 (Japan)

(Received 23 April 1986)

Key words: Leucine transport; cytoplasmic pH; Na^+-H^+ exchange; Na^+/K^+ pump; (Chang liver cell)

Fetal calf serum and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) increased the rate of leucine uptake by Chang liver cells in Na^+-containing medium. Addition of monensin to the incubation medium also increased the leucine uptake. All these agents were capable of raising the cytoplasmic pH, which was blocked by a prior addition of amiloride or removing Na^+ from assay medium, suggesting activation of Na^+-H^+ exchange across the cell membrane by fetal calf serum and TPA. The stimulation of leucine uptake by monensin and fetal calf serum was blocked completely or incompletely by addition of ouabain or amiloride. The basal and fetal-calf-serum- or TPA-stimulated leucine uptake was extensively depressed by the presence of an excess of 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid in the incubation medium. Based on these results it is proposed that the transport of leucine by the system L is stimulated by fetal calf serum and TPA with a high concentration of Na^+ outside the cells as a result of alkalinization of the cytoplasm and coordinated activation of ($Na^+ + K^+$)-ATPase by these stimulators to maintain the transmembrane Na^+ gradient and also hyperpolarize the cell membrane.

Introduction

Leucine and other branched-chain amino acids such as isoleucine, valine and phenylalanine have been elucidated generally to have affinity for the Na^+-independent amino-acid transport system (system L) in the cell membrane of many mammalian cells [1–3] and the transport of leucine has been shown to be driven by protonmotive force in the Chang liver cell [4]. Recently we have demonstrated in this cell [5] that addition of amiloride to a Na^+-containing medium is inhibitory on the uptake of leucine by cells and that acidification of the cytoplasmic pH accompanying the leucine uptake is intensified by either removing Na^+ from or adding amiloride to the medium, suggesting the contribution of an Na^+-H^+ antiport system [6–8] in pumping H^+ out of cells.

On the other hand, the mitogenic action of epidermal growth factor [6], platelet-derived growth factor [7,9], TPA [8,9] and fetal calf serum [7,9] is proposed to involve a common mechanism that effects alkalinization of the cytoplasm, i.e., a stimulation of H^+ discharge from cells by Na^+-H^+ antiport system [10–12]. There is a line of evidence indicating that growth factors stimulate the activity of Na^+/K^+ pump at the cell membrane secondarily to the increase of Na^+ entry into cells

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; FITC-dextran, fluorescein isothiocyanate-dextran; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence address: Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan.

caused by activation of the Na^+-H^+ exchanger [13,14].

We have therefore studied the effect of fetal calf serum, TPA and monensin on the uptake of leucine by the Chang liver cell and propose in this communication as a mechanism of the stimulation by fetal calf serum and TPA of leucine transport a coordinated activation of the two membrane systems of Na^+-H^+ exchanger and ($Na^+ + K^+$)-ATPase to alkalinize the cytoplasm and probably polarize the membrane.

Materials and Methods

Materials

A dried preparation of Eagle's minimum essential medium for monolayer culture was purchased from Nissui Seiyaku Co. Ltd. (Tokyo, Japan). L-[U-^{14}C]Leucine (342 Ci/mol) was purchased from Amersham International (Amersham, U.K.). Fluorescein isothiocyanate-dextran (FITC-dextran, average M_r 70 000), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and monensin were purchased from Sigma (St. Louis, MO, U.S.A.). Fetal calf serum was obtained from Maruzen Oil Co. (Tokyo, Japan). Amiloride was obtained by a courtesy of Merck, Sharp and Dohme. 2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid was purchased from Calbiochem-Behring Corp. (San Diego, U.S.A.). Other reagents used here were of special grade from Wako Pure Chemical Co. (Tokyo, Japan).

Cell culture

Chang liver cells were grown as monolayer culture in culture dishes (35 mm or 60 mm) with a medium composed of Eagle's minimum essential medium, Hanks' balanced salt solution containing 0.4% (v/v) lactalbumin hydrolysate and bovine serum (45 : 45 : 10) under 5% CO_2 in air at 37°C.

