

details). The model correctly reproduced the voltage-dependent slow increase in experimental current relaxation (figure 3c(ii)). The time constant of relaxation in the model was also calculated and compared with the experimental data (figure 3c(iii)). The time constants of relaxation in simulated data did not show clear voltage dependence and coincided with the experimental data well except at  $-60$  and  $-80$  mV in  $0.3 \mu\text{M}$  ACh where the amplitudes of the relaxation were too small to be accurately assessed.

Figure 3d shows the voltage dependence of the  $K_{\text{ACh}}$  channel relative  $NP_o$  in the presence of  $0.1$  and  $0.3 \mu\text{M}$   $[\text{ACh}]_0$ . The symbols correspond to the experimental results and the lines are the results calculated from the integrated model. As  $[\text{ACh}]_0$  was decreased, the  $K_{\text{ACh}}$  channel relative  $NP_o$  decreased especially at depolarized potentials because of the modulated action of RGS proteins represented by the voltage-dependent  $k_6$ . The integrated model developed in this study can therefore quantitatively account for the voltage- and ACh-dependence of the  $K_{\text{ACh}}$  current relaxation behaviour.

Figure 4a shows experimental data examining the effects of prepulse ( $+40$  mV) duration on the relaxation behaviour of the  $K_{\text{ACh}}$  current at  $-100$  mV. As  $[\text{ACh}]_0$  was increased, the instantaneous component increased and the time-dependent relaxation component became smaller. This may indicate that the effects of RGS proteins are attenuated as  $[\text{ACh}]_0$  is increased. At each  $[\text{ACh}]_0$ , as the duration of prepulse was prolonged, the instantaneous component decreased and the relaxation component increased. This implies that the effect of the RGS protein action develops in a time-dependent manner during the prepulse, because the instantaneous component reflects the  $K_{\text{ACh}}$  channel  $NP_o$  at the end of the prepulse. In GTP $\gamma$ S-loaded atrial cells, the prepulse duration did not affect the  $K_{\text{ACh}}$  current. This is consistent with the idea that the effect of prepulse duration on  $K_{\text{ACh}}$  current is mediated by the voltage- and time-dependent action of RGS proteins.

In figure 4b(i)(ii), the temporal characteristics were extracted from the experimental data by normalizing relative to the data obtained with GTP $\gamma$ S. The normalized currents clearly demonstrated the prepulse duration-dependent development of relaxation (figure 4b, dotted lines). The model correctly reproduces the experimental results (figure 4b(iii)(iv)). Both the model and experimental results also showed that the alterations of the instantaneous and relaxation components depend on  $[\text{ACh}]_0$ . Therefore, the model successfully replicates the characteristics of  $K_{\text{ACh}}$  channel current relaxation.

### (iii) *The temporal and steady-state behaviour of ACh-induction of $K_{\text{ACh}}$ current*

Figure 5a shows the temporal behaviour of the ACh-induced  $K_{\text{ACh}}$  channel current simulated with the improved integrated model. When ACh was applied, the  $K_{\text{ACh}}$  current was induced with a time constant of 2–8 s. Upon removal of ACh from the external solution, the  $K_{\text{ACh}}$  current was deactivated with a time constant of 6–12 s. These values are similar to those in physiological activation and deactivation and much smaller than those calculated with the previous models which do not take the voltage-dependent RGS action into account. In the simulated  $K_{\text{ACh}}$  currents, differences in the activation time course at different membrane potentials were not obvious, while those in the deactivation time

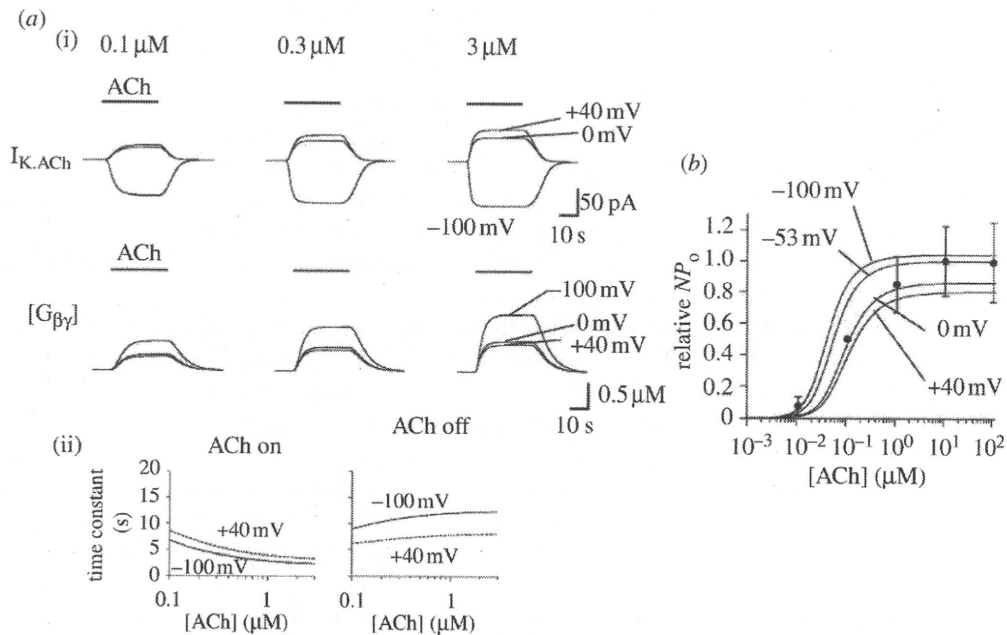


Figure 5. The relationship between  $K_{ACh}$  channel open probability and the concentrations of GTP and ACh obtained by the improved integral model. (a) (i) The simulated time courses of activation and deactivation phases of 0.1, 0.3 and 3.0  $\mu\text{M}$  ACh-induced  $K_G$  channel currents and  $G_{\beta\gamma}$  on steps of various membrane voltages. (ii) Simulated time constants for activation and deactivation phases. (b) The relationship between the whole cell  $K_{ACh}$  current and ACh concentration. Symbols represent data from Kurachi *et al.* (1987), lines represent data obtained from the model at the indicated voltages. See text for further details.

course were evident. The deactivation of  $K_{ACh}$  current was faster at depolarized potentials than at hyperpolarized potentials. This phenomenon agrees with experimental results (half deactivation times of 6.3 ms at 0 mV and 15.7 ms at -80 mV; Ishii *et al.* 2001).

In figure 5b, the  $[ACh]_0$  dependence of the steady state  $K_{ACh}$  current was calculated with the improved integrated model at various membrane potentials. The experimental data at -53 mV were taken from Kurachi *et al.* (1987; figure 5b, circles). Experimentally, as  $[ACh]_0$  was increased, the whole cell  $K_{ACh}$  current was augmented in a concentration-dependent manner. The concentration-response relationship in the model was similar to the experimental data except at intermediate ACh concentrations (figure 5b, line -53 mV). Because the quasi-steady-state current was measured approximately 1 min after the onset of ACh application, the experimental data contain the effect of short-term desensitization. This may explain at least in part the difference between experimental data and the simulation data with the new model. The model results also suggest a novel possibility that the membrane potential affects the steady-state relationship between  $K_{ACh}$  current and  $[ACh]_0$ : as the membrane is more depolarized, the sensitivity of the  $K_{ACh}$  channel to ACh decreases. This possibility should be examined experimentally.

