Molecular Mechanisms for $\alpha_2$-Adrenoceptor-Mediated Regulation of Synoviocyte Populations

Katsuyuki Mishima$^1$, Hitomi Otani$^1$, Takatoshi Tanabe$^2$, Hiroshi Kawasaki$^1$, Akihiro Oshiro$^1$, Naoaki Saito$^3$, Ryokei Ogawa$^2$ and Chiyoko Inagaki$^{1,*}$

$^1$Department of Pharmacology, and $^2$Department of Orthopaedic Surgery, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570-8506, Japan
$^3$Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

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ABSTRACT—The sympathetic nervous system has been indicated to influence the severity of inflammatory disease including rheumatoid arthritis. In this study, we elucidated the effects of catecholamine on the synovial cell populations. Stimulation with epinephrine or norepinephrine for 1–2 weeks dose- and time-dependently increased the number of synovial A (macrophage-like) cells but decreased that of B (fibroblast-like) cells. These responses in A and B cells were inhibited by the $\alpha_2$-antagonist yohimbine, the G-protein inactivator pertussis toxin and the phospholipase C (PLC) inhibitor U-73122. Furthermore, the protein kinase C (PKC) inhibitor calphostin C and mitogen-activated protein (MAP) kinase inhibitors PD98059 and wortmannin also abolished the norepinephrine effects on A and B cell numbers. In A cells cloned from an A and B cell mixture, norepinephrine also increased the cell number. In immunoblotting and immunocytochemical analyses, among the PKC isozymes, only PKC $\beta II$ immunoreactivity was observed in the cytoplasm of unstimulated A and B cells. After $\alpha_2$-adrenoceptor stimulation, PKC $\beta II$ immunoreactivity increased in the plasma membranes of both A and B cells with decreases in the cytoplasm. These findings indicated that $\alpha_2$-adrenoceptor stimulation of type A and B synoviocytes produced an increase and a decrease in the respective cell number, probably through Gi-coupled PLC activation and the resulting stimulation of the PKC $\beta II$/MAP kinase.

Keywords: Synoviocyte, $\alpha_2$-Adrenoceptor stimulation, Cell population, Protein kinase C $\beta II$

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by synovial hyperplasia. Synovial tissues, highly innervated by sympathetic and sensory nerve fibers (1), consist of at least 2 types of cells, type A (macrophage-like) and type B (fibroblast-like) cells. In the RA synovium, both cell types contribute to joint destruction via secretion of various substances such as cytokines, prostaglandins and free radicals (2–4). In addition, type A cells have been shown to have phagocytic properties and to contain lysosomal enzymes, while type B cells are fibroblasts or fibroblast-like cells that are locally derived, possibly by proliferation of underlying connective tissue cells and produce proteoglycans (2). The degree of uncontrolled accumulation and/or proliferation of these synovial cells strongly correlates with the progress of cartilage and bone erosion, where platelet-derived growth factor (PDGF), tumor necrosis factor $\alpha$ (TNF-$\alpha$), interleukin (IL) 1 and neuropeptides (substance P and calcitonin gene-related peptide) act as potent cell growth factors (5, 6).

Recently, the sympathetic nervous system has been demonstrated to play some roles in the pathogenesis of autoimmune diseases including RA (7, 8). Particularly, important clinical evidence that emotional stimuli such as anxiety and environmental stress aggravate RA symptoms (9, 10) is a well-known example of this “neuro-immuno-modulation”. Psychological stimuli occasionally induce oversecretion of norepinephrine and epinephrine (11) and a previous report by Levine et al. demonstrated that guanethidine-induced sympathectomy and reserpine-induced depletion of catecholamine attenuated joint destruction in the arthritic rat (12). Thus, catecholamines appear to influence the severity of joint injury in patients with RA. In addition, epinephrine has been shown to induce hypertrophy and proliferation in a variety of tissue types through $\alpha$-adrenoceptor-mediated activation of protein kinase C (PKC).
and mitogen-activated protein (MAP) kinase (13, 14). From these information, we hypothesized that catecholamine modulates the growth of synovial cells in the joint destruction process.

In the present study, we examined whether chronic stimulation with epinephrine or norepinephrine modulates the synoviocyte population by using cultured rabbit synovial A and B cells. The signalling pathways of the catecholamine effects were also analyzed with respect to receptor/G protein-mediated PKC activation.

