Extracellular Ca^{2+} stimulates the activation of mitogen-activated protein kinase and cell growth in human fibroblasts

Shixia HUANG, Veronica M. MAHER and J. Justin McCORMICK*

Carcinogenesis Laboratory, Fee Hall, Department of Microbiology and Department of Biochemistry, the Cancer Center, Michigan State University, East Lansing, MI 48824, U.S.A.

In serum-free medium containing serum replacements but totally lacking in protein growth factors, diploid human fibroblasts remained quiescent if the extracellular Ca^{2+} concentration was only 0.1 mM. However, when the Ca^{2+} concentration in this medium was increased to 1 mM, the cells replicated as rapidly as they do in medium supplemented with protein growth factors. When quiescent cells in medium with only 0.1 mM Ca^{2+} were exposed to 1 or 10 mM Ca^{2+} or 100 ng/ml epidermal growth factor (EGF), the 42 kDa and 44 kDa forms of mitogen-activated protein kinase (MAPK) were rapidly activated, as demonstrated by a characteristic electrophoretic mobility shift of these proteins and by their enhanced ability to phosphorylate myelin basic protein (MBP). Analysis of fractions from Mono Q anion-exchange chromatography of lysates of cells exposed to 10 mM Ca^{2+} or 100 ng/ml EGF revealed a peak of MBP phosphorylation activity that was coeluted with p42 and p44 MAPK as shown by immunoblot analysis. Activation of MAPK by extracellular Ca^{2+} was dose-dependent and biphasic, with a peak of activation at 5–10 min after exposure, followed by a period of sustained activation of MAPK at a lower level. This pattern has been shown [Vouret-Craviari, Van Obberghen-Schilling, Scimeca, Van Obberghen and Pouysségur (1993) Biochem J. **289**, 209–214] to correlate with the re-entry of mammalian cells into the cell cycle.

INTRODUCTION

It is known that human fibroblasts in culture will replicate in medium containing specific protein growth factors [1], and that Ca^{2+} is an important factor in cell metabolism [2]. Studies show that such protein growth factors as epidermal growth factor (EGF), platelet-derived growth factor, insulin, thrombin, nerve growth factor, tumour necrosis factor and bombesin can initiate a phosphorylation cascade in various cell types, including human fibroblasts, and that one of the key events of this cascade is the activation of mitogen-activated protein kinase (MAPK) by phosphorylation [3–9]. Several closely related MAPK isoforms, referred to by their molecular masses, i.e. p40, p42, p44 and p54 [10], have been identified in various cell types. In human fibroblasts, EGF [5], tumour necrosis factor [7,11] and interleukin [11] have been shown to cause activation of p42 and/or p44 MAPKs. Activated MAPK can, in turn, phosphorylate myelin basic protein (MBP), microtubule-associated protein 2, the EGF receptor, phospholipase A_2 and the nuclear transcriptional factors c-*myc*, c-*fos* and c-*jun* [10,12,13].

Morgan et al. [14] in this laboratory showed that diploid human fibroblasts can replicate rapidly in serum-free McM medium [15], a modified version of MCDB 110 [1], if supplied with the serum replacements, e.g. lipids, iron, attachment factors, etc., specified by Ryan et al. [15] and with insulin as the only protein growth factor, instead of the two growth factors specified by Ryan et al. [15], e.g. insulin and EGF. McM medium, as ordinarily prepared [15], contains 1 mM Ca^{2+}. Morgan et al. [14] also showed that the cells do not replicate if the Ca^{2+} concentration is reduced from 1 mM to 0.1 mM, suggesting that extracellular Ca^{2+} stimulates cell growth. We tested this hypothesis by removing all protein growth factors from the medium and seeing if Ca^{2+} alone could cause the cells to replicate for an extended period. Since MAPK is known to integrate the signals from various growth factor pathways, we also tested whether Ca^{2+} causes the activation of MAPK in these cells as protein growth factors do. The answer to both questions was positive. In the total absence of protein growth factors, 1.0 mM extracellular Ca^{2+} supported growth of the cells during the 2 weeks of the experiment. When quiescent cells were stimulated with Ca^{2+} or EGF, MAPK activation was observed as demonstrated by a characteristic shift in the electrophoretic mobility of MAPK and an enhanced ability of MAPK to phosphorylate MBP.