#### 4. Discussion

This study presents the further development of a mathematical model to simulate the kinetics of G<sub>K</sub>-protein signalling and the K<sub>ACH</sub> current in the heart. This integrated model reproduced major characteristics of the K<sub>ACH</sub> current, including steady-state relationships and temporal behaviour. Two experimental results played essential roles in the improvement of this model: (i) the biochemical assay of G<sub>βγ</sub> binding to K<sub>ACH</sub> channels and (ii) the discovery of the involvement of RGSs in the control of the K<sub>ACH</sub> channel activity.

Using these two key experimental advances, we improved the integrated model for ACh activation of the K<sub>ACH</sub> channel (Hosoya *et al.* 1996; Hosoya & Kurachi 1999). Now with the new integrated model, we can reproduce the fast onset and offset of the K<sub>ACH</sub> current upon application and removal of ACh. The calculated time constants for activation and deactivation are close to the physiological experimental data. However, we recognize that the activation of K<sub>ACH</sub> current in experiments is yet faster than that of the model. We currently hypothesize that this may be related to the high affinity and low affinity properties of muscarinic receptors.

The deactivation time course of the simulated K<sub>ACH</sub> current is quite in the physiological range. In simulation, as the membrane is depolarized, the deactivation becomes faster. This is also consistent with experimental observations reported in Ishii *et al.* (2001), and may be due to the higher GTPase accelerating activity of RGS proteins at depolarized potentials.

The simulated steady-state relationship between the K<sub>ACH</sub> current and [ACh]<sub>0</sub> is shown in figure 5*b*. As the membrane is depolarized, the relationship shifts to the right and the maximal K<sub>ACH</sub> channel activity is attenuated. This is also due to the higher activity of RGS proteins at depolarized potentials. But such an effect of the membrane potential on the relationship has not been yet reported *in vitro* and should be examined experimentally.

Models of processes associated with G-protein intracellular signalling may have practical applications. For example, an estimated 50 per cent of current pharmaceuticals target GPCR (Howard *et al.* 2001) and models may be useful tools to examine drug effects on G-protein signalling. The contributions of G-protein signalling to atrial fibrillation (AF) may be a good example. Hirose *et al.* showed that while G<sub>αq</sub>-TG mice activate the IP3 and DAG pathways and other intracellular signalling pathways to promote cellular hypertrophy, the over-expression of DAG kinase isoform DGKζ in the hearts of these mice prevented the generation of atrial arrhythmias including AF (Hirose *et al.* 2009). This suggests that therapies targeting G<sub>αq</sub> and/or DAG-mediated signalling may prevent atrial arrhythmogenic remodelling and models of the appropriate signalling pathways in the atria may help to examine theoretical possibilities. The K<sub>ACH</sub> current has been proposed to contribute to AF. Dobrev *et al.* (2005) and Cha *et al.* (2006) suggest that a constitutively active K<sub>ACH</sub> current contributes to enhanced basal K<sup>+</sup> conductance and action potential shortening in chronic AF and blockade of the K<sub>ACH</sub> current has become a new therapeutic target in AF. K<sub>ACH</sub> channel blockers are under development and among them tertiapin and NIP-151 show effectiveness in terminating AF (Hashimoto *et al.* 2006, 2008). Differential phosphorylation-dependent regulation of the ion channel might underlie this constitutively active K<sub>ACH</sub> current (Voigt *et al.* 2007). It will be interesting to use our model to

investigate possible sources of constitutive  $K_{ACh}$  channel activity—activation of  $K_{ACh}$  channels by GTP in the absence of ACh (Ito *et al.* 1991) and the loss of  $Ca^{2+}$ -CaM regulation of RGS proteins might prove to be attractive targets for further investigation. In which context it is interesting to note that knock-out of the RGS4 protein abolished desensitization of the response to ACh in the SA node in mice (Cifelli *et al.* 2008). A major limitation of the model presented here is that it cannot reproduce desensitization, and this after considerable effort. This may be because the model consists of linear systems subjected to a simple input (ACh concentration) which cannot reproduce a complex response such as desensitization. Further investigation of the function of RGS proteins may contribute to the understanding of sinus bradycardia.

Another limitation is that the experimental data used to develop the model were from different species (rat, guinea pig). Though the different expression levels of ion channels between species lead to different action potential forms, the characteristics of  $K_{ACh}$  channels in mammalian atrial myocytes do not seem to be so different and the species difference needs to be noted but it should not cause significant problems in the present modelling study which focuses on ion channel level phenomena.

In conclusion, progress in experimental research, such as the assay of  $G_{\beta\gamma}$  binding to the  $K_{ACh}$  channels and the discovery of RGS proteins' action upon the  $K_{ACh}$  current, was essential for the development of this mathematical model for ACh activation of the  $K_{ACh}$  current. The simulation analyses raise further questions concerning the physiological behaviour of the  $K_{ACh}$  current, which indicates the necessity of new experiments. Experiments and simulation thus complement each other for the further understanding of physiological systems.

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## Appendix A

### (a) Allosteric model

The  $G_{K\beta\gamma}$  generated by the  $G_K$  protein cycle model was taken into the allosteric model and then the channel availability was calculated with the following equation:

$$NP_o = \frac{(1 + L(K_R/K_T)^4)(1 + ([G_{\beta\gamma}]/K_R))^4}{L(1 + (K_R/K_T)([G_{\beta\gamma}]/K_R))^4 + (1 + ([G_{\beta\gamma}]/K_R))^4}. \quad (A 1)$$

### (b) G-protein cycle model

In the  $G_K$ -protein cycle model (figure 1*d(ii)*), six values of concentrations in the G-protein cycle are calculated with the following six ordinary differential

equations:

$$\frac{d[R]}{dt} = -k_1[A][R] + k_{-1}[AR], \quad (\text{A } 2)$$

$$\begin{aligned} \frac{d[AR]}{dt} = & k_1[A][R] + k_{-2}[ARG-GDP] + k_3[ARG-GTP] \\ & - k_{-1}[AR] - k_2[AR][G-GDP], \end{aligned} \quad (\text{A } 3)$$

$$\begin{aligned} \frac{d[ARG-GDP]}{dt} = & -k_{-2}[ARG-GDP] + k_2[AR][G-GDP] \\ & - k_{3,4}[ARG-GDP][GTP] \\ & + k_{-4,-3}[ARG-GTP][GDP], \end{aligned} \quad (\text{A } 4)$$

$$\begin{aligned} \frac{d[ARG-GTP]}{dt} = & k_{3,4}[ARG-GDP][GTP] \\ & - k_{-4,-3}[ARG-GTP][GDP] \\ & - k_5[ARG-GTP], \end{aligned} \quad (\text{A } 5)$$

$$\frac{d[G-GTP]}{dt} = k_5[ARG-GTP] - k_6[G-GTP] \quad (\text{A } 6)$$

and

$$\begin{aligned} \frac{d[G-GDP]}{dt} = & k_6[G-GTP] + k_{-2}[ARG-GDP] \\ & - k_2[AR][G-GDP], \end{aligned} \quad (\text{A } 7)$$

where A is ACh, R is m<sub>2</sub>-receptor, G is G<sub>K</sub> protein, and *k* are reaction rate constants. The value of each rate constant was fixed, except for *k*<sub>6</sub> (table 1). Since GTPase activity of G<sub>Kα</sub> in atrial myocytes can be modulated by RGS proteins in a voltage-dependent manner (Ishii *et al.* 2001), *k*<sub>6</sub> is defined as a function of membrane voltage as follows:

$$k_6 = 1.13 \left( 1 + \frac{1.30}{1 + e^{(-0.042(V+25))}} \right), \quad (\text{A } 8)$$

where *V* is the membrane potential.