MATERIALS AND METHODS

Preparation of synovial cells

All animals were handled in accordance with “Rules of the Animal Experimentation Committee, Kansai Medical University”. Synovial cells were isolated as described by Tanabe et al. (4). Surface parts of synovial tissues dissected from the knee joints of conventional Japanese white rabbits (2.8 to 3.0 kg body wt.) were minced into 1 mm³ pieces. The minced tissues were incubated in Hank’s balanced salt solution (136.8 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 0.8 mM MgSO₄, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.1 mM NaHCO₃, 20 mM HEPES, 5.5 mM glucose, pH 7.4) containing 0.3% collagenase (type II) for 60 min with occasional stirring. Samples were centrifuged for 5 min at 600×g, and the resulting pellets were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM); and they were then plated on 35-mm culture dishes at 10⁴ /dish. The isolated cells were cultured for 7 days at 37°C in an incubator with 5% CO₂. Viabilities of cells (tested by trypan blue exclusion) were unchanged (≥95%) in all vehicle and inhibitor treatments.

Immunocytochemistry

Synovial cells were sedimented on 12-mm glass coverslips. On culture day 7, cells were fixed with 1% paraformaldehyde for 5 min in a phosphate-buffered salt (PBS) solution containing 136.9 mM NaCl, 2.1 mM KCl, 3.2 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4. Fixed cells were permeabilized in 1% Triton X-100 for 5 min, washed twice with PBS solution, and then incubated for 1 h with specific monoclonal mouse antibody against human CD68 (15) or monoclonal mouse antibody against human pro-collagen I in PBS containing 2 mg/ml bovine serum albumin. After washing with PBS solution, cells were incubated for 20 min with rhodamine red-conjugated goat anti-mouse IgG or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, washed again with PBS solution, mounted with Slow-Fade to prevent photobleaching, and then viewed using confocal microscopy (GB200; Olympus, Tokyo) on an Olympus ×60 SPlanApo oil objective for high resolution. Aperture, gain and black level for imaging acquisition were maintained at a constant level.

Cell counting studies

After 7-day incubation periods, the cells were pretreated with various inhibitors or vehicles for 1 h at 37°C, and then stimulation with epinephrine or norepinephrine was started. Pertussis toxin (PTX) was applied 12 h before the stimulation to inactivate inhibitory G protein (Gi) (16). Cells were incubated for 3 weeks in the total kinetic studies, and the media were changed every 3 or 4 days with the same DMEM containing the respective drugs. The stimulants with or without inhibitors were present throughout the whole experimental period. Phorbol ester, U-73122, U-73343, calphostin C and PD98059 were dissolved in dimethyl sulfoxide (DMSO) as stocks and the final concentration of DMSO was less than 0.01%, which did not show any toxic effects and did not affect the changes in synoviocyte populations. On the 7th and 14th day after stimulation, A and B cells were counted by direct observation with phase-contrast microscopy (Olympus) within a fixed area (1 × 2 mm²) marked on the scaled outer surface of the dish bottom.

Western blot analysis of PKC translocation

On culture day 7, synovial cells were stimulated with norepinephrine for the indicated durations followed by harvest. Preparation of membranes and cytosol was performed at 4°C, as described previously (17) with a slight modification. The cells were homogenized in a 0.25 M sucrose buffer (pH 7.4) containing 20 mM Tris, 1 mM EDTA, 10 mM EGTA, 20 µg/ml leupeptin, 20 µg/ml aprotinin and 0.4 mM phenylmethylsulfonyl fluoride and then centrifuged at 100,000 × g for 5 min to remove cell debris. The upper fraction was centrifuged again at 100,000 × g for 10 min. The resulting pellet was resuspended in the homogenization buffer, the supernatant being concentrated using centrifugal filtration (ultrafree MC; Millipore, Bedford, MA, USA), and they were saved as membrane and cytosol fractions, respectively. Protein concentrations of the samples were measured by the method of Lowry et al. (18). The membrane and cytosol fractions were mixed with a SDS-Laemmli sample buffer and heated to 100°C for 2 min. Proteins (membrane: 50 µg, cytosol: 30 µg) were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to an immobilized P membrane (Millipore) using electroblotting apparatus. The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5) overnight at 4°C. After washing with TBS containing 0.1% Tween 20, the membrane was incubated with specific antibodies for 8 different PKC
isoforms for 2 h at room temperature. Anti-α-, βII-, γ-, δ-, ε-, ζ- and η PKC polyclonal antibodies were purchased from Oxford Biomedical Research (Oxford, MI, USA), and the anti-βIII polyclonal antibody was prepared as described in the previous report (19). The membrane was then washed and incubated for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulin. Reactive bands were visualized using the ECL-enhanced chemiluminescence method. The signal intensities were determined with a densitometer (DMU-33C; Advantec Digital Densitolor, Tokyo).