MATERIALS AND METHODS

Materials

MBP, EGF and β-glycerophosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Polyclonal anti-MAPK antibody Ab283 (from rabbit) was kindly provided by Dr. Marsha R. Rosner of the University of Chicago. Monoclonal anti-MAPK antibodies were purchased from Chemicon (Temecula, CA, U.S.A.) and Zymed Laboratories (South San Francisco, CA, U.S.A.). Horseradish peroxidase (HRP)-linked goat anti-mouse IgG was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and HRP-conjugated goat anti-rabbit IgG was from Boehringer-Mannheim Corp. (Indianapolis, IN, U.S.A.).

Cells and cell culture

Diploid human fibroblast cell lines LG1 and SL80, derived in this laboratory from neonatal foreskins, were used at cell population doubling 15 to 28. The *in vitro* life span of LG1 cells is 40 population doublings, and that of SL80 cells is 80 population doublings. Cells were routinely grown in Eagle's minimum

Abbreviations used: EGF, epidermal growth factor; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; SCS, supplemented calf serum.

* To whom correspondence should be addressed.

essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, 0.2 mM serine, 10% supplemented calf serum (SCS) (Hyclone Laboratories, Logan, UT, U.S.A.), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml) (complete medium) at 37 °C in a humidified incubator with 5% CO_2.

Media for testing for growth stimulation

To assay for growth stimulation we used McM medium [15], a modified version of MCDB 110 [1] which was developed for the continuous proliferation of human fibroblasts. We used the serum replacements specified by Ryan et al. [15] but omitting the protein growth factors, i.e. EGF and insulin. The medium was prepared to contain 0.1 mM or 1 mM Ca^{2+} as indicated.

Preparation of cell lysates

Cells were grown in 100 mm-diameter dishes in complete medium until subconfluent or confluent as desired. To make the cells quiescent, the medium was replaced for 24–30 h with McM medium containing serum replacements but no protein growth factors and with the Ca^{2+} concentration reduced to 0.1 mM or to zero where indicated. Unless otherwise indicated, increased Ca^{2+} or EGF was then added directly to the medium on the cells with a gentle swirling, and the incubation at 37 °C was continued for the indicated time. To harvest the cells, the medium was removed, the cell sheet was quickly washed twice with cold Ca^{2+}-free Mg^{2+}-free PBS, and the cells were lysed in 0.3–0.5 ml of buffer composed of 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P40, 10% glycerol, 1 mM PMSF, 5 mM EDTA, 0.15 units/ml aprotinin and 1 mM sodium orthovanadate. The cells were collected with a rubber scraper and kept on ice for 20 min. The lysates were centrifuged for 10 min at 4 °C in a Microfuge, and the protein concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA as the protein standard. The cell lysates were used immediately after preparation or stored at −80 °C until used.

Immunoblot analysis of MAPK

Cell lysates containing 25 μg of protein were dissolved in buffer containing 0.05 M Tris/HCl, pH 6.9, 0.72 M 2-mercaptoethanol, 9% glycerol, 2.3% SDS and 0.1% Bromophenol Blue (sample buffer), and the proteins were separated by SDS/PAGE (10% gels). They were then transferred from the gels on to Immobilon poly(vinylidene difluoride) transfer membranes (Millipore Corp., Bedford, MA, U.S.A.) overnight at 35 mA, and the blots blocked by incubation for 1 h at room temperature in Tris-buffered saline, pH 7.6 (20 mM Tris/HCl, 137 mM NaCl) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk. To test for MAPK, the membranes were incubated for 1 h with the indicated anti-MAPK antibodies in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk, washed several times in this Tris-buffered saline, and then incubated with goat anti-rabbit IgG or goat anti-mouse IgG as desired. Enhanced chemiluminescence (Amersham, Arlington Heights, IL, U.S.A.) was used as a detection system according to the manufacturer's instructions.