Amino-acid transport assay

Cells grown in 35 mm culture dishes as monolayers were washed twice, each time with 2 ml of Hepes-buffered saline (140 mM NaCl/1.5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose/10 mM Hepes (pH 7.4)) and incubated with the same medium for 30 min at 37°C. They were then incubated in the presence or absence of growth-promoting agents (fetal calf serum and TPA), monensin, ouabain or amiloride in Hepes-buffered saline or Hepes-buffered choline medium (made by replacing the NaCl and NaOH of Hepes-buffered saline by choline chloride and KOH, respectively) as specified in Results for 10 min at 37°C. TPA :(10^{-4} M) and monensin (10 mM) were dissolved in ethanol, and amiloride (0.5 M) in dimethylsulfoxide, to make stock solutions. Cells were incubated with Hepes-buffered saline containing 1 mM of a total of L-leucine and ^{14}C-labeled (0.17 μCi/ml) leucine at 37°C for 1 min to determine the leucine uptake. To terminate incubation, the medium was aspirated and the cell layers were rinsed three times with ice-cold phosphate-buffered saline. The dishes were kept on ice until the cells in each were collected with a rubber policeman and transfer pipette into 1.0 ml of 0.2 M NaOH. The mixture was incubated at 70°C for 10 min. The solution was vortexed and 1.0 ml of it was taken, neutralized with 0.5 ml of 0.4 M perchloric acid and subjected to assay of radioactivity using 10 ml of a Triton-toluene scintillator cocktail. When monensin, TPA and amiloride were used in experiments, identical concentrations of vehicle were added to the control. Zero-time values of uptake were obtained by terminating incubation immediately after addition of labeled leucine and treating cells the same way. Protein determination was carried out using 0.1 ml of the solution.

FITC-dextran scrape-loading method and cytoplasmic pH monitoring

Subconfluent cells grown in 60 mm tissue-culture-grade polystyrene dishes were utilized for the scrape-loading method [15] as described below. FITC-dextran (100 mg) dissolved in Eagle's minimum essential medium (10 ml) was divided among 20 of the culture dishes at 37°C. The cells in the dishes were immediately scraped from the plastic surface in the medium with a rubber policeman and collected into a tube by centrifugation at 200 × *g*. FITC-dextran-loaded cells were washed twice with a culture medium consisting of ice-cold minimum essential medium and 10% (v/v) bovine serum, suspended in the same medium and then cultured on the coverglass slides (11 × 22 mm) settled in the bottom of culture dishes for 20 h as monolayers. After terminating the culture by wash

with either Hepes-buffered saline or Hepes-buffered choline medium, one of the glass slides from each culture dish was inserted into the thermostatted sample cuvette together with the medium specified in Results and its fluorescence intensity was measured with a Hitachi MPF-4 fluorescence spectrophotometer. Cytoplasmic pH was monitored by the fluorescence intensity obtained with excitation at 495 nm and emission at 520 nm, slit 5 × 5 nm [16].

Protein determination

Cellular protein was determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

Results

Na^+ requirement in the cytoplasmic pH change induced by fetal calf serum and TPA

Fig. 1 shows alkalinization of the cytoplasm of Chang liver cells after addition of fetal calf serum or TPA to the medium and the requirement of extracellular Na^+ for this effect. The pH-dependence profile of the fluorescence intensity of intracellularly trapped FITC-dextran was found to be sigmoidal, showing a nearly proportional relation between pH 6.0 and 7.4 (data not shown), very similar to that reported previously [16]. The cytoplasmic pH increased gradually after addition of the stimulators, attaining a plateau within 10 min, in Hepes-buffered saline. This stimulation effect was completely cancelled by removal of Na^+ from the medium or addition of 1 mM amiloride to Hepes-buffered saline prior to either fetal calf serum or TPA. Amiloride was used as a specific inhibitor of the Na^+-H^+ antiporter in the cell membrane. These results suggest that both fetal calf serum and TPA alkalinize the cytoplasm by stimulation of the Na^+-H^+ exchange system in the plasma membrane. Activation of Na^+-H^+ exchange by phorbol ester would result in cell swelling, but this welling can be considerably diminished in nominally HCO_3^--free medium [20]. Therefore, we used nominally HCO_3^--free medium (Hepes-buffered saline or Hepes-buffered choline medium) in the experiments for leucine uptake and fluorescence assay.