**Immunostaining of PKC and confocal laser microscopy**

Synovial cells were sedimented on 12-mm glass coverslips. On culture day 7, cells were exposed to epinephrine, with or without yohimbine, for 10 min, and fixed for 5 min in PBS solution containing 3.7% formalin. Fixed cells were permeabilized in 1% Triton X-100 for 5 min, washed twice with PBS solution, and then incubated for 1 h with specific antibodies for PKC isoforms in PBS containing 2 mg/ml bovine serum albumin. After washing with PBS solution, cells were incubated for 20 min with FITC-conjugated goat anti-rabbit immunoglobulin, washed again with PBS solution, mounted with Slow-Fade and then viewed using confocal microscopy (GB200) as described in “immunocytochemistry”.

**Cloning of synovial A cells**

Synovial A and B mixed cells prepared from rabbit knee joints were suspended in the DMEM as mentioned in “preparation of synovial cells” and placed on 100-mm culture dishes. Aliquots of the floating cells were microscopically aspirated with a micropipette and dropped onto culture dishes. Aliquots of the floating cells were microscopically aspirated with a micropipette and dropped onto 60-mm culture dishes. After a week, cells, except for scopically aspirated, were all punctuated with a 27-gauge needle under 60-mm culture dishes. After a week, cells, except for

**Drugs**

Epinephrine, norepinephrine, prazisin, propranolol and yohimbine were obtained from Wako Pure Chemical (Osaka). Phentolamine, oxymetazoline, UK14304 (5-bromo-6-(2-imidazolin-2-ylamino) quinoxaline), PTX, phorbol ester and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). U-73122 (\{1-[6-((17β-3-methoxy-estra-1,3,5(10)-tri-en-17-yl)amino)hexyl]-1H-pyrole-2,5-dione\}), U-73343 (\{1-[6-((17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)amino)hexyl]-2,5-pyrrolidinedione\}), calphostin C, wortmannin and PD98059 (2'-amino-3'-methoxyflavone) were purchased from Calbiochem (La Jolla, CA, USA). Collagenase (type II) was obtained from Worthington Biochemical Co. (Malvern, PA, USA). DMEM and fetal calf serum were obtained from Flow Laboratories (Irvine, Scotland). Monoclonal mouse antibodies against human CD68 and against human procollagen I were purchased from Pharmingen-Fujisawa Co. (Tokyo) and Chemicon International, Inc. (Temecula, CA, USA), respectively. Rhodamine red-conjugated goat anti-mouse IgG and Slow-Fade were obtained from Molecular Probes (Eugene, OR, USA). FITC-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-rabbit immunoglobulin were Cappel, ICN Pharmaceutical (Aurora, OH, USA). ECL-enhanced chemiluminescence was from NEN Life Science Product (Boston, MA, USA).

**Statistical analyses**

Statistical analysis was performed by an analysis of variance (ANOVA) followed by the Fisher PLSD test using statistical data analysis software (Stat View; Abacus Concepts, Inc., Berkeley, CA, USA). The differences with \( P \) values of less than 0.05 were considered significant.

**RESULTS**

**Effects of epinephrine on the numbers of synovial A and B cells**

Figure 1 (upper panels) shows the typical cellular images of 7-day-old cultured rabbit synovial A type (round macrophage-like) and B type (elongated fibroblast-like) cells morphologically evaluated with phase-contrast microscopy. Lower panels in Fig. 1 show the typical immunofluorescence stainings of A and B cells with their markers, anti-CD68 (15) and anti-pro-collagen I antibodies, respectively. A-cell-like cells were CD-68-positive (lower, left panel) and pro-collagen I-negative (lower, right panel), B-cell-like cells vice versa. Thus, the optical identification of A and B cells was rationalized. The contamination of dendritic cells was not observed within the visual field.