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## ANG II inhibits insulin-mediated production of PI 3,4,5-trisphosphates via a $\text{Ca}^{2+}$ -dependent but PKC-independent pathway in the cardiomyocytes

Masashi Ikushima,<sup>1,2</sup> Masaru Ishii,<sup>1</sup> Mitsuru Ohishi,<sup>2</sup> Koichi Yamamoto,<sup>2</sup> Toshio Ogihara,<sup>2</sup> Hiromi Rakugi,<sup>2</sup> and Yoshihisa Kurachi<sup>1</sup>

Departments of <sup>1</sup>Pharmacology and <sup>2</sup>Geriatric Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

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**Ikushima M, Ishii M, Ohishi M, Yamamoto K, Ogihara T, Rakugi H, Kurachi Y.** ANG II inhibits insulin-mediated production of PI 3,4,5-trisphosphates via a  $\text{Ca}^{2+}$ -dependent but PKC-independent pathway in the cardiomyocytes. *Am J Physiol Heart Circ Physiol* 299: H680–H689, 2010. First published July 2, 2010; doi:10.1152/ajpheart.00220.2009.—Insulin resistance (IR) is a condition where different organs are refractory to insulin stimulation of glucose uptake. ANG II has been suggested to be involved in the development of IR in the heart. The precise mechanism by which this occurs is still unknown. Here we have used dynamic fluorescent imaging techniques to show that ANG II inhibits insulin production of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>] in cardiac myocytes. Fluorophore (Venus)-conjugated cAMP-dependent protein kinase-pleckstrin homology domain, which specifically binds to PI(3,4,5)P<sub>3</sub>, was transfected in neonatal rat cardiac myocytes. Insulin induced a robust increase in the fluorescence intensity at the cell surface, which was diminished by application of ANG II. The inhibitory action of ANG II was antagonized by RNH-6270 (an angiotensin type 1 receptor antagonist) but not by PD-122370 (an angiotensin type 2 receptor antagonist). BAPTA-AM ( $\text{Ca}^{2+}$  chelator) largely attenuated the ANG II effect, whereas K-252b (PKC inhibitor) did not. Furthermore, an elevation of intracellular  $\text{Ca}^{2+}$  induced by ionomycin mimicked the ANG II effect. Therefore, it is suggested that ANG II antagonizes insulin-mediated production of PI(3,4,5)P<sub>3</sub> via a  $\text{Ca}^{2+}$ -dependent but PKC-independent pathway in cardiac myocytes.

protein kinase C; angiotensin type 1 receptor; heart; adenosine 3',5'-cyclic monophosphate-dependent protein kinase; real-time imaging; fluorophore

INSULIN IS ESSENTIAL FOR CONTROL OF energy metabolism to maintain appropriate cellular homeostasis. A number of pathological factors severely attenuate the sensitivity to insulin in a variety of tissues, including skeletal muscle (43), adipocytes (6), and heart (1, 30, 39). This abnormal condition, called “insulin resistance” (IR), is known to be a predisposition or even a direct genesis of various common diseases, such as diabetes mellitus and atherosclerosis. In the heart, IR is reported to be critically involved in dysfunction of cardiac myocytes and closely related to some cardiac disorders (17, 30, 39).

The downstream signaling of insulin stimulation has been extensively studied (15, 23, 38). The stimulation of insulin receptor induces activation of phosphatidylinositol 3-kinase (PI 3-kinase) via insulin receptor substrate-1 (IRS-1). The active form of PI 3-kinase then generates phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>] from phosphatidylinositol 4,5-bisphosphate on the membrane (10). PI(3,4,5)P<sub>3</sub> can interact with the pleckstrin

homology (PH) domain of a Ser/Thr kinase cAMP-dependent protein kinase (Akt)/PKB, which phosphorylates various downstream target molecules.

Recent studies have suggested that activation of ANG II type 1 receptor, AT<sub>1</sub>, attenuates insulin signaling, and thus contributes to the pathogenesis of IR in various tissues (7, 36). Stimulation of AT<sub>1</sub> leads to activation of phospholipase C- $\beta$  (PLC- $\beta$ ) (9) via a subtype of heterotrimeric G proteins, G<sub>q</sub> (28, 35), and thus triggers phosphoinositide signaling cascades, which finally result in the elevation of intracellular  $\text{Ca}^{2+}$  concentration and activation of protein kinase C. A recent report showed that ANG II inhibits the insulin-mediated phosphorylation of Akt, a downstream signaling of PI(3,4,5)P<sub>3</sub>, through PKC- $\alpha$  in vascular smooth muscle cells (VSMC) (22). The interaction between insulin and ANG II in cardiac myocytes, however, has not been well studied.

In this study, we examined the effects of insulin and ANG II on PI(3,4,5)P<sub>3</sub> in cardiac myocytes with dynamic visualization of PI(3,4,5)P<sub>3</sub>. PI(3,4,5)P<sub>3</sub> is the product generated by insulin-mediated activation of PI 3-kinase and thus serves as a good indicator of insulin-mediated signaling cascades (5, 25, 27). For this purpose, we exploited a real-time imaging technique using a fluorophore-conjugated Akt-PH domain, which specifically binds to PI(3,4,5)P<sub>3</sub> in living cells. In result, we detected that ANG II competes insulin-mediated PI(3,4,5)P<sub>3</sub> production in a  $\text{Ca}^{2+}$ -dependent mechanism in cardiac myocytes. Application of BAPTA-AM ( $\text{Ca}^{2+}$  chelator), but not K-252b (PKC inhibitor), blocked the effect of ANG II. Ionomycin but not phorbol ester mimicked the effect of ANG II. Therefore, different from VSMC, ANG II may antagonize insulin action via a  $\text{Ca}^{2+}$ -dependent and PKC-independent pathway in cardiac myocytes.

### MATERIALS AND METHODS

**Reagents and plasmid.** Insulin, ANG II, wortmannin, ionomycin, phorbol 12-myristate 13-acetate (PMA), and PD-123319 were all purchased from Sigma-Aldrich (St. Louis, MO). K-252b was purchased from BIOMOL International. 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Peptide Institute (Minoh, Osaka, Japan). RNH-6270, an active metabolite of olmesartan medoxomil, a specific AT<sub>1</sub> receptor antagonist, was supplied by Daiichi Sankyo (Tokyo, Japan). A fluorophore, Venus (kindly provided from Dr. Atsushi Miyawaki, RIKEN-BRC, Wako, Japan) fused construct with Akt-PH domain, was the generous gift from Dr. Tadaomi Takenawa (University of Tokyo, Tokyo, Japan). H9c2 cells, a clonal line derived from embryonic rat heart, were obtained from American Type Culture Collection (CRL-1446).