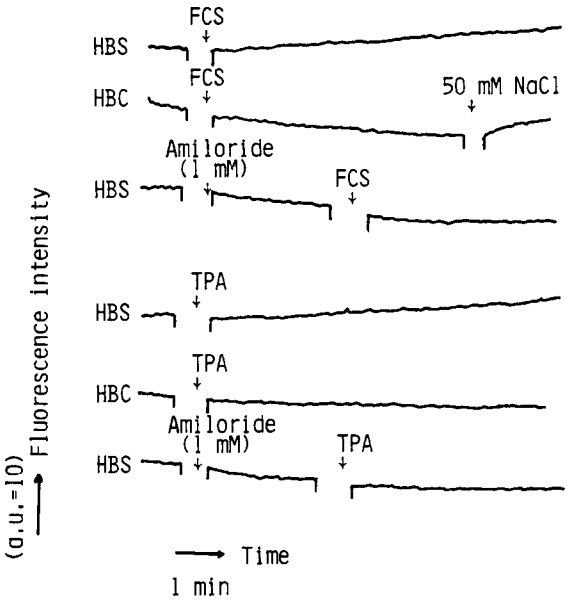

Fig. 1. Cytoplasmic pH changes induced by fetal calf serum and TPA. Cells were loaded with the fluorescent pH indicator FITC-dextran using the scrape-loading method and the fluorescence was recorded as described in Materials and Methods. The fluorescence trace of typical experiments are depicted before and after addition of 5% (v/v) fetal calf serum (FCS) or $1 \cdot 10^{-6}$ M TPA at the arrows. HBS, Hepes-buffered saline; HBC, Hepes-buffered choline.

Na^+ requirement in the stimulation of leucine uptake by fetal calf serum and TPA

Table I shows the effect of 10 min pretreatment of cells with fetal calf serum or TPA in Hepes-

TABLE I

EFFECT OF FETAL CALF SERUM AND TPA PRETREATMENT ON LEUCINE UPTAKE IN THE PRESENCE OR ABSENCE OF Na^+

Cells were incubated with addition of 5% (v/v) fetal calf serum, $1 \cdot 10^{-6}$ M TPA or neither of them to Hepes-buffered saline (A) or Hepes-buffered choline medium (B). After 10 min of incubation, 1 min uptake of leucine (1 mM) was determined in Hepes-buffered saline. Each value represents the mean ± S.E. ($n = 4$).

Condition during pretreatment	Leucine uptake (nmol/mg protein per min)		
	basal	fetal calf serum	TPA
A	15.55 ± 1.08	24.23 ± 0.89	18.53 ± 0.82
B	16.43 ± 1.31	16.02 ± 0.70	15.34 ± 0.60

buffered saline or Hepes-buffered choline medium on 1 min uptake of leucine (1 mM) in the presence of Na^+. Preincubation with fetal calf serum (5%) and TPA ($1 \cdot 10^{-6}$ M) induced 56 and 20% stimulation of the leucine uptake, respectively, when the preincubation was performed in the presence of 140 mM NaCl or Hepes-buffered saline. Isoosmotic replacement of Na^+ of Hepes-buffered saline by choline in the pretreatment with fetal calf serum and with TPA cancelled the stimulation of the subsequent uptake by the cells.

The ineffectiveness of using Hepes-buffered saline in the incubation on the action of these stimulators after the treatment in Hepes-buffered choline medium is interpreted as an indication that the incubation period is insufficient for recovery of the cytoplasmic pH or alkalinizing it over the basal level (cf. Fig. 1).

This result indicates the essentiality of Na^+ in the medium and alkalinization of the cytoplasm to the stimulation of leucine uptake by fetal calf serum and TPA.

Inhibitory effect of amiloride and ouabain on the fetal-calf-serum-stimulated leucine uptake

Table II shows the effect of amiloride (2.5 mM) and of ouabain (0.13 mM) added to the preincubation medium on the basal and fetal-calf-serum-stimulated leucine uptakes in Hepes-buffered saline. The stimulated uptake with fetal calf serum was largely retarded by the addition of amiloride as well as ouabain. But the inhibitions of the fetal calf serum effect by these specific inhibitors on the Na^+-H^+ antiporter and ($Na^+ + K^+$)-ATPase were all incomplete, i.e., the leucine uptake after treatment with fetal calf serum was still significantly above the control value, even upon addition of the inhibitors. These two inhibitors, when used simultaneously in the pretreatment, were not additive in their inhibitory effect on the stimulation (data not shown). Ouabain was stimulatory on the basal uptake of leucine, although no explanation can be afforded so far of this phenomenon.