Figure 2 shows the changes in the synoviocyte population in the presence or absence of epinephrine. On the 7th culture day (the starting point of stimulation), synoviocytes were grown at a low density (41 ± 3.8/1 × 2 mm\(^2\) area, \( n = 10 \)) and the percentages of A and B cells within this area averaged 35% and 65%, respectively. In unstimulated cell groups (control), total synoviocytes time-dependently proliferated with a decrease in the number of A cells and a marked increase in the number of B cells, reaching a plateau level (that is confluent state) at 3 weeks after plating. In contrast, epinephrine at a concentration of 10\(^{-7}\) M reduced the increase in total cell number by approximately one half throughout the treatment period. However, when characterized with each change in the A or B cell popula-
tion, this stimulant was found to induce a time-dependent increase in the number of A cells and a reduction in B cell proliferation. Therefore, we analyzed the effect of epinephrine on the cell population of each cell type (Fig. 3). The data in Figs. 2 and 3 were derived from the same experimental series. Type A and B cell mixture cultured at low density (A cell, 18 ± 2.3/2 mm² area; B cell, 23 ± 4.3 /2 mm² area) were treated with epinephrine (10⁻⁸ to 10⁻⁶ M) for 2 weeks. In A cells, epinephrine inhibited the decrease in cell number observed in the control and conversely, increased this cell population in a dose- and time-dependent manner. In contrast, this agent at concentrations ≥10⁻⁸ M inhibited the spontaneous increase in the number of B cells, with the near maximum effect at 10⁻⁷ M. The level of proliferative activity of B cells was 40% and 55% of each control at 1 and 2 weeks after the treatment, respectively.

Adrenoceptor subtype involved in synovial cell number modulation

To determine the adrenoceptor subtype responsible for epinephrine action on the synoviocyte population, we first examined the effect of the α- or β-adrenoceptor antagonists on the epinephrine-induced changes in the type A and B cell numbers/2 mm² area (Fig. 4: A and B). In the A and B cell mixture, both the increasing (type A) and decreasing (type B) effects on the cell number were blocked by the α-antagonist phentolamine (1 μM), but not by the β-antagonist propranolol (1 μM), indicating the involvement of the α-adrenoceptor in these responses. Therefore, in the following experiments, we used norepinephrine instead of epinephrine as an α-stimulator. Norepinephrine at 10⁻⁷ M, a submaximum concentration (10⁻⁸ to 10⁻⁶ M), also increased and decreased the numbers of synovial A and B cells, respectively, with almost the same potency as epinephrine. To further determine whether such norepinephrine actions were mediated by the α₁- or α₂-adrenoceptor, the effects of specific receptor antagonists for these receptor subtypes were applied (Fig. 4: C and D). Both responses in type A and type B cells were blocked by the α₂-antagonist yohimbine (1 μM), but not by the α₁-antagonist prazosin (1 μM). Furthermore, the selective α₂-adrenoceptor agonist UK14304 (1 μM) and the α₂₃-selective agonist oxymetazolin (1 μM)

Fig. 1. Typical images of rabbit synoviocytes. Upper panels: cellular images of synovial A (left) and B (right) cells with phase-contrast microscopy. Lower panels: immunofluorescence staining of synovial A (left) and B (right) cells for respective marker CD68 and pro-collagen I with confocal laser microscopy. The horizontal bars represent 10 μm.
showed the similar effects on both A and B cells as observed with norepinephrine. Thus, norepinephrine appeared to increase and decrease the number of synovial A and B cells, respectively, via $\alpha_2$-adrenoceptor (probably $\alpha_2A$)-stimulation.

Effects of the $G$ protein and phospholipase $C$ inhibitors on norepinephrine-induced modulation of cell number

Since the $\alpha_2$-adrenoceptor is coupled to Gi, which in turn triggers multiple signal transduction systems, phospholipase C (PLC) activation and reduction in adenylate cyclase (AC) activity (20, 21), we then examined the Gi inactivator PTX and the PLC inhibitor U-73122 on the norepinephrine-induced changes in each cell number in the type A and B cell mixture. As shown in Fig. 5, the effects of norepinephrine on cell number/2 mm$^2$ area were abolished by the pretreatment with PTX (20 ng/ml) or U-73122 (1 $\mu$M), suggesting the involvement of Gi-coupled PLC stimulation. The effects of the structurally related inactive compound U-73343 (1 $\mu$M), which lacks PLC inhibitory activity, was found not to affect the norepinephrine action (data not shown).