MBP phosphorylation as an assay for MAPK activity

This was assayed essentially as described previously [16]. Briefly, cell lysates containing 2.5 μg of protein were incubated with MBP at 0.5 mg/ml for 10 min at room temperature in a final volume of 20 μl containing 18 mM Hepes, pH 7.4, 10 mM magnesium acetate and 2 μCi of [γ-^{32}P]ATP (2–10 Ci/mmol) (DuPont, New England Nuclear Research Products, Boston, MA, U.S.A.). The reaction was stopped by the addition of 5 × sample buffer. After 5 min at 95 °C, proteins were separated on a 10% polyacrylamide gel. The dried gel was used to expose Kodak X-Omat film and a phosphor screen. The phosphor screen was scanned and the amount of MBP phosphorylation was quantified using a PhosphorImager (model 400B; Molecular Dynamics, Sunnyvale, CA, U.S.A.) according to the manufacturer's instructions.

Purification of MAPK by Mono Q column chromatography

Cell lysates from Ca^{2+}-stimulated or EGF-stimulated cells or untreated cells were prepared and normalized for protein concentration. MAPK was purified by Mono Q anion-exchange chromatography as described [17]. Before purification, lysates were thawed and centrifuged for 5 min in a Microfuge, and the supernatant containing 1.5–2 mg of protein was diluted 2-fold in column buffer containing 50 mM 2-glycerophosphate, pH 7.2, 100 μM sodium orthovanadate, 1 mM EGTA and 1 mM dithiothreitol. The diluted sample was filtered through a 0.2 μm filter and loaded on to a Mono Q HR5/5 FPLC column (Pharmacia, Piscataway, NJ, U.S.A.) (1.5 ml bed volume) at a flow rate of 0.4 ml/min. Using a linear gradient of 0–0.4 M NaCl for elution, 1 ml fractions were collected. Aliquots of 10.5 μl were assayed for MAPK activity using the MBP phosphorylation assay, and quantified using a PhosphorImager. Aliquots of 110 μl were assayed by immunoblot analysis, as described above.

RESULTS AND DISCUSSION

Evidence that extracellular Ca^{2+} stimulates cell growth

To determine if extracellular Ca^{2+} could stimulate diploid human fibroblasts to replicate in the total absence of protein growth factors, we plated LG1 and SL80 cells in McM medium containing 0.1 mM Ca^{2+} and 1% SCS. The next day, the number of attached cells was determined, and the medium was replaced with McM medium containing the serum replacements of Ryan et al. [15] but minus the protein growth factors. The rate of replication of the cells in this medium supplemented with Ca^{2+}, EGF or 10% SCS is shown in Figure 1. Under the most stringent condition (0.1 mM Ca^{2+}) the number of cells increased only slightly or at most doubled. In the presence of 1 mM Ca^{2+}, the LG1 cells doubled every 48 h, and the SL80 cells every 36 h. This rate of growth was as fast as or faster than that in medium containing EGF (10 ng/ml), and in the case of SL80 cells, almost as fast as that with 10% SCS. Higher concentrations of EGF did not increase the growth rate. These results indicate that 1 mM Ca^{2+} stimulates human fibroblasts to proliferate in the same way as they do in response to protein growth factors.