**Cell dissociation and culture.** All animal experiments were performed in accordance with the guidelines for the use of laboratory animals of the Osaka University Graduate School of Medicine. The

Address for reprint requests and other correspondence: H. Rakugi, Dept. of Geriatric Medicine, Osaka Univ. Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan (e-mail: rakugi@geriat.med.osaka-u.ac.jp).

study protocol was approved by our institution. Primary cultures of neonatal rat cardiac myocytes were prepared from the hearts of 1- to 2-day-old Wistar rats (Nippon Dobutsu, Osaka, Japan) as previously described (32). In brief, hearts were quickly removed from deeply anesthetized neonatal rats, minced, and digested with 0.125% trypsin (Becton-Dickinson, Sparks, MD) and 0.025% collagenase (Sigma-Aldrich) at 37°C for 1 h. Myocyte-enriched culture was achieved by preplating for 60 min to minimize the contamination of fibroblasts. Nonattached cells were then suspended in Hanks' medium-199 (M-199) (PAA Laboratories, Linz, Austria), supplemented with 10% FCS (Hyclone, Logan, UT), and cultured at 37°C in 99.5% air and 0.5% CO<sub>2</sub> for 1 day. The culture medium was changed to M-199 containing gentamycin and kanamycin (25 mg/l each) and then maintained for 2 days for the purpose of serum deprivation. On the next day, the medium was changed again and used for gene transfection. We performed immunostaining with  $\alpha$ -actinin to confirm whether the isolated cells were cardiac myocytes. More than 95% of isolated cells were cardiac myocytes (data not shown).

**Gene transfection.** We applied Nucleofector (Amaxa Biosystems) for transient transfection of the Akt-PH-Venus gene. In brief, cardiac myocytes in medium  $2 \times 10^6$  cells/ml were spun for 1 min at 340 g, and the cell pellet was resuspended in 100  $\mu$ l Nucleofector solution. We mixed 100  $\mu$ l of cell suspension with 2  $\mu$ g of the Akt-PH-Venus plasmid, placed the sample in Nucleofector, and transfected the gene. Experiments evaluating insulin-mediated production of PI(3,4,5)P<sub>3</sub> under several conditions were performed within 24–48 h postnucleo-

fection. Transfection efficiency of primary neonatal rat cardiac myocytes during this time period was ~50%, which was evaluated with cell numbers that were significantly positive with a fluorescence microscope.

**Confocal microscopy imaging.** Dynamic imaging was performed using laser scanning confocal microscopy (model LSM510; Carl Zeiss, Jena, Germany). The Axiovert 100 M microscope is equipped with a  $\times 40$  oil immersion objective (numeric aperture ~1.3). The Venus (a variant of yellow fluorescent protein) channel used the 488-nm line of an argon laser for excitation, and the emission wavelength was collected by a 500- to 535-nm bandpass filter for Venus.

**Western blotting.** Cell lysate was subjected to SDS-PAGE and was electrophoretically transferred to a nitrocellulose membrane (42). The membranes were then exposed to primary antibodies for Akt or phosphorylated (p)-Akt (dilution at 1:1,000; Cell Signaling Technology) overnight at 4°C. After incubation with the peroxidase-linked secondary antibody (dilution at 1:2,000; Cell Signaling Technology) for 1 h at room temperature, immunoreactive proteins were visualized by ECL PLUS reagent (Amersham Life Sciences).

**Measurement of glucose uptake.** To measure glucose uptake in rat cardiomyocytes (H9c2 cells), a nonradioactive assay was performed using a fluorescent D-glucose analog, 2-NBDG, as previously described (19, 41). Briefly, the cells plated on a 96-well plate at a density of 5,000 cells/well were serum depleted for 24 h. Next, the cells were incubated with the indicated drugs for 10 min, and 100  $\mu$ M 2-NBDG

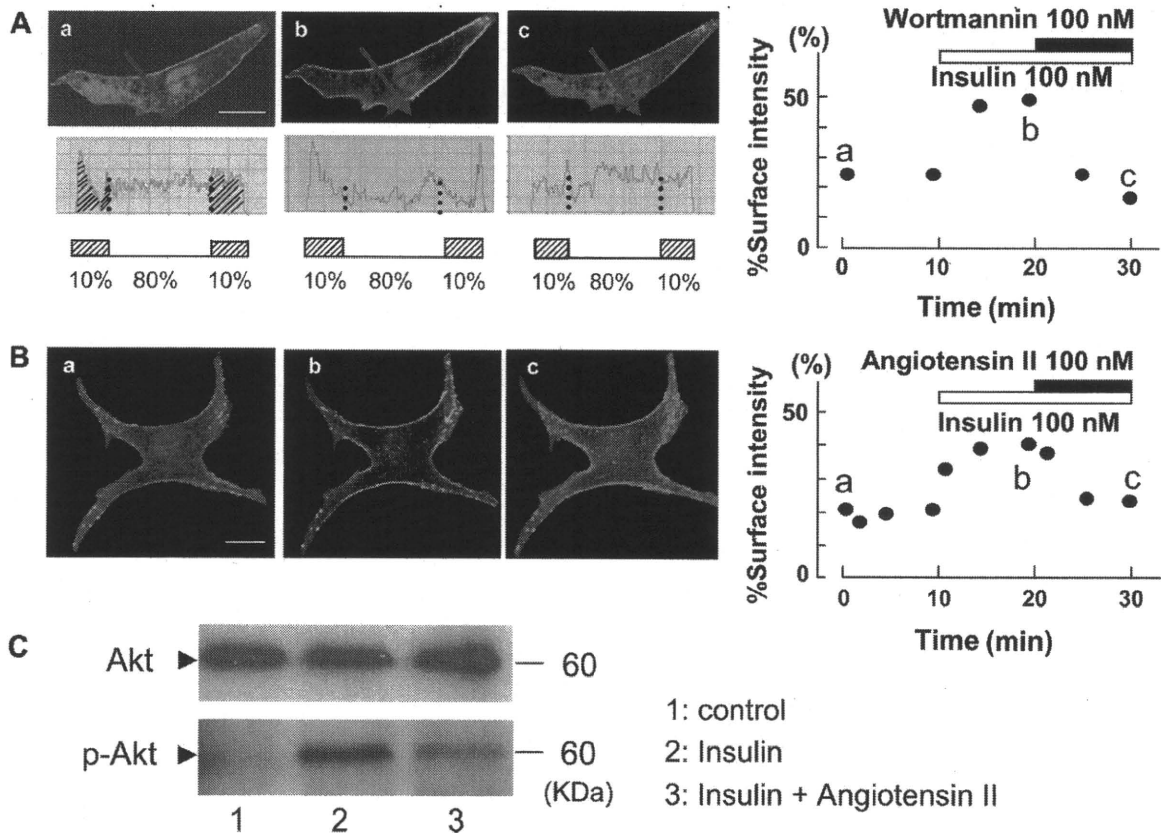


Fig. 1. Effects of ANG II on insulin signaling in cardiac myocytes. **A:** rat neonatal cardiac myocytes transfecting a fluorophore (Venus)-conjugated pleckstrin homology (PH) domain of cAMP-dependent protein kinase (Akt)/PKB were observed using a laser confocal microscope. Wortmannin prevents phosphatidylinositol trisphosphate production. *a*, In the basal state; *b*, cardiac myocyte after insulin (100 nM) application for 10 min; *c*, cardiac myocytes following wortmannin (100 nM) application for 10 min. **B:** fluorescence of Venus concentrated on application of insulin and ANG II. *a*, Cardiac myocyte in the basal state; *b*, cardiac myocyte after insulin (100 nM) application for 10 min; *c*, cardiac myocyte after ANG II (100 nM) application for 10 min. **C:** Western blotting of Akt or phosphorylated (p)-Akt in the cardiac myocyte on the application of insulin and ANG II. Panels on *right* indicate semiquantitative assessment of fluorescence. Surface intensity = intensity (10% from both edges)/total intensity. Scale bar indicates 10  $\mu$ m.

was administrated to each well. After 10 min incubation, the cells were washed with PBS two times, and fluorescence was measured using a microplate reader (ARVOsx; PerkinElmer).