TABLE II

EFFECT OF AMILORIDE AND OUABAIN ON BASAL AND FETAL-CALF-SERUM-STIMULATED LEUCINE UPTAKE

Cells were preincubated in the presence or absence of 5% (v/v) fetal calf serum with 2.5 mM amiloride, 0.13 mM ouabain or neither of them in Hepes-buffered saline for 10 min. 1 min uptake of 1 mM leucine was determined in Hepes-buffered saline. Each value represents the mean ± S.E. ($n = 4$).

Condition during pretreatment	Leucine uptake (nmol/mg protein per min)		
	–	amiloride	ouabain
–	7.28 ± 1.13	7.11 ± 0.29	10.13 ± 0.34
Fetal calf serum	19.61 ± 0.76	13.69 ± 0.85	13.00 ± 0.83

Effects of monensin and ouabain on leucine uptake

When monensin (10 μM) was added to preincubation medium (Hepes-buffered saline) leucine uptake was stimulated by more than 50%, and the addition of ouabain (0.25 mM) to the preincubation medium blocked completely the stimulatory effect of monensin. Monensin is an ionophore having activity in the cell membrane to exchange

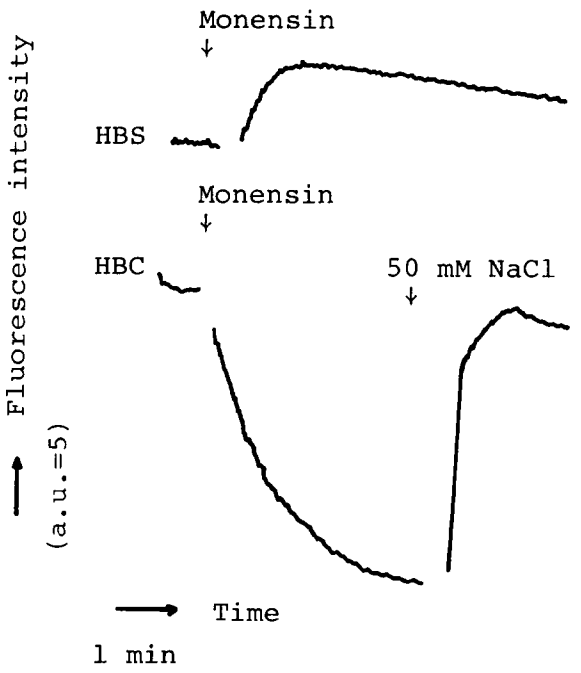

Fig. 2. Effects of monensin on the cytoplasmic pH. The fluorescence of FITC-dextran trapped in the cytoplasm as in Fig. 1 was recorded on addition of 10 μM monensin in Hepes-buffered saline (HBS) or Hepes-buffered choline (HBC). The data shown are specimens of at least triplicate samples.

electroneutrally H^+ for Na^+ along the electrochemical gradients of these ions across the membrane. The action of monensin on the cytoplasmic pH is shown in Fig. 2 as expressed by the change of fluorescence intensity of trapped FITC-dextran. Addition of monensin to Hepes-buffered saline medium promptly alkalinizes the cytoplasm, while it acidifies the cytoplasm when examined in Hepes-buffered choline medium, capable of reversing the pH on rendering the Na^+ gradient inward by a supply of NaCl.

It can be stated that an Na^+-H^+ exchanger like monensin stimulates leucine uptake by cells, provided an inward Na^+ gradient is maintained across the cell membrane, and $(Na^+ + K^+)$-ATPase is important in simultaneous facilitation of Na^+ recycling and polarization at the membrane.

Inhibition by 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid of the stimulated leucine uptake

2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid is a model substrate specific for the system L of amino-acid transport in mammalian cells [18]. Table III shows the inhibitory effect of the presence of 5 mM 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid in the incubation medium (Hepes-buffered saline) on the fetal-calf-serum- and TPA-stimulated leucine uptake. The data shown in the table indicate that a large fraction of the leucine uptake susceptible to inhibition by 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid is specifically activated by fetal calf serum and TPA, although some 20% or less of the minor, non-inhibited fraction is also stimulated by these activators.