In contrast, preaddition of dibutyric cAMP (1 mM) did not affect the norepinephrine-induced changes in cell number (data not shown), suggesting that Gi-mediated inhibition of the AC/cAMP system had no role in the norepinephrine action.

Effects of the PKC and MAP kinase inhibitors on norepinephrine-induced modulation of cell number

Activation of PLC results in phosphatidylinositol 4,5-bisphosphate (PI-4,5-P$_2$) hydrolysis leading to diacylglycerol-stimulated PKC activation. Furthermore, previous reports suggested that cell growth in response to various proliferative stimuli is, at least in part, mediated by the PKC/MAP kinase signalling pathway (22, 23). We there-
fore examined the effects of the PKC and MAP kinase inhibitors on the norepinephrine-induced changes in each cell number/2 mm² area using the mixed cultures of type A and B cells. As shown in Fig. 6, A and B, the PKC inhibitor calphostin C (1 μM) reduced both stimulatory (A cell) and inhibitory (B cell) effects of norepinephrine on cell number to nearly the respective control level at each time point. The PKC activator phorbol 12,13-dibutyrate (0.1 μM) caused
similar effects to norepinephrine on both A and B cells. MAP kinase inhibitors wortmannin (1 µM) and PD98059 (50 µM) also abolished the norepinephrine-induced increase and decrease in A and B cell numbers, respectively (Fig. 6: C and D). These inhibitors did not affect the control responses in either cell type by themselves (data not shown). These data suggest that α2-adrenoceptor stimulation by norepinephrine produced increase and decrease in A and B cell populations, respectively, probably through PKC and MAP kinase(s) activation.

**Determination of PKC βII activation in response to norepinephrine using Western blot and immunocytochemistry**

Among the antibodies for 8 different PKC isozymes (α, βI, βII, γ, δ, ε, ζ, η) that recognized the corresponding PKC proteins in the brain or heart cytosol fractions, only PKC βII immunoreactivity was observed in the cytosol of synoviocytes as a single 79-kDa band (Fig. 7A), suggesting that PKC βII is the main isozyme in synoviocytes. Using the anti-PKC βII antibody, Western blot analyses were performed on membrane (particulate: P) and cytosolic (C) fractions from norepinephrine-treated or untreated synovial cells (Fig. 7B). Abundant expression of PKC βII was detected in the cytosolic fractions of untreated cells (control). In contrast, exposure to 10^{-7} M norepinephrine for 1 to 3 h increased the amount of immunoreactive PKC βII in the membrane fraction associated with a concomitant decrease in the cytosolic fraction. However, prolonged stimulation for a further 3 h induced loss of PKC immunoreactivity from the membrane fraction. As shown by the densitometric analyses (Fig. 7C), an approximately sevenfold increase in the particulate-to-cytosol density ratio (an index of PKC translocation) was induced by norepinephrine stimulation for 1 h, and it was completely inhibited by 5-min pretreatment with yohimbine (1 µM), suggesting that norepinephrine activated PKC βII via α2-adrenoceptor stimulation.

Since the materials used for the Western blot contained both A and B cells, we could not distinguish the cells responsible for PKC redistribution. Therefore, immunocytochemical imaging analyses at the single cell level were carried out to determine whether such a PKC βII activation occurred in type A or B cells or in both. In the preliminary study, we found that among the various PKC isozymes, βII was the main isozyme present in the synoviocyte. As the positive controls, immunoreactivities against anti-PKC α-, δ-, ε antibodies were found in the cardiac myocytes with the present fixation procedure, excluding an inhibitory effect of 3.7% formalin on the immunoreactivities of these PKC isozymes. As shown in Fig. 8, transverse optical sections observed with confocal laser microscopy, positive immunostainings for the PKC βII antibody were observed in the cytoplasmic area of both unstimulated A and B cells. In both type A and B cells stimulated with 10^{-7} M norepinephrine for 30 min, PKC βII immunoreactivities in the plasma membrane areas increased concomitantly with a decrease in those in the cytoplasmic area, and these responses were attenuated by 5-min pretreatment with yohimbine (1 µM). These findings suggest that α2-adrenoceptor-
mediated PKC/βII activation occurred in both cell types.