**Statistical analysis.** Statistical analysis was performed by Student's *t*-test. Statistical analysis for glucose uptake was performed by ANOVA, followed by Fisher's protected least-significant difference test using StatView (Abacus Concepts, Berkeley, CA). Results are expressed as means  $\pm$  SE. A value of  $P < 0.05$  was regarded significant.

## RESULTS

**Insulin-mediated PI(3,4,5)P<sub>3</sub> production on the cell membrane of cardiac myocytes.** To visualize insulin signaling and to test the effects of ANG II on it in living cardiac myocytes, we used a real-time imaging technique that monitors the dynamics of PI(3,4,5)P<sub>3</sub>, a membrane phospholipid generated by the insulin-signaling pathway. PH domain of Akt/PKB has been reported to strongly and specifically bind PI(3,4,5)P<sub>3</sub> (16, 31, 34). We therefore monitored the intensity/distribution of the fluorescence of a bright fluorophore, Venus, conjugated to the Akt-PH domain. Because of the improved speed and efficiency of maturation, the signal intensity of Venus is not affected by intracellular pH and not quenched by chloride ion. Therefore, the fluorophore is regarded as a powerful tool for tracking the movement of various substances in living cells (24).

We detected the fluorescence of the Venus-PH domain on the plasma membrane as well as in the cytosolic region in the control condition (Fig. 1Aa). This may indicate that a certain

amount of PI(3,4,5)P<sub>3</sub> is constitutively present on the plasma membrane, even in the resting state. To evaluate the distribution of PI(3,4,5)P<sub>3</sub> in the cell, we vertically scanned the cell along a single line, as shown in Fig. 1A, and measured the intensity of fluorescence as the sum of pixels on the line (Fig. 1A, bottom). Next, we divided the sum of pixels within 10% from both edges with the total number of pixels on the line. We referred to this value as "percent surface intensity" (%SI). This value was used as a semiquantitative indicator reflecting the distribution of the fluorescence, i.e., PI(3,4,5)P<sub>3</sub>, on the plasma membrane.

Application of insulin to the bath prominently enhanced the fluorescent signal at the cellular surface and its vicinity, although decreasing it in the cytosol (Fig. 1, Aa and Ab). This alteration gradually reached a plateau within 5 min, and %SI increased from 24 to 50 in this example (Fig. 1A, right). The increase of %SI was  $95.2 \pm 10.3\%$  ( $n = 10$ ,  $P < 0.0001$ ). This translocation of the fluorescence of the Venus-PH domain from the cytosol to the plasma membrane region may be interpreted as indicating that insulin stimulates production of PI(3,4,5)P<sub>3</sub> in the close vicinity of plasma membrane in cardiac myocytes.

The plateau level of fluorescence was maintained constant during continuous presence of insulin at least for 40 min (data not shown). Treatment of the cardiac myocytes with wortmannin (100 nM), a blocker for PI 3-kinase, caused rapid redistribution of the fluorescence from the surface to the cytosol (Fig. 1Ac) (33, 37), and the %SI decreased to the level less than the control. The decrease of %SI was  $46.1 \pm 3.9\%$  ( $n = 5$ ,  $P <$

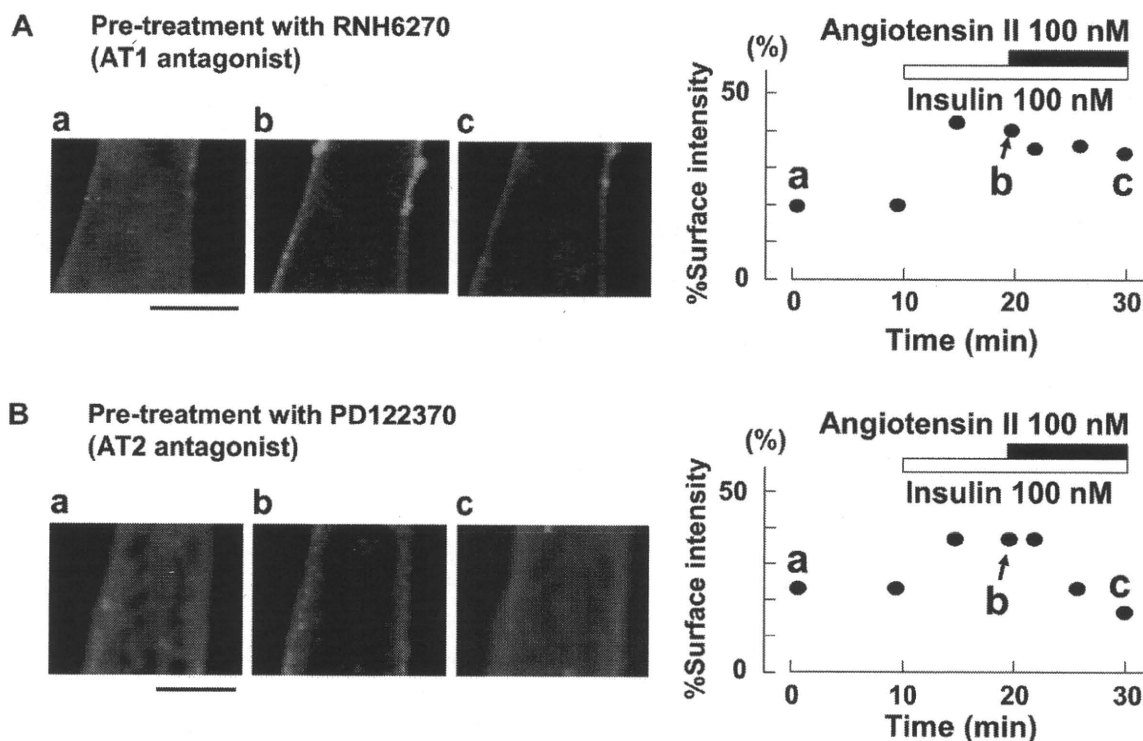


Fig. 2. ANG II antagonizes insulin signal via the angiotensin type 1 (AT<sub>1</sub>) receptor. ANG II inhibits production of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>] by insulin through AT<sub>1</sub> receptor but not the angiotensin type 2 (AT<sub>2</sub>) receptor. Insulin (100 nM) was added at 30 min after RNH-6270 or PD-123319 application in the transfected myocyte medium, followed by ANG II (100 nM). A and B: effect of RNH-6270 (10 nM) and PD-123319 (10 nM). a, In the basal state; b, cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocyte following ANG II (100 nM) application for 10 min. Panels on right indicate quantitative assessment of fluorescence. Scale bar indicates 10  $\mu$ m.

0.0001). This suggests that PI 3-kinase continued to produce PI(3,4,5)P<sub>3</sub> as long as insulin was present.

**Inhibitory effect of ANG II on insulin-mediated production of PI(3,4,5)P<sub>3</sub> and phosphorylation of Akt through AT<sub>1</sub>.** We next examined the effect of ANG II on insulin-mediated translocation of the Venus-PH domain in cardiac myocytes (Fig. 1B). Insulin again caused the translocation of the Venus-PH domain from the cytosol to the plasma membrane region (Fig. 1B, *a* and *b*), and the %SI increased from 20 to 40 in this myocyte. ANG II (100 nM) further added to the bath reduced the fluorescence on the membrane region within 10 min almost to the initial level, and the %SI decreased to 25 in this example (Fig. 1Bc, *right*). The decrease of %SI after ANG II stimulation was  $36.2 \pm 3.9\%$  ( $n = 6$ ,  $P < 0.0001$ ).

