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Inhibition of Epidermal Growth Factor-induced Cell Transformation and Akt Activation by Caffeine

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Abbreviations: AP-1, activator protein 1; ATM, ataxia-telangiectasia-mutated protein kinase; ATR, ATM/Rad3-related protein kinase; BME, basal medium Eagle; cAMP, cyclic adenosine monophosphate; DNM-Akt1, dominant negative mutant of Akt1; EGF, epidermal growth factor; Erks, extracellular signal-regulated kinases; FBS, fetal bovine serum; MAP, mitogen activated protein; MDM2, murine double minute 2; MEM, minimal essential medium; mTOR, mammalian target of rapamycin; P+1-1, JB6 Cl 41 stable AP-1 reporter transfectant cell line; p70 S6K, ribosomal p70 S6 protein kinase; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-monophosphate; PIKK, PI3K-related kinase; 8-PTH, 8-phenyltheophylline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Running Title: *CAFFEINE INHIBITS CELL TRANSFORMATION AND AKT*

Key words: caffeine, EGF, cell transformation, Akt, adenosine receptor

We found that caffeine significantly inhibited epidermal growth factor (EGF)- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation in the JB6 mouse epidermal cell line. The tumor promoter-induced cell transformation was also blocked by treatment with an adenosine A1 receptor antagonist, 8-phenyltheophylline. Caffeine slightly attenuated activation of EGF-induced activator protein 1 (AP-1) activation, which play important roles in cell transformation, but only at the highest concentration examined (1 mM). Interestingly, pretreatment with caffeine suppressed EGF-induced phosphorylation and activation of Akt and ribosomal p70 S6 protein kinase, a target of Akt, without inhibiting phosphatidylinositol 3-kinase activation. The inhibition of Akt activation of caffeine was not a result of its adenosine receptor antagonism. Because Akt plays a key role in signal transduction pathways leading to cell proliferation and apoptosis, our results provide novel insight into possible mechanisms of the chemotherapeutic effect of caffeine.

INTRODUCTION

Methylxanthines, such as caffeine, have a number of biological effects, including stimulation of muscle contraction [1], alterations in glucose metabolism [2,3], immunomodulatory effects [4], induction of apoptosis [5], and anti-cancer or anti-carcinogenic effects [6-8]. These pleiotropic effects may be explained by inhibition of phosphodiesterase (PDE) and an antagonistic effect on adenosine receptors. On the other hand, caffeine potentiates the sensitivity of tumor cells to ionizing radiation or anti-tumor agents [8,9]. In our university hospital, radiochemotherapy or chemotherapy combined with caffeine is used to treat high-grade soft tissue sarcomas in the Department of Orthopedic Surgery [10,11]. Although the chemotherapeutic effect of caffeine is concentration-dependent, the plasma concentration in patients is limited to 80 μg/ml (412 μM) to avoid toxicity. The effect of caffeine may result from disruption of the G2/M DNA damage checkpoint by the inhibition of ataxia-telangiectasia-mutated protein kinase (ATM) and/or ATM/Rad3-related protein kinase (ATR) and their downstream signaling effectors [12,13]. ATM and ATR are members of the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family [14]. The PIKK

family members share a C-terminal kinase domain bearing significant sequence homology to the catalytic domains of mammalian and yeast PI3K. Methylxanthines are known to inhibit phosphoinositide metabolism [15,16] and the PI3K signaling pathway [2,17]. Thus, caffeine's effects may involve other mechanisms than PDE inhibition and adenosine receptor antagonism at cancer chemotherapeutic concentrations. We were therefore interested in the role of altered signal transduction pathways in the biological mechanisms of caffeine.

The JB6 cell system of clonal genetic variants, which are promotion-sensitive (P+) or promotion-resistant (P-), is an excellent model for studying signal transduction at the molecular level in various stages of carcinogenesis [18-23]. The JB6 P+, P- and transformed variants are a series of cell lines representing 'earlier-to-later' stages of preneoplastic-to-neoplastic progression [19,21]. JB6 P+ cells transform when stimulated with epidermal growth factor (EGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), forming colonies in soft agar [19,21]. The transformation is thought to involve activation of activator protein 1 (AP-1) [18,22-25], which regulates the transcription of various genes related to cellular

inflammation, proliferation and apoptosis [26]. It plays a key role not only in neoplastic transformation in tumor promotion [24,25], but also in tumor progression and metastasis [27]. In addition, we have demonstrated that phosphatidylinositol 3-kinase (PI3K) and its downstream effector, Akt, are closely involved in epidermal growth factor (EGF)-induced cell transformation in JB6 P+ cells [28]. PI3K is central to the coordinated control of multiple cell-signaling pathways leading to tumor development, including cell proliferation, apoptosis and migration [29,30], and Akt plays a pivotal role in the PI3K-signaling pathway [31]. In this study, we found that caffeine inhibited EGF-induced cell transformation. To test the hypothesis that caffeine might modulate the AP-1 and/or the PI3K/Akt signaling pathways, we investigated the effect of caffeine on the signaling pathways induced by EGF in JB6 P+ cells.

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium (MEM), L-glutamine and basal medium Eagle (BME) were from Life Technologies (Rockville, MD); fetal bovine serum (FBS), gentamicin, ribonuclease A and caffeine were from Sigma (St. Louis, MO, USA); the PI3K inhibitor, LY29402, was from Calbiochem (La Jolla, CA, USA); the Akt immunoprecipitation kinase and S6 kinase assay kits were from Upstate Biotechnology Inc. (Lake Placid, NY, USA); the Akt antibody and phospho-specific Akt (serine 473), ribosomal p70 S6 protein kinase (p70 S6K) antibody and phospho-specific p70 S6K (threonine 389), and PhosphoPlus p44/42 mitogen activated protein (MAP) kinase antibody kits were from Cell Signaling Technology Inc. (Beverly, MA, USA); the anti-Akt1/2 antibody and agarose conjugated with monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz (Santa Cruz, CA, USA); propidium iodide and 8-phenyltheophylline (8-PTH) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The cyclic adenosine monophosphate (cAMP) EIA system was purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA).

Cell Culture

The stable transfectant cells expressing dominant negative mutant Akt1 (DNM-Akt1) were obtained as reported previously [28]. The mouse epidermal JB6 Cl 41 cell line and its stable transfectants, P+1-1 (AP-1 reporter transfectant) and DNM-Akt1, were grown at 37°C in MEM supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin.

Anchorage-independent Growth Assay

JB6 Cl 41 cells and its transfectant cells, DNM-Akt1, in aliquots of $1x10^4$ cells, were exposed to EGF (10 ng/ml) or TPA (10 ng/ml) with or without the indicated concentrations of caffeine or 8-PTH in 1 ml of 0.33% BME agar containing 10% FBS over 3.5 ml of 0.5% BME agar medium containing 10% FBS. The cultures were maintained in a 37°C, 5% CO_2 incubator and the cell colonies were scored at 14 days after exposure of the cells to EGF or TPA.