Effects of norepinephrine on the numbers of cloned synovial A cells

Finally, we examined whether α2-adrenoceptor-mediated increase in A cell number occurred depending on the presence of B cells, using cloned A cells. We successfully isolated synovial A cells from the rabbit synovial A and B cell mixture as ascertained by acid-phosphatase staining (24) (Fig. 9A). Under 2 weeks’ stimulation with 10^{-7} M norepinephrine, the A cell population markedly increased (Fig. 9C), compared with unstimulated cells (Fig. 9B). This
stimulatory response was significantly attenuated in the presence of the α2-agonist yohimbine (1 μM) (Fig. 9D).

Figure 10 shows the changes in cell population in the presence or absence of reagents. The populations of the cloned A cells initially grown in a low density of 18 ± 2.3 /1 x 2 mm² area were unchanged during the 2-week experimental periods. In contrast, exposure to 10⁻⁷ M norepinephrine increased the population of A cells in a time-dependent
DISCUSSION

The present study is the first report suggesting the pathophysiological role of epinephrine and/or norepinephrine in RA, especially in the synoviocyte population. In the primary cultured rabbit synovial cells with initial subpopulations of type A (macrophage-like) and type B (fibroblast-like) cells (44% and 56%), an increase in the number of total cells (Fig. 2), with a time-dependent decrease in the number of type A cells, was observed, consistent with previous reports (25, 26). A similar proliferation profile was observed in long-term-cultured synoviocytes from patients with RA (25).

Exposure of synoviocytes to epinephrine or norepinephrine apparently inhibited the spontaneous increase in total cell number in a dose- and time-dependent manner. However, these reagents differently affected each type of cell, i.e., an increase in the A cell population and a decrease in the B cell population (Fig. 3). These responses were blocked by phentolamine or yohimbine, but not by propra-
nolol or prazosine (Fig. 4: A – D) and were mimicked with selective α₂-adrenoceptor agonists UK14304 and oxymetazolol indicating the involvement of α₂-adrenoceptor (probably α₂A) stimulation in this response. The actions of epinephrine/norepinephrine did not differ between cultures containing 10% and 1% serum (data not shown); therefore, we excluded the influence of serum on the present experiments.

To further clarify the intracellular mechanisms for this α₂-adrenoceptor-mediated cell number regulation, we evaluated the effects of several kinds of agents that interfere with signalling effectors such as G protein, PLC and AC. Both the increasing (A cell) and decreasing (B cell) effects of norepinephrine on the cell number were almost completely inhibited by the G protein (Gi/o) inactivator (PTX) and the PLC inhibitor (U-73122) (Fig. 5: A and B). These findings suggest that α₂-adrenoceptor stimulation regulates the populations of type A and B synoviocytes through PTX-sensitive G protein (Gi) mediated PLC activation. Recently, βγ subunits from heterotrimeric PTX-sensitive G protein have been shown to stimulate PLC β under the stimulation of various Gi protein-coupled receptors, including the α₂-receptor (21, 27, 28). In synoviocytes, we speculate that the βγ subunit dissociated from α₂-adrenoceptor-coupled Gi may stimulate PLC, resulting in phosphoinositide hydrolysis and PKC activation.

PKC has been implicated in the regulation of cell proliferation by triggering the activation of downstream signalling transducers including P42-40 MAP kinase (22, 23, 29). In the present study, norepinephrine-induced up- and down-regulation of type A and B cell numbers were completely inhibited by not only the PKC inhibitor calphostin C, but also the MAP kinase(s) inhibitors PD98059 and wortmannin. In addition, a phorbol ester mimicked these norepinephrine actions (Fig. 6). From these findings, activation of PKC/MAP kinase(s) signalling appears to be involved in α₂-adrenoceptor-mediated regulation of synoviocyte populations. However, we cannot completely exclude the possibility that additional cell-cycle regulatory signalling, such as phosphatidylinositol 3-kinase (PI3-kinase) (30) is involved, since wortmannin also inhibits this kinase at the phosphatidylinositol 3-kinase (PI 3-kinase) (30) is involved in this study (31).