TABLE III

INHIBITION BY 2-AMINOBICYCLO[2.2.1]HEPTANE-2-CARBOXYLIC ACID OF THE FETAL-CALF-SERUM- AND TPA-STIMULATED LEUCINE UPTAKE IN Na^+-CONTAINING MEDIUM

Cells were preincubated in the presence of 5% (v/v) fetal calf serum, $1 \cdot 10^{-6}$ M TPA or neither of them in Hepes-buffered saline for 10 min. 1 min uptake of 1 mM leucine was determined in Hepes-buffered saline with (B) or without (A) 5 mM 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid. Each value represents the mean ± S.E. ($n = 4$)

Condition during incubation	Leucine uptake (nmol/mg protein per min)		
	–	fetal calf serum	TPA
A	6.23 ± 0.17	12.97 ± 0.32	10.11 ± 0.57
B	1.54 ± 0.17	2.78 ± 0.08	2.31 ± 0.28

Discussion

The fraction of leucine transport which can be greatly inhibited by a high concentration of 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid in Chang liver cell was greatly increased by addition of fetal calf serum or TPA to Na^+-containing incubation medium (Table III). That fraction of leucine transport is assumed to correspond to the Na^+-independent amino-acid transport operated by the system L in Ehrlich ascites tumor cells [1] and has been shown to be driven by a protonmotive force [4].

The occurrence of amiloride-sensitive Na^+-H^+ exchange system in Chang liver cell has been suggested by our previous work [5]. Therefore, extracellular Na^+-dependent alkalinization induced by fetal calf serum or TPA (Fig. 1) of the cytoplasm of this cell would result from activation of the Na^+-H^+ exchange system as suggested by Moolenaar et al. [7] in human fibroblast cells.

Consequently, we believe that alkalinization of the cytoplasm, potentially reversing the basal proton gradient across the cell membrane (cf. Table I), is a rationale of the enhancement of the Na^+-independent, proton-coupled leucine uptake by fetal calf serum and TPA in the presence of Na^+ in medium. A large inhibition by amiloride of the effect of fetal calf serum on leucine uptake (Table II) and the stimulatory effect of monensin, which mimics the electroneutral Na^+-H^+ exchange system in the cell membrane and alkalinizes effectively the interior of cells with a high concentration of Na^+ in the exterior, on the uptake in Hepes-buffered saline support the participation of the Na^+-H^+ antiport system in the stimulation of leucine transport by fetal calf serum and TPA.

It has been reported by Rozengurt and his colleagues [8,14,19] and Grinstein et al. [20] in mammalian cells that mitogens including fetal calf serum and cell growth factors such as platelet derived growth factor stimulate the $(Na^+ + K^+)$-ATPase activity in response to activation of the Na^+-H^+ exchange system, i.e., discharge of pro-

tons from cells and efflux of Na^+ are simultaneously accelerated. And TPA has been shown to hyperpolarize mammalian cells in such a manner as to be sensitive to ouabain and amiloride [20,21].

The necessity of activating ($Na^+ + K^+$)-ATPase for the stimulatory effect of fetal calf serum on leucine uptake has been shown in the present experiment (Table II). Therefore it is reasonable to infer that hyperpolarization of the cell membrane resulting from activation of the ($Na^+ + K^+$)-ATPase additionally contributes to the stimulation of leucine uptake by fetal calf serum and TPA. A complete inhibition of the stimulatory effect of monensin on leucine uptake upon addition of ouabain is considered to support this notion, because monensin was also found to hyperpolarize a neuroblastoma hybrid cell [22].

The actions of ouabain and amiloride on the stimulation by fetal calf serum were incomplete (Table II) and could not be further reinforced even by a simultaneous addition of these two inhibitors. Fetal calf serum is supposed at present to have some additional action(s) on the cell to promote leucine transport. One of possible mechanisms for the extra action(s) of fetal calf serum is the increase of intracellular Ca^{2+} concentration [10]. Our colleagues have found in the cell that an increase in intracellular free Ca^{2+} induces stimulation of leucine uptake [23].

Petronini et al. [24] have shown a stimulatory effect of intracellular Na^+ on phenylalanine uptake. Any substantial accumulation of Na^+ was, however, scarcely detected in this cell after treatment with serum, TPA and monensin without addition of amiloride or ouabain (data not shown). We think the increase of intracellular Na^+ concentration could be counteracted by stimulation of Na^+ pumping out of cells due to a quick reaction of ($Na^+ + K^+$)-ATPase, because the Na^+/K^+ pump is highly sensitive to a small change in cellular Na^+ [19].