Cell Proliferation Assay

JB6 Cl 41 cells were suspended in 5% FBS/MEM at a density of $1x10^4$ cells/ml. The cell suspension (1 ml) was placed in each well of a 24-well plate and incubated for 24 h. Then, the indicated concentrations of caffeine were added and incubation was continued for 48 h at 37°C. After incubation for the indicated periods, the cell number was counted under a microscope.

Flow Cytometry

The cell cycle of JB6 Cl 41 cells treated with EGF (10 ng/ml) and/or caffeine (1 mM) for 24 h or 48 h was examined by flow cytometry using a FACS Calibur (Becton Dickinson Co. Ltd., San Jose, USA) as described previously [32].

Assay of AP-1 Activity

JB6 AP-1 reporter stable P+1-1 cells ($7x10^3$) were seeded in a 96-well plate. After 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM for 24 h. Then, the cells were pretreated with the indicated concentrations of caffeine

for 1 h and cultured with 10 ng/ml EGF in the presence or absence of caffeine for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured with a BioOrbit 1253 luminometer (BioOrbit , Turku, Finland). Relative AP-1 activity was calculated as described previously [33].

Assay for cAMP Production

JB6 Cl 41 cells ($7x10^3$) were seeded in a 96-well plate. After a 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM for 24 h. Then, the cells were treated or not treated with caffeine (1 mM) in the absence or presence of EGF (10 ng/ml) or forskolin (100 nM) for the indicated periods. The medium was aspirated, and the cells were lysed using a lysis buffer (Amersham Biosciences Corp.). Intercellular cAMP content was determined by a cAMP EIA system (Amersham Biosciences Corp.) according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was carried out as described previously [33]. In brief, JB6 Cl 41

cells were cultured to 80% confluence. The cells were starved in 0.1 % FBS/MEM for 24 h at 37°C. Then the medium was changed to fresh 0.1% FBS/MEM and the cells were incubated for another 2-4 h at 37°C. Before exposure of the cells to EGF, they were treated or not treated with caffeine for 1 h. Then, EGF (10 ng/ml) was added and cells were subsequently incubated for an additional 30 min at 37°C. The cells were then lysed, and immunoblot analysis was performed by using antibodies against Akt, p70 S6K and Erks or phospho-specific antibodies against the phosphorylated proteins. Antibody-bound proteins were detected by luminescence assay (ECL Western Blotting Kit, Amersham Biosciences Corp.) and analyzed using a BAS-1000 lumino-imaging analyzer (Fuji Film, Tokyo, Japan).

p70 S6K and Akt Kinase Immunoprecipitation Assay

JB6 Cl 41 cells were treated or not treated with caffeine or 8-PTH before treatment with EGF (10 ng/ml) or TPA (10 ng/ml) for the indicated time. Lysates were prepared from the cells and immunoprecipitation was carried out using 20 μl of anti-Akt1/2 (Santa Cruz) or 3 μg of anti-p70 S6K antibodies (Cell Signaling). The enzyme

immune complex was washed three times with 0.5 ml of lysis buffer, and once with 100 μl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, pH 7.0, 1 mM sodium orthovanadate, 1 mM DTT). For the Akt kinase assay, the enzyme immune complex was added to 10 μl of assay dilution buffer, 40 μM protein kinase A inhibitor peptide, 0.4 mM Akt substrate peptide, and 1 μCi of [γ-^{32}P]ATP. For the p70 S6K assay, the enzyme immune complex was added to 20 μl of assay dilution buffer, 10 μl of inhibitor cocktail, 50 μM S6 kinase substrate peptide, and 1 μCi of [γ-^{32}P] ATP. The respective reaction mixtures were incubated for 10 min at 30°C, then centrifuged, and 30 μl of the supernatant fraction was transferred onto P81 phosphocellulose paper. The P81 paper was washed three times in 0.75% phosphoric acid and then once in acetone, and the γ-^{32}P incorporation was measured by scintillation counting.

PI3K Assay

JB6 Cl 41 cells pretreated with caffeine for 1 h were exposed to EGF (10 ng/ml) for 5 min. The cells were lysed in 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM sodium

orthovanadate, 1 mM PMSF, 10 μM aprotinin, 10 μM leupeptin). The lysate was sonicated and centrifuged, and the supernatant fraction was incubated with 20 μl of agarose conjugated with a monoclonal anti-phosphotyrosine antibody (PY99), with gentle rocking overnight at 4°C. Then, PI3K activity was determined as described previously [34]. The radiolabeled spots of phosphatidylinositol 3-monophosphate (PI3P) that indicates PI3K activation were quantified using the BAS-2000 bio-imaging analyzer (Fuji Film).

Statistical Analysis

The significance of differences in this study was determined by using Student's *t* test.

RESULTS

Inhibition of tumor promoter-induced cell transformation by caffeine

In the JB6 Cl 41 promotion-sensitive (P+) cell line, in which transformed colonies are induced by EGF or TPA, caffeine significantly blocked EGF- and TPA-induced cell transformation in a concentration-dependent manner (Figure 1A). The inhibition of cell transformation by caffeine was not due to growth inhibition, because the concentration range of caffeine that blocked cell transformation had no effect on cell proliferation (Figure 1B).

We also analyzed the cell cycle in cells treated with EGF and/or caffeine for 24 hr or 48 hr. EGF tended to increase the proportion of cells in S-phase, but caffeine had no effect on the cell cycle distribution (data not shown).

Involvement of intracellular cAMP and adenosine receptor antagonism in EGF-induced cell transformation

As shown in Figure 2A, the cAMP content was not enhanced in cells treated with caffeine (1 mM) and/or EGF (10 ng/ml) for the indicated time. In contrast, when the

cells were treated with caffeine in the presence of forskolin (100 nM), a cAMP elevator, caffeine significantly enhanced the increase of cAMP content induced by forskolin.

We also assessed the effect of 8-phenyltheophylline (8-PTH), a selective A1 adenosine receptor antagonist [35], on EGF- and TPA-induced cell transformation. Treatment with 8-PTH significantly inhibited tumor promoter-induced cell transformation (Fig. 2B).

Effect of caffeine on EGF-induced AP-1 activation

Caffeine slightly attenuated EGF-induced AP-1 activation, but only at the highest concentration examined (1 mM) (Figure 3A). Extracellular signal-regulated kinases (Erks) have been shown to be necessary components in the pathway leading to AP-1 activation and neoplastic transformation, and caffeine also tended to inhibit EGF-induced Erks phosphorylation at 1 mM (Figure 3B).

Caffeine inhibited Akt activation induced by EGF

Expression of dominant negative mutant Akt1 (DNM-Akt1) in JB6 Cl 41 cells

inhibited cell transformation induced by EGF, but not TPA (Figure 4A). Akt was activated by treatment with EGF, whereas TPA had no effect (Figure 4B).

We then investigated whether caffeine modulates EGF-induced Akt activation. Treatment with caffeine inhibited EGF-induced phosphorylation at serine 473 and activation of Akt in the same concentration range as that causing inhibition of EGF-induced cell transformation (Figure 5). Furthermore, caffeine suppressed EGF-induced phosphorylation at threonine 389 and activation of ribosomal p70 S6 protein kinase (p70 S6K), a target of Akt (Figure 6).