We next analyzed the phosphorylation of Akt/PKB (p-Akt) in cardiac myocytes induced by insulin by the Western blotting technique using an antibody specific to p-Akt. Because the phosphorylation of Akt requires the presence of PI(3,4,5)P<sub>3</sub>, the p-Akt should be a good indicator for the active state of insulin signaling (3). Little signal of p-Akt was visible under control conditions (Fig. 1C, *bottom*, *lane 1*). The intensity of the band of p-Akt clearly increased in the presence of insulin (Fig. 1C, *bottom*, *lane 2*). When we treated the myocytes with the mixture of insulin (100 nM) and ANG II (100 nM), we detected a much less amount of p-Akt (Fig. 1C, *bottom*, *lane 3*). This result may indicate that ANG II inhibited the insulin-mediated production of PI(3,4,5)P<sub>3</sub> and thus decreased the amount of p-Akt. This is consistent with the fluorescent data using the Venus-Akt-PH domain in Fig. 1B. It is of note that

neither insulin nor ANG II changed the amount of total Akt (Fig. 1C, *top*).

ANG II is known to exert its specific action by binding to either AT<sub>1</sub> receptor or angiotensin type 2 receptor (AT<sub>2</sub>) that are coupled to G proteins (12, 20). When we preincubated the cells with a selective antagonist for AT<sub>1</sub> receptor, RNH-6270 (10 mM), ANG II could not interfere with the effect of insulin on PI(3,4,5)P<sub>3</sub> production on the plasma membrane (Fig. 2A, *a-c*). The %SI only marginally decreased from 40 to 35 in this example. Similar results were obtained in five different cardiac myocytes. The decrease of %SI was  $4.5 \pm 6.0\%$  ( $n = 6$ ,  $P = 0.49$ ). On the other hand, ANG II could still block the insulin-mediated redistribution of the Venus-PH domain in the presence of PD-123319 (10 mM), a blocker specific for AT<sub>2</sub> (Fig. 2B, *a-c*). The %SI decreased from 40 to 18 in the example of Fig. 2B. The decrease of %SI was  $32.9 \pm 4.9\%$  ( $n = 5$ ,  $P < 0.003$ ). These results strongly suggest that the inhibitory reaction of ANG II on insulin-mediated production of PI(3,4,5)P<sub>3</sub> occurs through AT<sub>1</sub> receptor, but not through AT<sub>2</sub>.

**Intracellular Ca<sup>2+</sup> mobilization by ANG II is critically involved in inhibitory effects of ANG II on insulin-mediated PI(3,4,5)P<sub>3</sub> production.** Compared with VSMC, little is known about the mechanism of IR mediated by ANG II signaling in cardiac myocytes. To clarify the mechanism of ANG II inhibition of insulin-mediated PI(3,4,5)P<sub>3</sub> production, we examined the role of inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), which are known to be produced by ANG II via activation of PLC- $\beta$ . We first examined the role of intracellular Ca<sup>2+</sup>, which is released from endoplasmic reticulum by IP<sub>3</sub>.

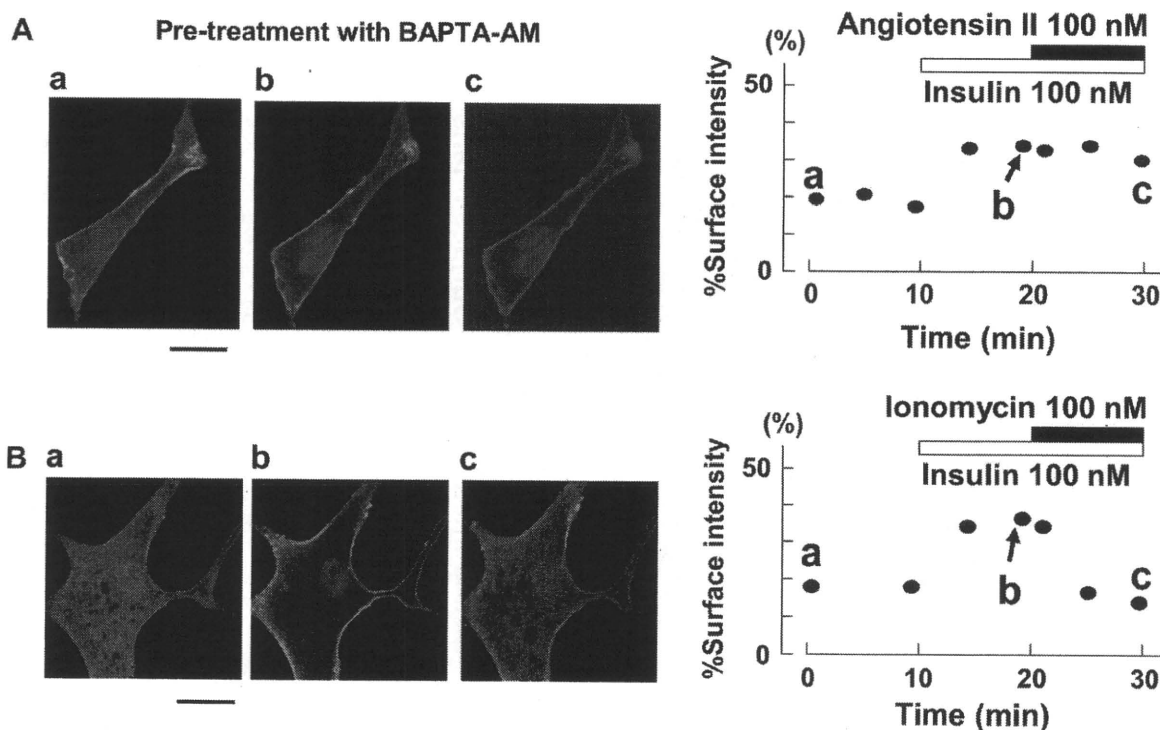


Fig. 3. ANG II antagonizes insulin signal through a Ca<sup>2+</sup>-dependent pathway. **A:** intracellular Ca<sup>2+</sup> chelator, BAPTA-AM (1 mM), was pretreated in the cardiac myocyte. *a*, In the basal state; *b*, cardiac myocyte after insulin (100 nM) application for 10 min; *c*, cardiac myocyte following ANG II (100 nM) application for 10 min. **B:** effect of ionomycin. *a*, In the basal state; *b*, cardiac myocyte after insulin (100 nM) application for 10 min; *c*, cardiac myocyte following ionomycin (100 nM) application for 10 min. Panels on *right* indicate quantitative assessment of fluorescence. Scale bar indicates 10  $\mu$ m.