To assess the potential specificity of the PKC isozyme mediating the norepinephrine-induced regulation of synoviocyte populations, immunoblotting and immunocyto-staining with confocal microscopy were employed. Recent biochemical and molecular analyses have revealed that PKC consists of several isozymes classified into the conventional Ca²⁺-dependent- (the cPKCs, α, βII, βI, γ) and the novel Ca²⁺-independent-forms (the nPKCs, δ, ε, ζ, η) (14, 19). When activated by extracellular stimuli, these kinases are translocated from the cytosol to plasma membranes to exert kinase activities. Using two analysis systems, we have shown for the first time that the main PKC isozyme present in rabbit synovial cells was the βII type and that this isozyme was translocated to the membrane fraction under α₂-adrenoceptor stimulation in both A and B type cells (Figs. 7 and 8). Such α₂-adrenoceptor-mediated activation of PKC was also reported in a recent study using chick-sympathetic neurons (32). As shown in Fig. 7A, activation of PKC βII in both A and B cells occurred transiently in the early stimulation period. Since calphostin C, PD98059 and wortmannin completely inhibited the cell number responses to norepinephrine, such activation of PKC βII appeared to trigger the downstream signaling cascade, including MAP kinase(s) activation which subsequently induced long-term regulation of the respective populations of A and B cells. How PKC βII/MAP kinase(s) signaling differentially regulates type A or B cell numbers is still unknown. More recently, in various cell types, this PKC isozyme has been reported to stimulate cell growth, i.e., proliferation or hypertrophy through progression of the cell cycle from the G2 phase to the M phase (33) or enhanced expression of transcription factors including immediate early gene products such as c-fos (34). In addition, PKC has been shown to inhibit the induction of apoptosis via a MAP kinase-dependent pathway (35). Either mechanism may be responsible for the stimulatory effects on the type A cell population. On the other hand, PKC is also reported to induce cell-cycle arrest correlated with the enhanced induction of the cyclin-dependent kinase inhibitors P21waaf1 and P27kip1 in intestinal epithelial cells (36) and of apoptotic signalling in thymocytes exposed to dexamethasone (37). Such mechanisms may be involved in the norepinephrine-induced inhibition of B cell proliferation. There is a further possibility that the increase in type A cell population inhibits proliferation of B cells through enhanced secretion of certain cytokines. These are subjects for future study.

The final experiment was carried out to find out whether the norepinephrine-induced increase in the number of A cell depends on the presence of B cells. In the cloned A cells, exposure to norepinephrine caused a time-dependent increase in cell number (Fig. 10). In this culture system, the time-dependent decrease of the number of A cells as seen in mixed culture with B cells (Fig. 2A) was not observed. The reason for this difference is unclear. However, it may be explained by the absence of B cells, since B cells reportedly release IL-6 that induces apoptosis of macrophages (38). Although the existence of the α₂-adrenoceptor in synovial cells has not been clarified yet, the antagonistic effect of yohimbine on this norepinephrine action of norepinephrine suggests that the number of type A cells increased through the stimulation of their own α₂-adrenoceptors. Such an increased number of A cells is consistent with the pathohistological features of synovial tissue during the
active phase of RA (2).

α₂-Adrenergic receptor-mediated modification of arthritis severity has also been demonstrated in arthritis rats, in which chronic treatment with a high (0.5 mg/kg) or low (20 µg/kg) dose of epinephrine reduced or exacerbated arthritis signs such as soft-tissue swelling and bone destruction, respectively (39, 40). Furthermore, catecholamine may also act on blood vessels as on the synovial cells and exacerbate arthritis by altering blood flow and/or vascular permeability. However, there is a conflicting evidence in rat paw that norepinephrine does not affect the inflammatory response such as afferent neuron-mediated plasma extravasation (41). Therefore, the contribution of norepinephrine in the inflammatory process may differ depending on the tissue type or drug concentration.

In conclusion, this study showed the effects of catecholamine on the synoviocyte population (Fig. 11, schematic illustration). Stimulation of type A and type B synoviocytes with norepinephrine or epinephrine produced an increase and decrease in the respective cell number, probably through their own α₂-adrenergic receptor-coupled G/PLC activation and resulting stimulation of the PKC βII/MAP kinase(s) signaling pathway. The selective inhibition of PKC βII in a limited area (for example, the synovial tissue) may be a new strategy to prevent catecholamine-mediated development of joint injury.

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