On the other hand, caffeine had no effect on EGF-induced PI3K activation (Figure 7). To test whether caffeine directly inhibits the kinase activities of Akt and p70 S6K induced by EGF, we performed Akt and p70 S6K kinase reactions in the presence of caffeine. The enzymes were immunoprecipitated from lysate of cells treated with EGF (10 ng/ml) by using antibody to Akt or p70 S6K. However, treatment with caffeine did not directly affect EGF-induced activation of either Akt or p70 S6K (data not shown).

Although several studies have shown that an adenosine A1 receptor-mediated signal transduction pathway is involved in Akt activation [36,37], 8-PTH did not block

EGF-induced Akt activation (Figure 8).

DISCUSSION

In this study, we investigated the influence of chemotherapeutic concentrations of caffeine on signal transduction in the mouse epidermal JB6 P+ cell line, JB6 Cl 41, stimulated with EGF. We found that caffeine inhibited EGF- and TPA-induced cell transformation in a concentration-dependent manner. The inhibitory effects of caffeine may be mediated by inhibition of PDE, an antagonistic effect on adenosine receptors, or G2/M arrest through ATM/ATR inhibition. Dong *et al*. [38] have reported that forskolin, a cAMP elevator, reversed the formation of transformed colonies of neoplastic epidermal JB6 RT101 cells. Treatment of cells with caffeine (1 mM) in the absence or presence of EGF did not change the intracellular cAMP content. An increased intracellular cAMP content in the presence of caffeine was observed only when the cells were treated with forskolin, suggesting that phosphodiesterase inhibitors are needed for cAMP elevators, such as forskolin, to stimulate increases in intracellular cAMP content. On the other hand, caffeine had no effect on cell proliferation or on the

cell cycle in cells treated with EGF (data not shown). Thus, the inhibition by caffeine of EGF-induced cell transformation is not mediated by cell growth inhibition or by induction of G2/M arrest through inhibition of ATM/ATR. In contrast, we found that 8-PTH, an adenosine A1 receptor antagonist, blocked EGF- and TPA-induced cell transformation. In addition to 8-PTH, a non-selective adenosine receptor antagonist, CGS-15943, also suppressed EGF-induced cell transformation (data not shown). Adenosine receptors are coupled to a complicated network of signaling pathways via G-proteins [39]. Therefore, the inhibitory effect of caffeine on tumor promoter-induced cell transformation appears to result from the modulation of adenosine receptor-dependent signaling pathways.

Many reports, including ours, have suggested that AP-1 activity is a critical factor for tumor promoter-induced cell transformation in JB6 P+ cells [18,22-25,33]. Therefore, we also assessed whether caffeine inhibits EGF-induced AP-1 activation. Caffeine slightly attenuated EGF-induced AP-1 activation and Erks phosphorylation, which are necessary components in the pathway leading to AP-1 transcriptional activation [40,41], but only at the highest concentration examined (1 mM), suggesting that AP-1 activation

may not be substantially involved in caffeine's inhibition of cell transformation.

In a wide range of cellular systems, Akt controls intracellular pathways responsible for preventing cell death, and promoting cell cycle progression, anchorage-independent growth and cell migration [28,31,42,43]. Akt was originally characterized as a cellular counterpart of the viral oncogene, *v-akt*, from the transforming retrovirus AKT8 [44]. It is overexpressed in a large proportion of ovarian, pancreatic, prostate and breast cancers [45-47]. Expression of DNM-Akt1 in JB6 Cl 41 cells significantly impaired EGF-induced cell transformation, in agreement with our previous report [28], whereas cell transformation induced by TPA, which did not activate Akt, was unaffected. Thus, Akt activation plays an important role in cell transformation induced by EGF, but not TPA, in JB6 P+ cells. Caffeine inhibited EGF-induced Akt activation and phosphorylation of Akt at serine 473 in the same concentration range as that causing inhibition of cell transformation, suggesting a relationship between these phenomena. The inhibition of Akt activation by caffeine is not specific to JB6 Cl 41 cells, because we have observed a similar effect in a mouse preadipose cell line, MC3T3-G2/PA6, stimulated with insulin [2].

The activation of p70 S6K is known to require signaling through mammalian target of rapamycin (mTOR) [48]. mTOR is reported to be essential for cell transformation induced by the oncogenes *p3k* and *akt* [49] and to be directly phosphorylated by Akt [50,51]. Caffeine inhibited EGF-induced p70 S6K activation and phosphorylation at threonine 389, a rapamycin-sensitive site. Thus, the inhibition of EGF-induced p70 S6K activation by caffeine may result from the impairment of mTOR through a blockade of Akt activation. PI3K and p70 S6K are constitutively activated in small cell lung cancer cells and anchorage-independent growth in the cells is mediated through Akt and p70 S6K-dependent pathways [52]. p70 S6K is also amplified or overexpressed in human breast cancer [53,54]. Therefore, blocking the activation of p70 S6K may also, in part, be involved in the inhibitory effect of caffeine on EGF-induced cell transformation.

On the other hand, the mechanism by which caffeine blocked activation of Akt is not clear. Foukas *et al.* [17] have shown by *in vitro* kinase assay that caffeine directly inhibited PI3K activation. Therefore, we assessed the influence of caffeine on PI3K activation induced by EGF and found that caffeine did not affect EGF-induced PI3K

activation. We previously showed that tea polyphenols directly inhibited ultraviolet B-induced p70 S6K activation in this cell line [34]. However, caffeine did not directly affect EGF-induced activation of either Akt or p70 S6K (data not shown). Furthermore, Akt is reported to be be activated through the adenosine A1 receptor [36,37], but 8-PTH, which blocked cell transformation, had no effect on EGF-induced Akt activation. Thus, inhibition of Akt activation by caffeine was not a result of its adenosine receptor antagonism. The mechanism through which caffeine blocked activation of Akt requires further study.

Caffeine, in addition to its effect on cell cycle regulation, has been reported to induce apoptosis in cells treated with or without DNA-damaging agents [13,55,56]. Akt inactivates the apoptotic function via phosphorylation of BAD, caspase-9, Forkhead family, IkappaB kinase, and cAMP-response element-binding protein [42]. Furthermore, it phosphorylates the murine double minute 2 (MDM2) protein, promoting its nuclear localization, and increasing p53 degradation [57]. The phosphorylation of MDM2 by activated Akt is suggested to play an important role in impairing p53 functions, including p53-dependent apoptosis. Therefore, the inhibition of Akt by

caffeine might induce apoptosis.

In this study, we found that caffeine blocked tumor promoter-induced cell transformation in JB6 cells, possibly through its adenosine receptor A1 antagonistic effect. We also found that caffeine significantly inhibited EGF-induced Akt activation. Akt plays a pivotal role in signal transduction pathways leading to preventing cell death, cell cycle progression, malignant transformation and cell migration [28,31,41,42]. Therefore, our results provide novel insight into the possible mechanisms of the cancer chemotherapeutic action of caffeine.