When we preincubated the cells with BAPTA-AM, a chelator of intracellular  $\text{Ca}^{2+}$ , ANG II could no longer inhibit the effect of insulin on the distribution of Venus-PH domain (Fig. 3A, *a-c*, *right*). The decrease of %SI after ANG II stimulation was  $8.2 \pm$

$7.6\%$  ( $n = 4$ ,  $P = 0.36$ ). Conversely, application of the  $\text{Ca}^{2+}$  ionophore ionomycin (100 nM) instead of ANG II abolished insulin-mediated translocation of the fluorescence signal to the plasma membrane region (Fig. 3B, *a-c*, *right*). The decrease of

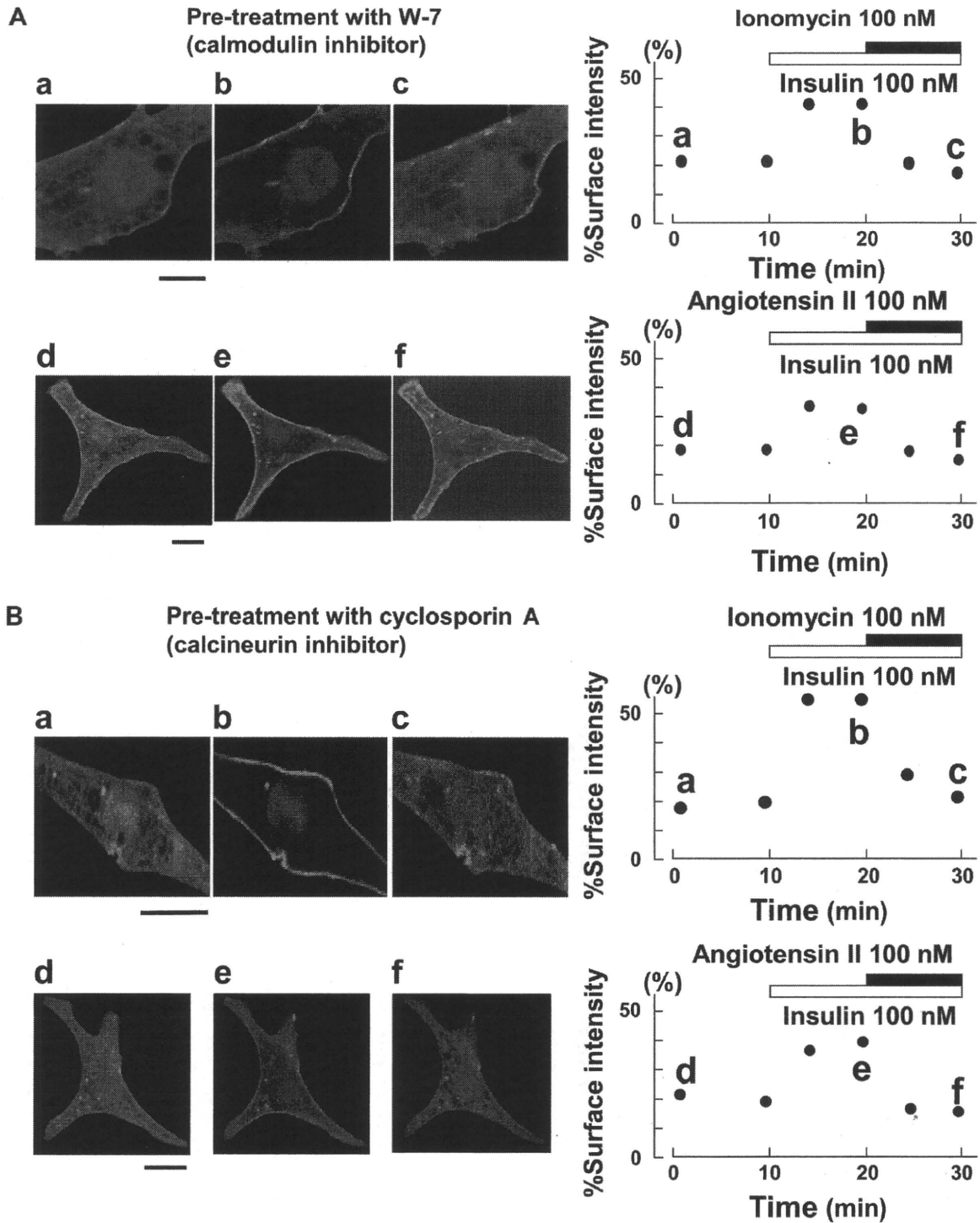


Fig. 4. Calmodulin and calcineurin have no effect of antagonizing insulin signaling. W-7 (calmodulin antagonist) or cyclosporin A (CyA, calcineurin antagonist) application was undertaken 30 min before insulin application. *A*: W-7 application (50  $\mu\text{M}$ ). *B*: CyA application (1  $\mu\text{M}$ ). *a* and *d*, In the basal state; *b*, cardiac myocyte after insulin application (100 nM) for 10 min; *c*, cardiac myocyte following ionomycin (100 nM) application; *e*, cardiac myocyte after ANG II application (100 nM) for 10 min; *f*, cardiac myocyte following ionomycin (100 nM) application. Panels on *right* indicate quantitative assessment of fluorescence. Scale bar indicates 10  $\mu\text{m}$ .

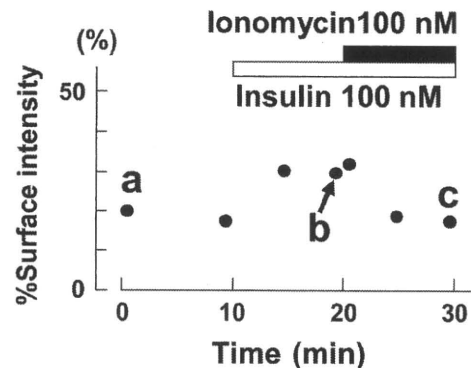
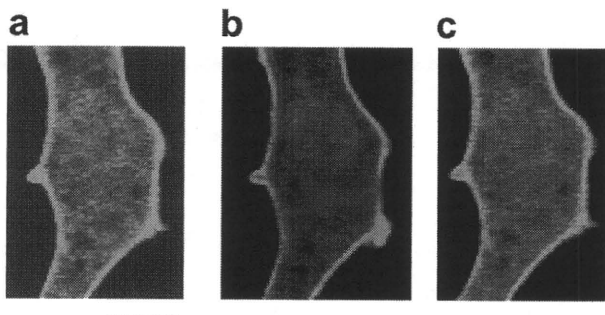
%SI was  $35.8 \pm 3.6\%$  ( $n = 5$ ,  $P < 0.001$ ). The time course of ionomycin action on insulin signaling resembled those of ANG II (see Fig. 1B). We also confirmed using the Ca<sup>2+</sup> indicator fluo 3 that the application of ANG II caused the prominent Ca<sup>2+</sup> mobilization in the cardiac myocytes (data not shown). Thus, it is suggested that an increase of intracellular Ca<sup>2+</sup> by ANG II is critically involved in insulin-mediated PI(3,4,5)P<sub>3</sub> production. W-7, a specific blocker for calmodulin, and cyclosporine, a blocker of calcineurin, did not influence inhibition of the %SI of Venus-PH domain by ionomycin (Fig. 4, A and B), suggesting that these downstream signals may not be involved in the ANG II effect.

ANG II-induced activation of PKC is not essential for the inhibitory effect of ANG II on insulin-mediated PI(3,4,5)P<sub>3</sub> production. In vascular smooth muscles, it was shown that DAG-induced PKC activation by ANG II directly inhibits activation of IRS-1, which is considered to be a cause of ANG II-mediated IR (22). In addition, it is known that localized changes of intracellular Ca<sup>2+</sup> determine the spatial and temporal targeting of PKC in smooth muscle cells (21). We therefore examined the effect of a PKC inhibitor, K-252b, on insulin-mediated PI(3,4,5)P<sub>3</sub> production in cardiac myocytes (Fig. 5). Pretreatment of the cells with K-252b (15 min) did not affect the inhibitory effect of either ionomycin or ANG II on the insulin-mediated increase of the %SI of Venus-PH domain (Fig. 5, A

and B, respectively). The decrease of %SI was  $29.6 \pm 1.3\%$  after stimulation with ionomycin ( $n = 3$ ,  $P < 0.002$ ) and  $36.2 \pm 3.3\%$  after stimulation with ANG II ( $n = 4$ ,  $P < 0.002$ ). Accordingly, it is likely that intracellular Ca<sup>2+</sup> elevation interferes with the action of insulin in a PKC-independent manner and that activation of PKC is not involved in ANG II-induced inhibition of insulin-mediated PI(3,4,5)P<sub>3</sub> production.