MAPK activation as a result of Ca^{2+} or EGF stimulation

To determine whether MAPK is involved in activation of the signal pathway induced by extracellular Ca^{2+} in human fibroblasts, we treated LG1 and SL80 cells for 5 min with 1 mM Ca^{2+}, 10 mM Ca^{2+} or 100 ng/ml EGF, and assayed the cell lysates for evidence of MAPK activation using immunoblot analysis. Protein growth factors are typically used at approx. 10-fold higher concentrations in MAPK assays than in growth studies. We used anti-MAPK polyclonal antibody Ab283 which was previously shown to detect both the 42 and 44 kDa isoforms of MAPK [5]. With the cell lysates prepared from untreated cells, this antibody

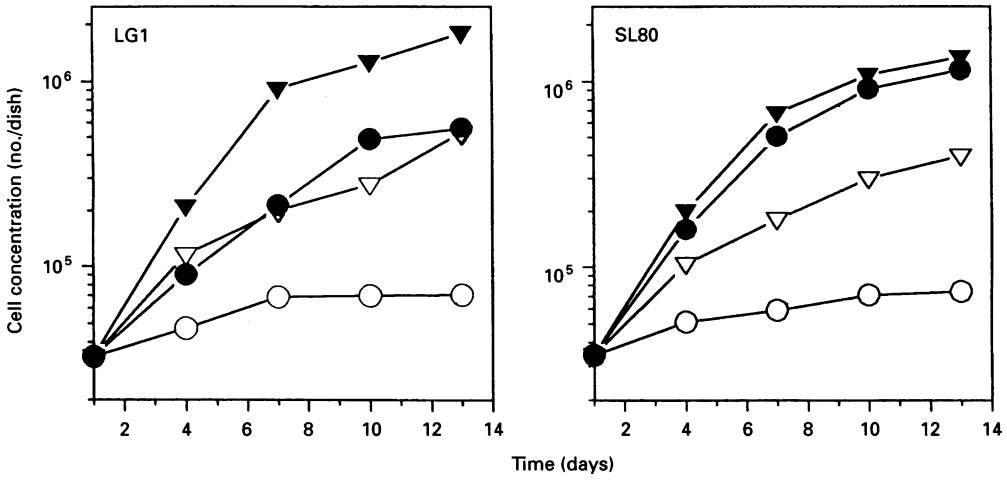

Figure 1 Growth-stimulating activity of Ca^{2+}

Cells were plated into 60 mm-diameter dishes in McM medium containing 0.1 mM Ca^{2+} and 1% SCS. The next day (day 1), the number of attached cells was determined, the medium was replaced with McM medium containing the serum replacements of Ryan et al. [15] minus insulin and EGF, but modified as follows: 0.1 mM Ca^{2+} (○); 1 mM Ca^{2+} (●); 0.1 mM Ca^{2+} with 10 ng/ml EGF (▽); 0.1 mM Ca^{2+} with 10% SCS (▼). The cells were re-fed with appropriate medium on days 4, 7 and 10, and the number of cells in four dishes each time for each condition was determined on days 4, 7, 10 and 13. Similar results were obtained in two separate experiments.

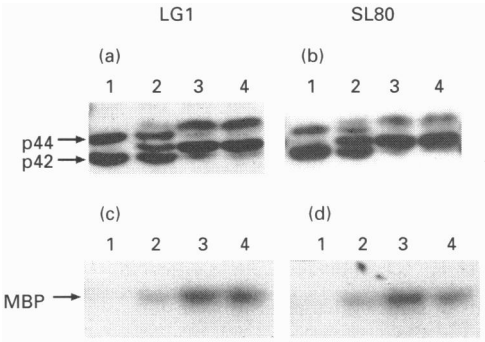

Figure 2 MAPK activation as a result of Ca^{2+} or EGF stimulation

Cells were grown to confluence in Eagle's medium with 10% SCS. The medium was changed to McM medium (containing 0.1 mM Ca^{2+}) with the serum replacements of Ryan et al. [15] but no protein growth factors. After 24 h, Ca^{2+} or EGF was added directly to the medium at the indicated concentrations, and 5 min later, cell lysates were prepared. (**a**, **b**) Immunoblot analysis of cell lysates. Proteins in cell lysates were separated by SDS/PAGE, and subjected to immunoblot analysis probed with anti-MAPK antiserum Ab283. (**c**, **d**) Phosphorylation of MBP by cell lysates. Samples of cell lysates were mixed with MBP and [γ-^{32}P]ATP for 10 min, and assayed as described. Lane 1, control, i.e. 0.1 mM Ca^{2+}; lane 2, 1 mM Ca^{2+}; lane 3, 10 mM Ca^{2+}; lane 4, 100 ng/ml EGF. The results shown are representative of three independently performed experiments.