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FIGURE LEGENDS

Figure 1. Inhibition of tumor promoter-induced cell transformation by caffeine. *A*, Aliquots of 10^4 JB6 Cl 41 cells were treated with EGF (10 ng/ml) or TPA (10 ng/ml) with or without the indicated concentration of caffeine in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after incubation for 14 days at 37˚C in a 5% CO_2 incubator. Data are expressed as means ± S.E. of independent three experiments. * Significantly different from EGF or TPA alone at $P < 0.05$. *B*, JB6 Cl 41 cells were suspended in 5% FBS/MEM at a density of $1x10^4$ cells/ml. The cell suspension (1 ml) was placed in each well of a 24-well plate and incubated for 24 h. Then, the indicated concentrations of caffeine were added and incubation was continued for 48 h at 37˚C in a 5% CO_2 incubator. After the indicated periods, cells were counted under a microscope. Data are expressed as means ± S.E. of four independent experiments.

Figure 2. Involvement of intracellular cAMP and adenosine receptor antagonism in EGF-induced cell transformation. *A*, JB6 Cl 41 cells ($7x10^3$) were

seeded in a 96-well plate. After a 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM, followed by incubation for 24 h. Then, the cells were treated or not treated with caffeine (1 mM) in the absence or presence of EGF (10 ng/ml) or forskolin (100 nM) for the indicated times. The cells were lysed, and the intercellular cAMP content was determined using a cAMP EIA system. Data are expressed as means ± S.E. of three independent experiments. # Significantly different from the untreated control at $P < 0.05$. * Significantly different from caffeine treatment only at $P < 0.05$. § Significantly different from forskolin treatment only at $P < 0.05$. *B*, Aliquots of 10^4 JB6 Cl 41 cells were treated with EGF (10 ng/ml) or TPA (10 ng/ml) with or without the indicated concentration of 8-PTH in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after incubation for 14 days at 37˚C in a 5% CO_2 incubator. Data are expressed as means ± S.E. of three independent experiments. * Significantly different from EGF or TPA alone at $P < 0.05$.

Figure 3. Effect of caffeine on EGF-induced AP-1 activation and Erks

phosphorylation. *A*, JB6 AP-1 reporter stable P+1-1 cells were seeded in a 96-well plate. After a 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM, and incubation was continued for an additional 24 h. Then, the cells were pretreated with the indicated concentration of caffeine for 1 h and cultured with 10 ng/ml EGF for an additional 24 h. The AP-1 activity was measured with a luciferase activity assay as described under ‘‘MATERIALS AND METHODS’’. The results are shown as relative AP-1 activity, expressed as means ± S.E. of five independent experiments. * Significantly different from EGF alone at $P < 0.05$. *B*, JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and incubation was continued for 24 h. The cells were pretreated with caffeine for 1 h at the indicated concentration, then treated or not treated with EGF (10 ng/ml) and further cultured for 30 min. The cells were lysed and the phosphorylation levels were estimated by immunoblotting with a phospho-specific p44/42 MAP kinases (Erks) antibody. The intensity of each phospho-specific band was normalized to the that of the band of the respective non-phosphorylated protein. The results are shown relative phosphorylation expressed as means ± S.E. of five independent experiments.

Figure 4. Inhibition of EGF-induced cell transformation by introduction of DNM-Akt1. *A*, Aliquots of 10^4 JB6 Cl 41 cells or DNM-Akt1 cells were treated with EGF (10 ng/ml) or TPA (10 ng/ml) in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after incubation for 14 days at 37˚C in 5% CO_2. Data are the means ± S.E. of three independent experiments. * Significantly different from EGF alone at $P < 0.05$. *B*, JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM, and incubation was continued for 24 h. The cells were then treated or not treated with EGF (10 ng/ml) or TPA (10 ng/ml) and further cultured for the indicated time. The cells were lysed and Akt was immunoprecipitated using an anti-Akt1/2 antibody. Activities were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as means ± S.E. of at least three independent experiments.

Figure 5. Inhibition of EGF-induced phosphorylation and activation of Akt by caffeine. JB6 Cl 41 cells (80% confluence) were starved by replacing the medium

with 0.1% FBS/MEM, and incubation was continued for 24 h. The cells were pretreated with caffeine for 1 h at the indicated concentration, then treated or not treated with EGF (10 ng/ml) and further cultured for 30 min. *A*, The cells were lysed and Akt was immunoprecipitated using an anti-Akt1/2 antibody. The respective activities were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as means ± S.E. of at least three independent experiments. * Significantly different from EGF alone at $P < 0.05$. *B*, The cells were lysed and the phosphorylation levels were estimated by immunoblotting with a phospho-specific antibody for Akt. The intensity of each phospho-specific band was normalized to the band of the respective non-phosphorylated protein. The results are shown as relative phosphorylation, expressed as means ± S.E. of four independent experiments. * Significantly different from EGF alone at $P < 0.05$.

Figure 6. Inhibition of EGF-induced phosphorylation and activation of p70 S6K by caffeine. JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM, and incubation was continued for 24 h. The cells were

pretreated with caffeine for 1 h at the indicated concentration, then treated or not treated with EGF (10 ng/ml) and futher cultured for 30 min. *A*, The cells were lysed and p70 S6K was immunoprecipitated using an anti-p70 S6K antibody. The respective activities were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as means ± S.E. of at least three independent experiments. * Significantly different from EGF alone at $P < 0.05$. *B*, The cells were lysed and the phosphorylation levels were estimated by immunoblotting with a phospho-specific antibody for p70 S6K. The intensity of each phospho-specific band was normalized to the band of the respective non-phosphorylated protein. The results are shown as relative phosphorylation, expressed as means ± S.E. of four independent experiments. * Significantly different from EGF alone at $P < 0.05$.

Figure 7. Effect of caffeine on EGF-induced PI3K activation. JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM, and incubation was continued for 24 h. The cells were pretreated with caffeine or LY294002 for 1 h at the indicated concentration, then treated or not treated with EGF

(10 ng/ml) and further cultured for 5 min. The cells were lysed and the activities of PI3K were determined as described in "MATERIALS AND METHODS''. PI3P, phosphoinositol 3-monophosphate.

Figure 8. Effect of 8-PTH on EGF-induced Akt activation. JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM, and incubation was continued for 24 h. The cells were pretreated with 8-PTH for 1 h at the indicated concentrations, then treated or not treated with EGF (10 ng/ml) and further cultured for 30 min. The cells were lysed and Akt was immunoprecipitated using an anti-Akt1/2 antibody. The activities were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as means ± S.E. of at least three independent experiments.