Most of essential amino acids, histidine and tyrosine share their transport system with L-leucine in mammalian cells [2,25]. And activation of ($Na^+ + K^+$)-ATPase has been reported to accelerate the uptake by fibroblasts [26,27] and hepatocytes [28] of α-aminoisobutyric acid, which is often used as a model compound for the type A transport of neutral amino acids such as alanine, glycine, proline and serine. Therefore it would be convincing that fetal calf serum and TPA are capable of promoting the uptake of many amino acids in mammalian cells, providing the cells with amino acids sufficient to support their stimulated growth.

References

1 Oxender, D.L. and Christensen, H.N. (1963) J. Biol. Chem. 238, 3689–3699
2 Shotwell, M.A., Kilberg, M.S. and Oxender, D.L. (1983) Biochim. Biophys. Acta 737, 267–284
3 Mohri, T., Yamaguchi, T. and Kitagawa, H. (1979) J. Biochem. (Tokyo) 85, 681–689
4 Mohri, T., Mitsumoto, Y. and Ohyashiki, T. (1983) Biochem. Int. 7, 159–167
5 Mitsumoto, Y., Sato, K., Ohyashiki, T. and Mohri, T. (1986) J. Biol. Chem. 261, 4549–4554
6 Rothenberg, P., Glasser, L., Schlesinger, P. and Cassel, D. (1983) J. Biol. Chem. 258, 12644–12653
7 Moolenaar, W.H., Tsien, R.Y., Van der Saag, P.T. and De Laat, S.W. (1983) Nature (Lond.) 304, 645–648
8 Burns, P.C. and Rozengurt, E. (1983) Biochem. Biophys. Res. Commun. 116, 931–938
9 Moolenaar, W.H., Tertoolen, L.G.J. and De Laat, S.W. (1984) Nature (Lond.) 312, 371–373
10 Moolenaar, W.H., Joosen, L. and De Laat, S.W. (1984) Peptide Hormones, Biomembrane and Cell Growth (Bolis, L. and Verna, R., eds.), pp. 215–231 Plenum Press, New York
11 L'Allemain, G., Paris, S. and Pouyssegur, J. (1984) J. Biol. Chem. 259, 5809–5815
12 Schuldiner, S. and Rozengurt, E. (1982) Proc. Natl. Acad. Sci. USA 79, 7778–7782
13 Moolenaar, W.H., Mummery, C.L., van der Saag, P.T. and De Laat, S.W. (1981) Cell 23, 789–798
14 Dicker, P. and Rozengurt, E. (1981) Biochem. Biophys. Res. Commun. 100, 433–441
15 McNeil, P.L., Murphy, R.F., Lanni, F. and Taylor, D.L. (1984) J. Cell Biol. 98, 1556–1564
16 Ohkuma, S., Moriyama, Y. and Takano, T. (1982) Proc. Natl. Acad. Sci. USA 79, 2785–2762
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Christensen, H.N. (1979) Adv. Enzymol. Relat. Mol. Biol. 49, 41–101
19 Smith, J.B. and Rozengurt, E. (1979) Proc. Natl. Acad. Sci. USA 75, 5560–5564
20 Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. and Gelfand, E.W. (1985) Proc. Natl. Acad. Sci. USA 82, 1429–1443
21 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature (Lond.) 295, 68–70
22 Lichtshtein, D., Dunlop, K., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 2580–2584
23 Takadera, T. and Mohri, T. (1985) Cell Struct. Funct. 10, 349–359

24 Petronini, P.G., Gandolfi, S.A. and Borghetti, A.F. (1985) Biochim. Biophys. Acta 815, 361–368
25 Kilberg, M.S. (1982) J. Membrane Biol. 69, 1–12
26 Smith, J.B. and Austic, R.E. (1980) Biochem. Biophys. Res. Commun. 93, 392–398
27 Villereal, M.L. and Cook, J.S. (1978) J. Biol. Chem. 253, 8257–8262
28 Wondergem, R. and Harder, D.R. (1980) J. Cell. Physiol. 104, 53–60