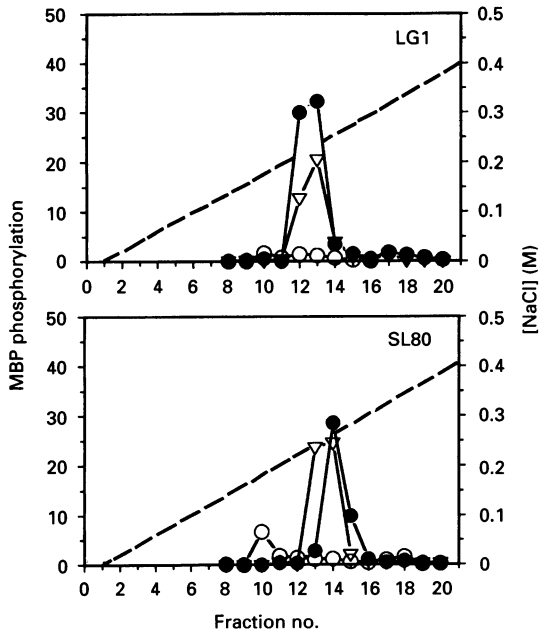

Figure 3 Assay of Mono Q fractions for ability to phosphorylate MBP

Cell lysates were prepared as described from cells exposed to 10 mM Ca^{2+} (●) or 100 ng/ml EGF (▽) for 5 min or left untreated (○). Lysate protein (1.5–2 mg) was diluted with column buffer and loaded on to a Mono Q column. The protein was eluted with a linear NaCl gradient, and 1 ml fractions were collected. Aliquots of fraction 8–20 were assayed for MBP phosphorylation. This was quantified using the PhosphorImager. The data are plotted as the sum of the pixel values in each band minus the background. The salt gradient is denoted by the broken line. Similar results were obtained in two experiments.

revealed two bands with molecular masses of approx. 42 and 44 kDa (lane 1 of Figures 2a and 2b). With cell lysates prepared from cells exposed for 5 min to 1 mM or 10 mM Ca^{2+} or to 100 ng/ml EGF (lanes 2, 3 and 4 of Figures 2a and 2b), slower moving bands were observed, one just above the 42 kDa band and one just above the 44 kDa band. This molecular-mass shift is characteristic of the phosphorylated form of the MAPK isoforms [18,19]. The percentage of each MAPK isoform that travelled more slowly was greater with lysate from cells exposed to 10 mM Ca^{2+} or 100 ng/ml EGF than from cells exposed to 1 mM Ca^{2+}. We also stripped the membrane using 62.5 mM Tris/HCl, pH 6.9, containing 2% SDS and 0.1 M 2-mercapto-ethanol for 30 min at 50 °C and reprobed it with monoclonal antibodies that recognize only the 42 kDa MAPK in human cell lines. The results indicated that the lower bands are the p42 form of MAPK (results not shown).

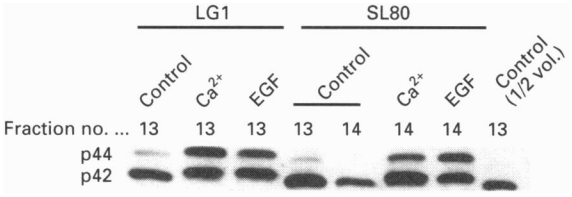

Figure 4 Immunoblot analysis of Mono Q fractions

Mono Q fractions were obtained as shown in Figure 3. Aliquots of the designated fractions were subjected to immunoblot analysis and probed as described with anti-MAPK antibody. The results shown are representative of three experiments.