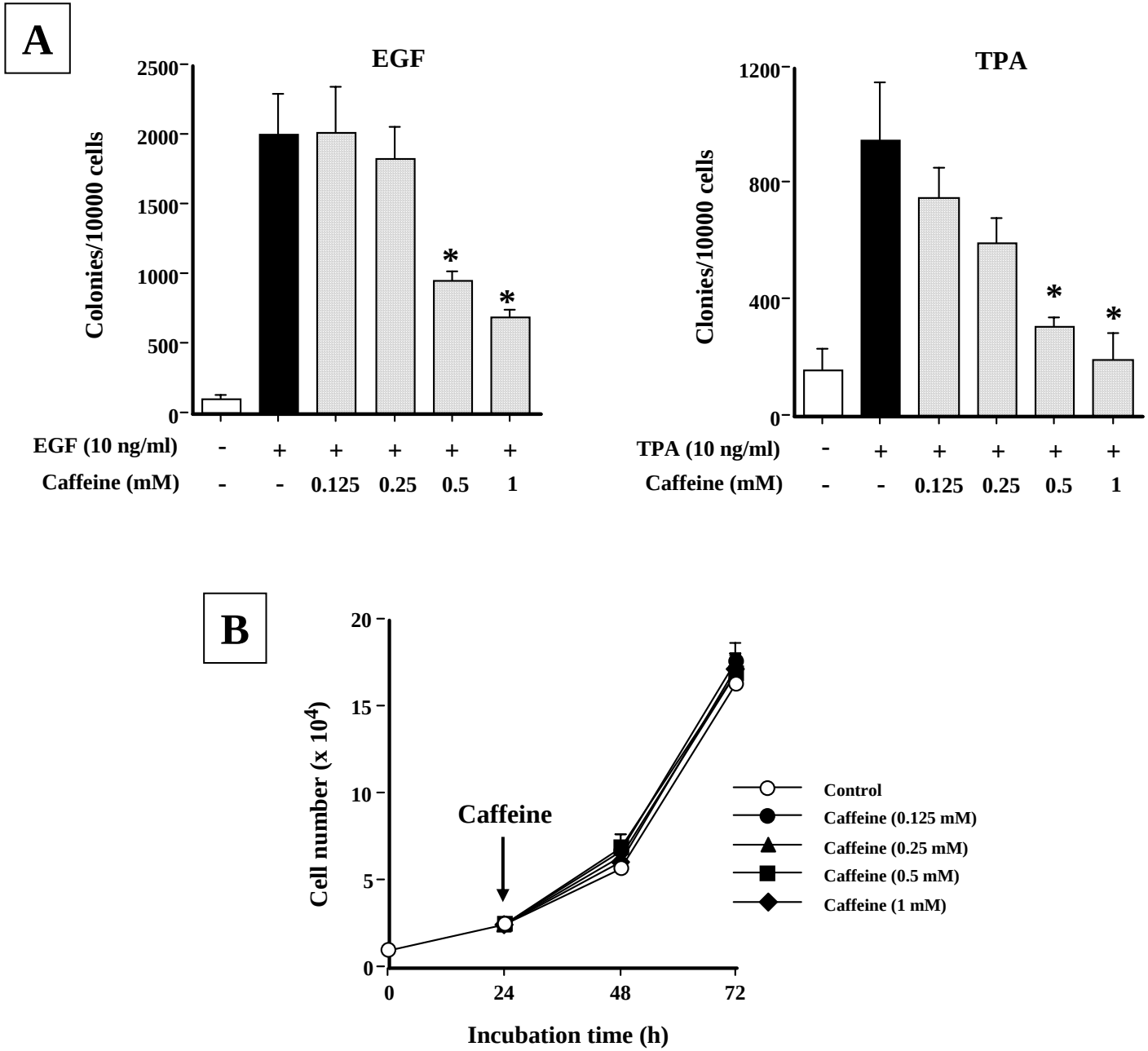
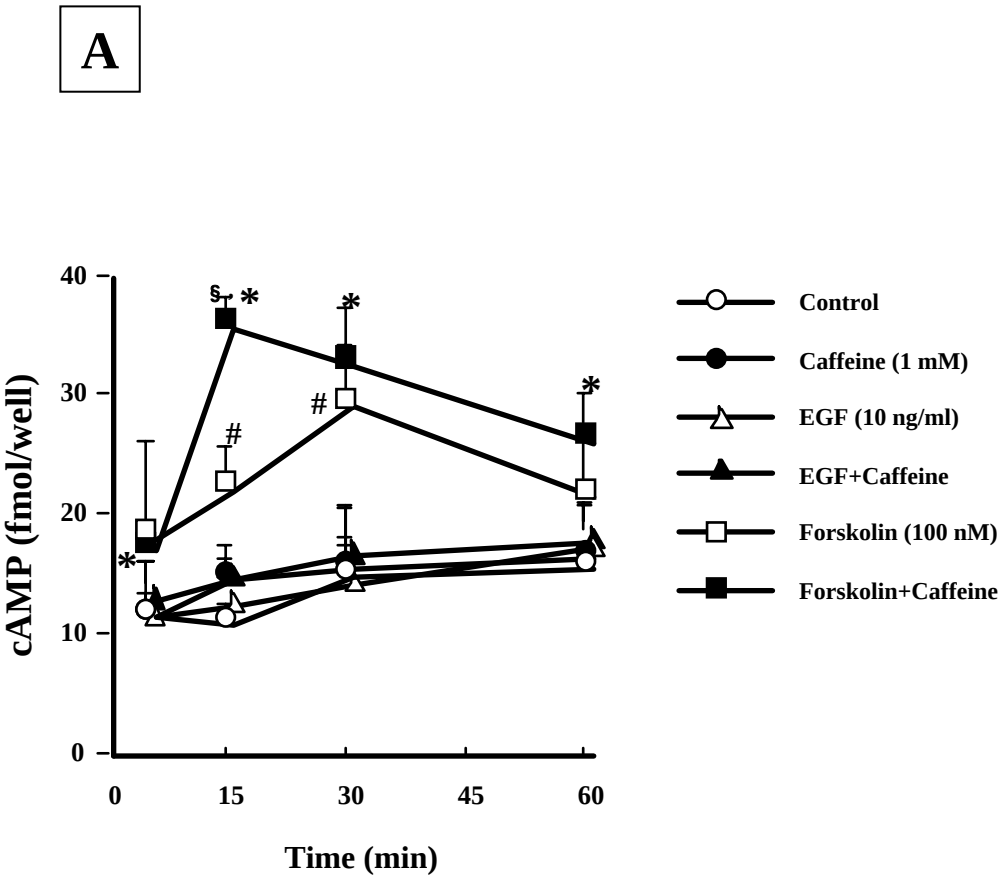
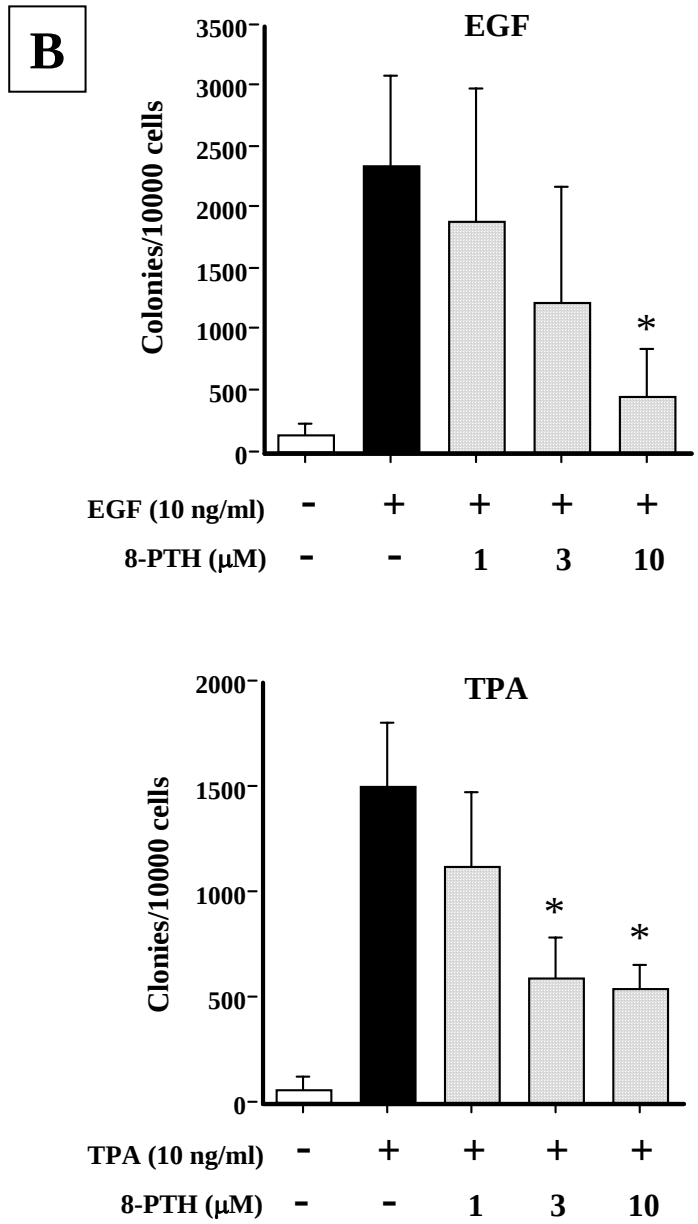


Figure 1. Nomura et al.





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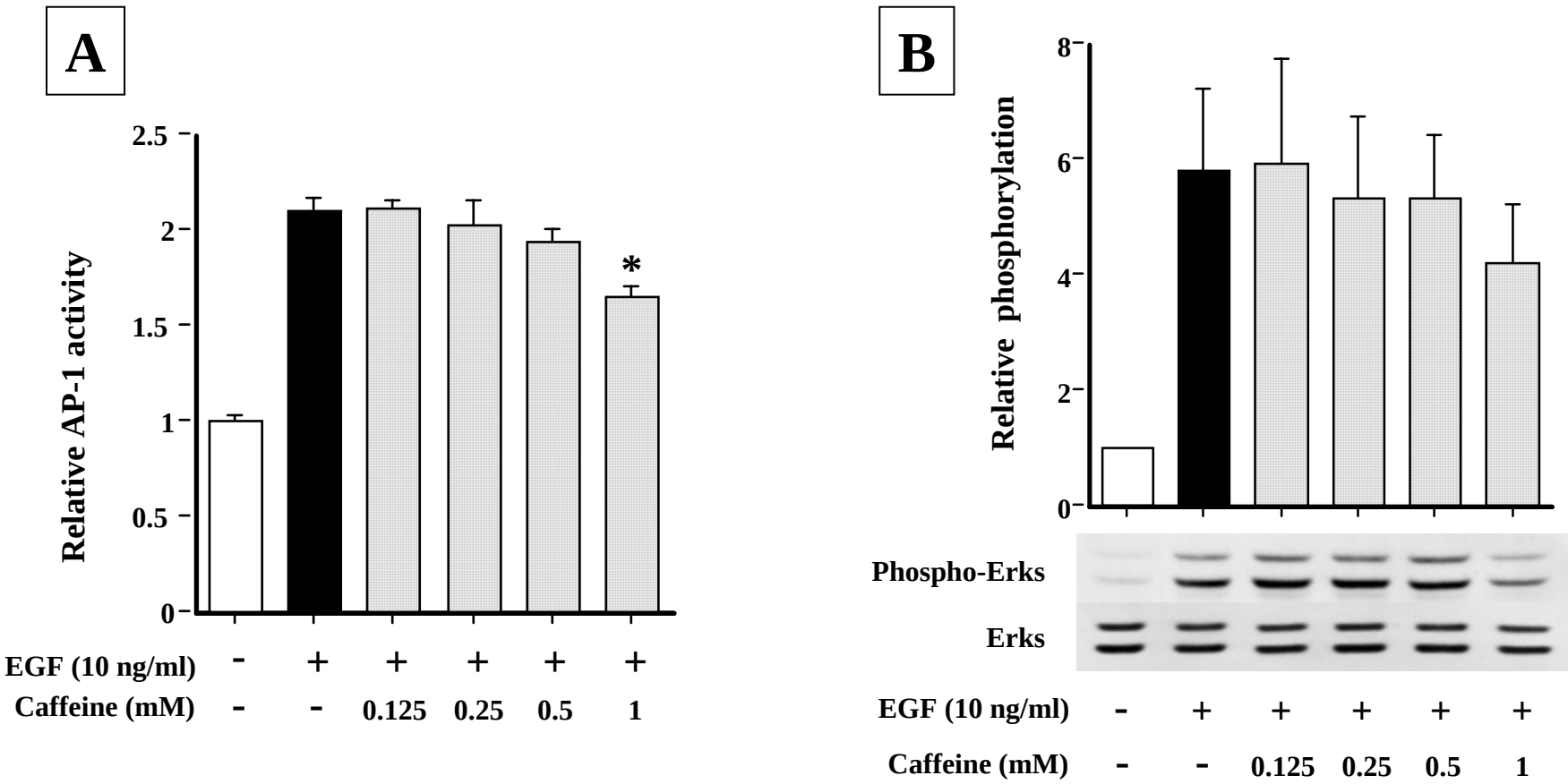