To determine whether this phosphorylation of MAPK resulted in a molecule with kinase activity, the cell lysates were also tested as described for their ability to phosphorylate MBP. As shown in Figures 2(c) and 2(d), EGF increased the degree of MBP phosphorylation. The degree of MBP phosphorylation produced by lysates from cells stimulated with 10 mM Ca^{2+} was much greater than that from cells given 1 mM Ca^{2+}, and approximately equal to that from the cells treated with 100 ng/ml EGF.

The activation of MAPK by Ca^{2+} or 100 ng/ml EGF was also evaluated on Mono Q column-purified MAPK from the cell lysates. The lysates from untreated cells or from cells exposed to 10 mM Ca^{2+} or EGF were fractionated by chromatography, and aliquots of fractions 8–20 were assayed for the ability to phosphorylate MBP (Figure 3). For LG1 cells, stimulated with either Ca^{2+} or EGF, high activity was observed in fractions 12 and 13, but not in the corresponding fractions from untreated cells. For SL80 cells, high activity was found in fractions 13 and 14 with EGF, and fraction 14 with Ca^{2+}. The presence of MAPK in those specific fractions and its absence from other nearby fractions was demonstrated by immunoblotting and probing with the polyclonal anti-MAPK antibody that recognizes both isoforms (Figure 4). The two forms were coeluted. Note that in the fractions from the Ca^{2+}-stimulated or EGF-stimulated cells, there was a shift in the electrophoretic mobility of the MAPK isoforms. Coelution of these two isoforms on Mono Q column chromatography has been previously reported [20] in a study using a similar volume of eluent. When a much larger volume of the eluent was used, the 42 kDa and 44 kDa isoforms showed a better separation, although some fractions still contained both isoforms [21]. As shown in Figure 3, the control sample from SL80 cells showed a small peak of phosphorylated MBP in fraction 10. However, the immunoblot analysis showed no detectable MAPK in that fraction (results not shown), and use of a second assay for MAPK activity (microtube-associated protein 2 phosphorylation) also showed no such activity in this fraction (results not shown).

To test whether albumin or other factors used as the serum replacements played a role in the activation of MAPK, we also assayed for Ca^{2+}-induced MAPK activation in confluent cells that had been incubated overnight in McM medium containing 0.1 mM Ca^{2+}, but no serum replacements, as well as in confluent cells similarly incubated overnight but then incubated for two additional hours in PBS. Under both circumstances, when 10 mM Ca^{2+} was added to the solution on the cells, MAPK activation was observed (results not shown).

To determine if there was a dose–response relationship for Ca^{2+}-induced MAPK activation, quiescent LG1 and SL80 cells in McM medium lacking Ca^{2+} and supplemented with serum replacements, but without protein growth factors, were exposed for 5 min to various concentrations of Ca^{2+}. Cell lysates were prepared and analysed for their ability to phosphorylate MBP. A dose–response relationship was observed with both cell lines (Figure 5). At high concentrations of Ca^{2+}, MAPK activation would be expected to exhibit saturation; however, at concentrations of 50 mM Ca^{2+} or higher, a precipitate formed, making it impossible to extend the curve.