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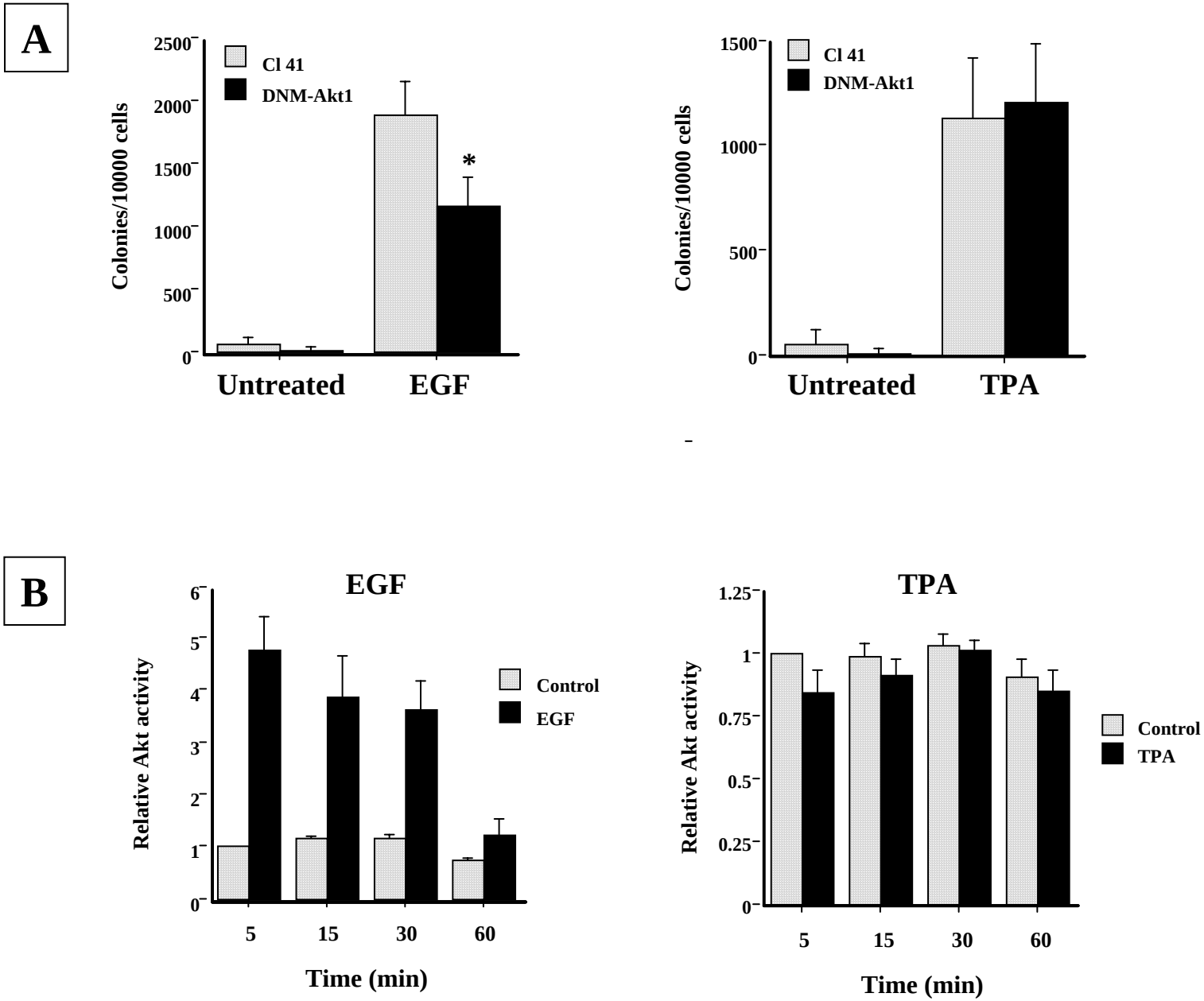

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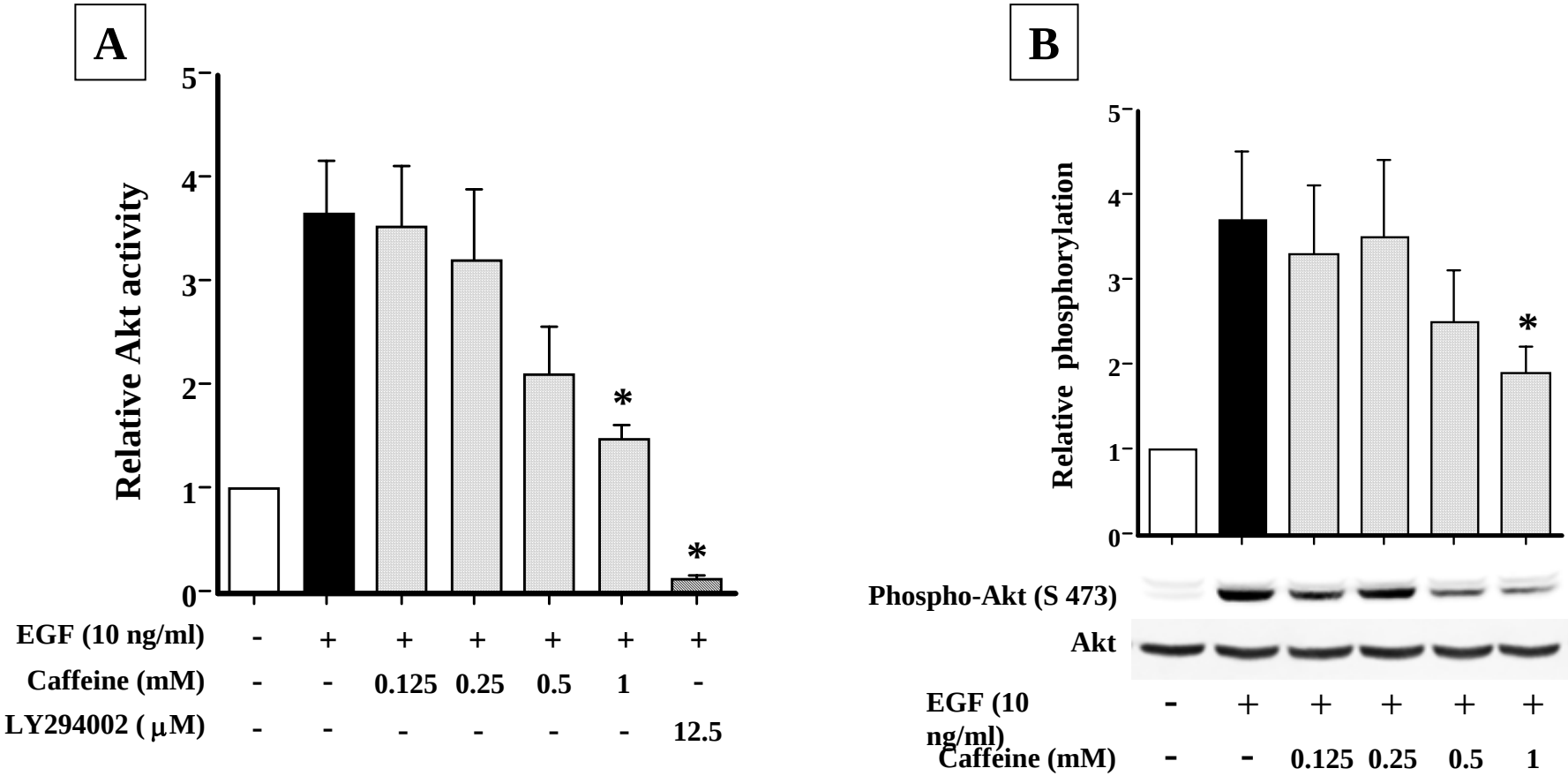