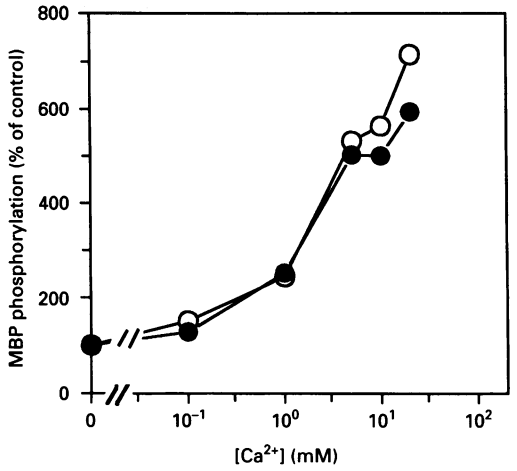

Figure 5 Dose–response relationship of Ca^{2+}-stimulated MAPK activity

Cells (○, LG1; ●, SL80) were grown to confluence and the medium was changed to McM medium with the serum replacements of Ryan et al. [15] but no protein growth factors, and modified to be without Ca^{2+}. After 24 h, the indicated concentration of Ca^{2+} was applied. After 5 min, cell lysates were prepared and assayed for MBP phosphorylation. Similar results were obtained in two separate experiments.

Time course for Ca^{2+}-induced MAPK activation

Pouysségur and his colleagues showed that in quiescent Chinese hamster fibroblasts (cell line CCL39), MAPK is activated by thrombin or basic fibroblast growth factor and that this activation exhibits a biphasic pattern, i.e. a rapid increase in activity followed by a rapid decrease and then a sustained level of lower activity [22,23]. They proposed that this sustained activity is required for the re-entry of these cells from G_0 into the cell cycle [23], i.e. for triggering the proliferative response, because, when they treated CCL39 cells with a thrombin analogue instead of thrombin or with carbachol, the second phase of MAPK activation was not observed and no DNA synthesis took place. To see if Ca^{2+}-stimulated MAPK activation in human fibroblasts also exhibits such a biphasic pattern, we carried out a time course study of Ca^{2+} stimulation, using phosphorylation of MBP as a measure of MAPK activation. The results are shown in Figure 6. Exposure of LG1 and SL80 cells to 10 mM Ca^{2+} rapidly activated MAPK, with a maximum peak of activation occurring at 5–10 min. After the initial burst of activation, a second wave of sustained activation was observed in both cell lines. The level of activation of MAPK did not come down to the basal level in either cell line during the 180 min of stimulation. This sustained activation, along with our finding that in the total absence of protein growth factors Ca^{2+} induces growth of human fibroblasts (Figure 1), suggests that Ca^{2+} stimulates cell proliferation by activating MAPK in a biphasic manner. Recently, Frost et al. [24] and Pagès et al. [25], using MAPK antisense RNA and/or MAPK kinase-deficient mutants to suppress MAPK activation, showed that MAPK activation is required for activation of

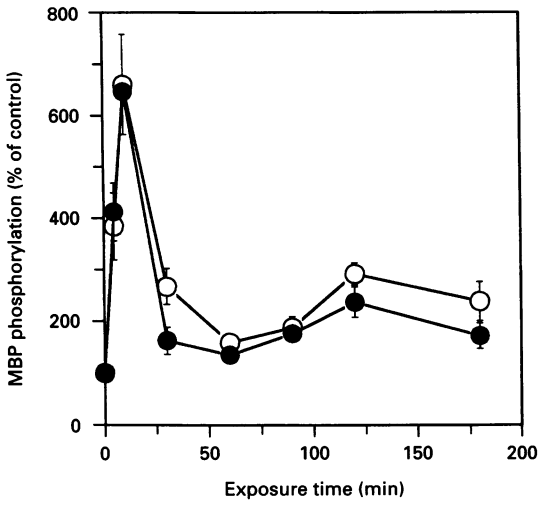

Figure 6 Time course for Ca^{2+}-induced MAPK activity

The medium on cells (○, LG1; ●, SL80) in exponential growth was changed to the McM medium with the serum replacements of Ryan et al. [15] but containing no protein growth factors and only 0.1 mM Ca^{2+}. After 24–30 h, the Ca^{2+} concentration was increased to 10 mM for the indicated time. Cell lysates were prepared and assayed for MBP phosphorylation. Results are expressed as means ± S.E.M. of triplicate determinations. Similar results were obtained in two separate experiments.

transcriptional factors [24] and for rodent fibroblast proliferation [25].

In summary, as far as we can determine, our study is the first to show that in the total absence of protein growth factors extracellular Ca^{2+} can stimulate sustained growth of diploid human fibroblasts and can induce the activation of MAPK. In the absence of protein growth factors, the 42 kDa and 44 kDa isoforms of MAPK are both activated, as they are by protein growth factors. A study by Chao et al. [5] indicates that various pathways can lead to MAPK activation in human fibroblasts. For example, EGF-mediated activation does not require intracellular Ca^{2+}, whereas thapsigargin-mediated activation depends on intracellular Ca^{2+}. We intend to determine whether extracellular Ca^{2+} stimulates MAPK activation by one of these pathways or by some alternative mechanism.

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REFERENCES

1 Bettger, W. J., Boyce, S. T., Walthall, B. J. and Ham, R. G. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5588–5592
2 Brown, E. M. (1991) Physiol. Rev. **71**, 371–411
3 Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K. and Krebs, E. G. (1991) J. Biol. Chem. **266**, 4220–4227
4 Nakielny, S., Cohen, P., Wu, J. and Sturgill, T. (1992) EMBO J. **11**, 2123–2129
5 Chao, T. O., Byron, K. L., Lee, K. M., Villereal, M. and Rosner, M. R. (1992) J. Biol. Chem. **267**, 19876–19883
6 Pelech, S. L. and Sanghera, J. S. (1992) Science **257**, 1355–1356
7 Vietor, I., Schwenger, P., Li, W., Schlessinger, J. and Vilcek, J. (1993) J. Biol. Chem. **268**, 18994–18999
8 Pang, L., Decker, S. J. and Saltiel, A. R. (1993) Biochem. J. **289**, 283–287
9 Burgering, B. M. T., de Vries-Smits, A. M. M., Medema, R. H., van Weeren, P. C., Tertoolen, L. G. J. and Bos, J. L. (1993) Mol. Cell. Biol. **13**, 7248–7256
10 Pelech, S. L. and Sanghera, J. S. (1992) Trends Biochem. Sci. **17**, 233–238
11 Guy, G. R., Chua, S. P., Wong, N. S., Ng, S. B. and Tan, Y. H. (1991) J. Biol Chem. **266**, 14343–14352
12 Sturgill, T. W., Ray, L. B., Erikson, E. and Maller, J. L. (1988) Nature (London) **334**, 715–718
13 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
14 Morgan, T. L., Yang, D., Fry, D. G. et al. (1991) Exp. Cell Res. **197**, 125–136
15 Ryan, P. A., McCormick, J. J. and Maher, V. M. (1987) Exp. Cell Res. **172**, 318–328
16 Zheng, C. F. and Guan, K. L. (1993) J. Biol. Chem. **268**, 11435–11439
17 Gupta, S. K., Gallego, C., Johnson, G. L. and Heasley, L. E. (1992) J. Biol. Chem **267**, 7987–7990
18 Welham, M. J., Duronio, V., Sanghera, J. S., Pelech, S. L. and Schrader, J. W. (1992) J. Immunol. **149**, 1683–1693
19 Posada, J. and Cooper, J. A. (1992) Science **255**, 212–215
20 Offermanns, S., Jones, S. V. P., Bombien, E. and Schultz, G. (1994) J. Immunol. **152**, 250–261
21 Boulton, T. G., Nye, S. H., Robbins, D. J. et al. (1991) Cell **65**, 663–675
22 Kahan, C., Seuwen, K., Meloche, S. and Pouysségur, J. (1992) J. Biol. Chem. **267**, 13369–13375
23 Vouret-Craviari, V., Van Obberghen-Schilling, E., Scimeca, J. C., Van Obberghen, E. and Pouysségur, J. (1993) Biochem. J. **289**, 209–214
24 Frost, J. A., Geppert, T. D., Cobb, M. H. and Feramisco, J. R. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3844–3848
25 Pagès, G., Lenormand, P., L'Allemain, G., Chambard, J. C., Meloche, S. and Pouysségur, J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8319–8323