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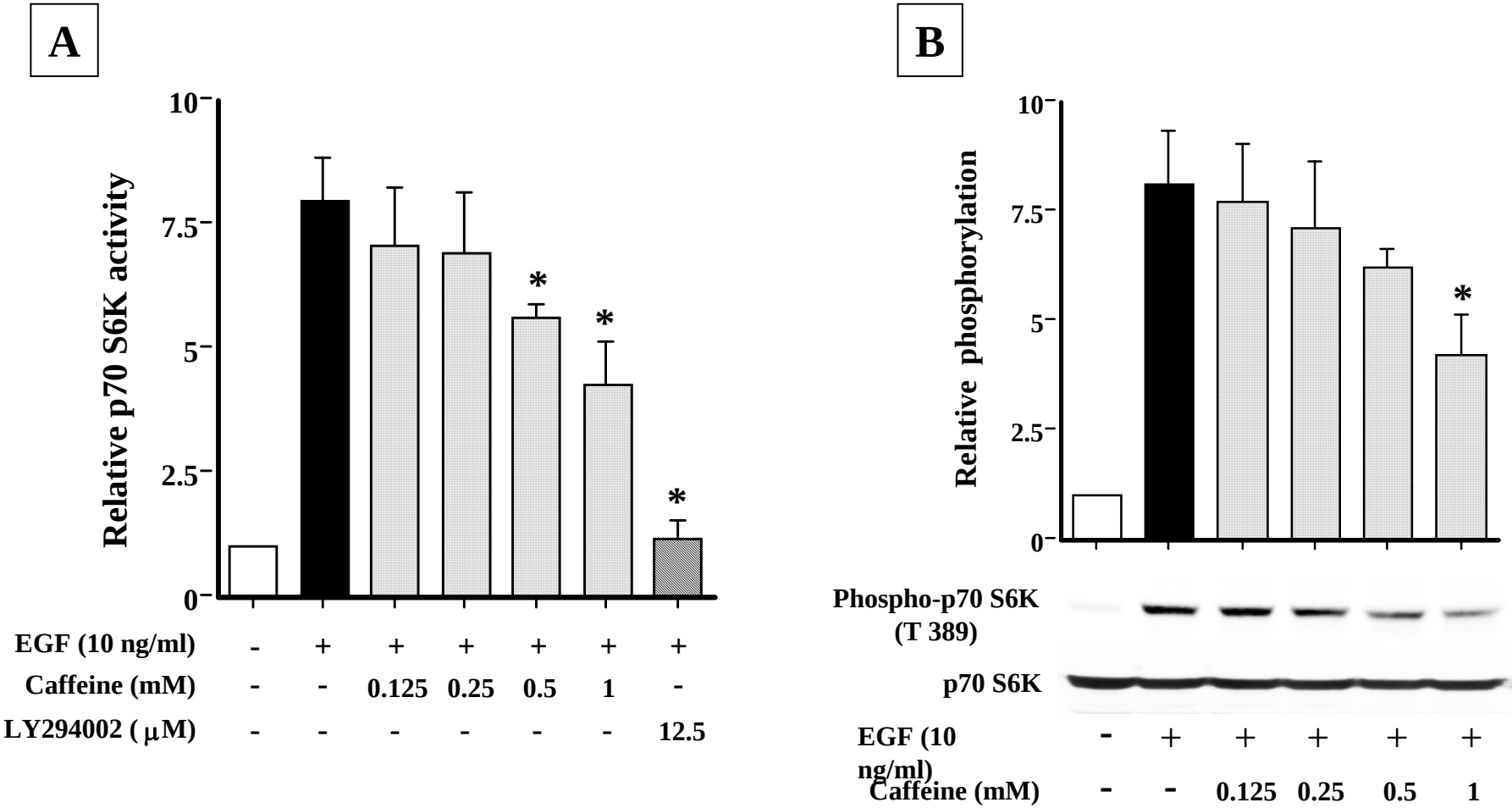

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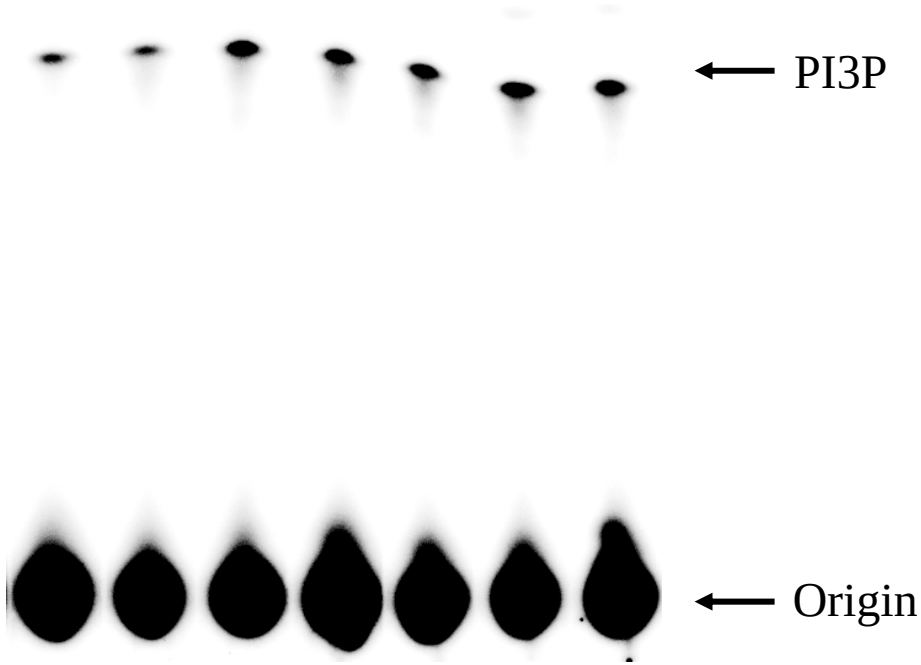

EGF (10 ng/ml)	+	-	+	+	+	+	+
Caffeine (mM)	-	-	-	0.125	0.25	0.5	1
LY294002 (μM)	12.5	-	-	-	-	-	-

PI3P, phosphatidylinositol 3-monophosphate.

Figure 7. Nomura et al.

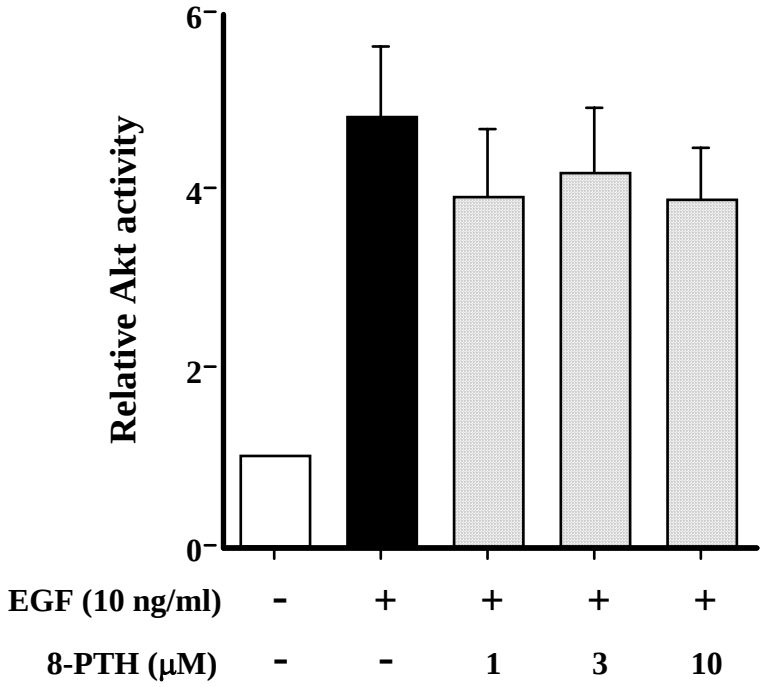

Figure 8. Nomura et al.