We next examined the effect of direct activation of endogenous PKC with the phorbol ester PMA. Insulin-mediated PI(3,4,5)P<sub>3</sub> production on the cell surface was only transiently inhibited by PMA (Fig. 6A). The transient decrease of %SI after PMA stimulation was  $39.6 \pm 5.7\%$  ( $n = 5$ ,  $P < 0.003$ ). This effect was not observed after the application of an inactive form of PMA, 4 $\alpha$ -PMA (data not shown). These results indicate that the activation of PKC has the rapid and transient interfering effect on the insulin-mediated PI(3,4,5)P<sub>3</sub> production. Chelating transient intracellular Ca<sup>2+</sup> elevation by BAPTA-AM (Fig. 6B) did not block the effect of PMA, suggesting that this effect of PKC occurs independent of Ca<sup>2+</sup> mobilization. The decrease of %SI after PMA stimulation was  $34.6 \pm 7.2\%$  ( $n = 3$ ,  $P < 0.04$ ). These results suggest that the activation of PKC is insufficient to explain the sustained inhibitory effect of ANG II against the insulin-mediated PI(3,4,5)P<sub>3</sub> production in cardiac myocytes.

#### A Pre-treatment with K-252b (PKC inhibitor)



#### B Pre-treatment with K-252b (PKC inhibitor)

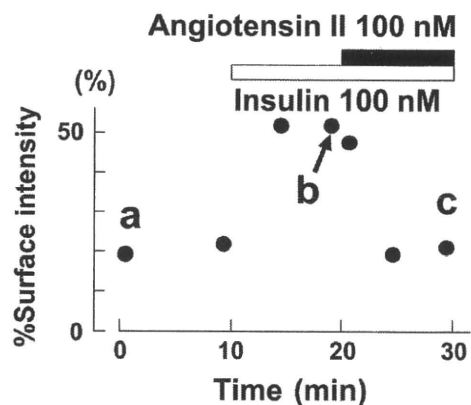
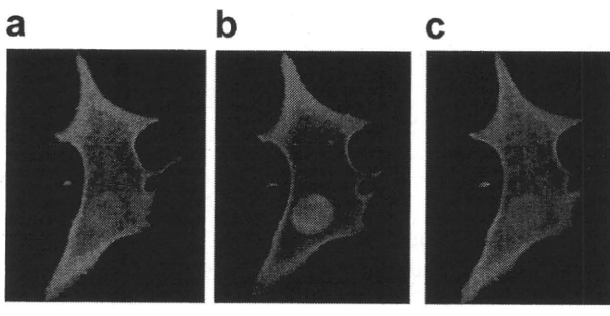


Fig. 5. Intracellular Ca<sup>2+</sup> mobilization reduces PI(3,4,5)P<sub>3</sub> production mediated by insulin through a PKC-independent pathway. A: K-252b (100  $\mu$ M) pretreatment 15 min before insulin application. a, In the basal state; b, cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocyte following ionomycin application (100 nM) for 10 min. B: K-252b (100  $\mu$ M) pretreatment 15 min before insulin application. a, In the basal state; b, cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocyte following ANG II application (100 nM) for 10 min. Panels on right indicate quantitative assessment of fluorescence. Scale bar indicates 10  $\mu$ m.

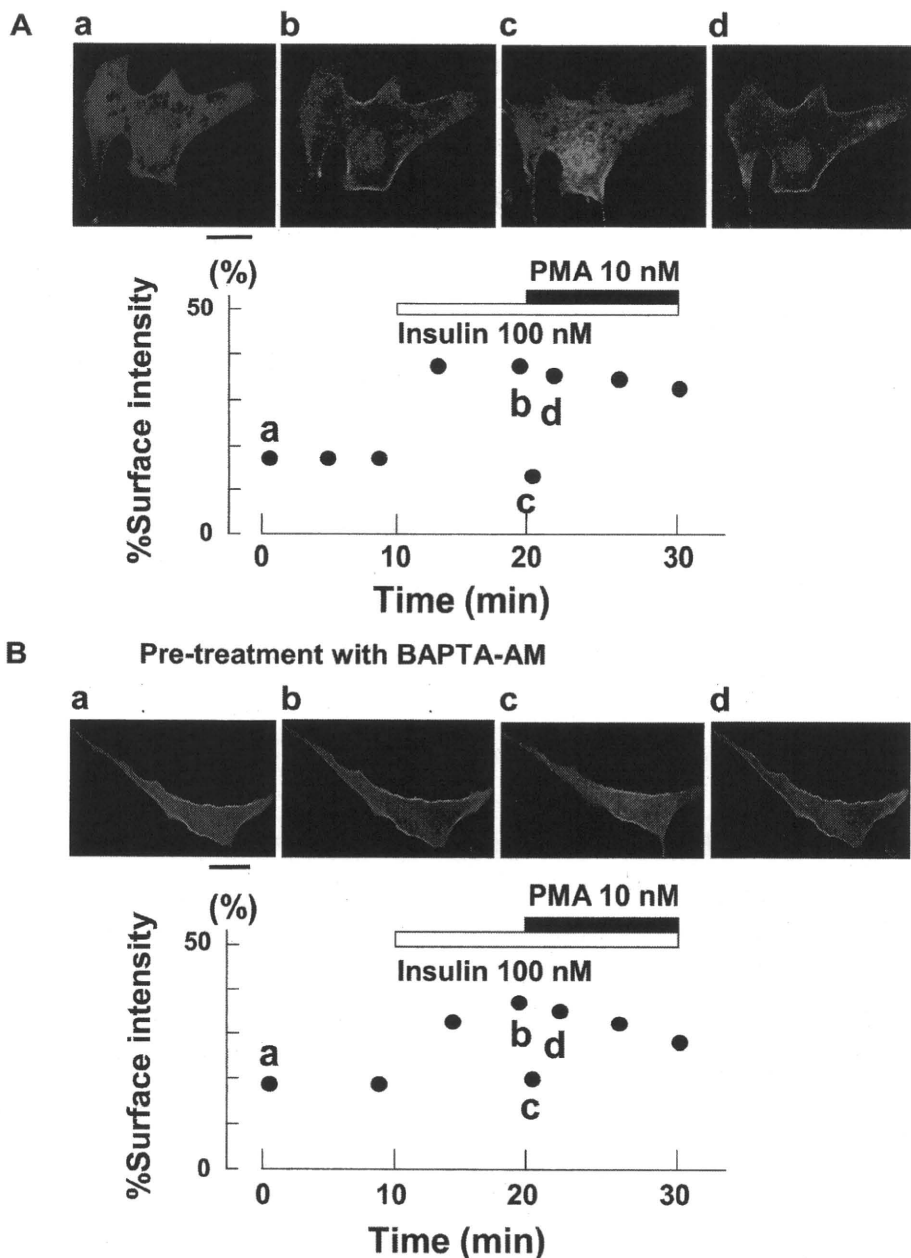


Fig. 6. Activation of PKC by phorbol 12-myristate 13-acetate (PMA) has rapid, transient antagonizing effects different from that observed with ANG II. *A*: no treatment. *B*: pretreatment with BAPTA-AM (1 mM). *a*, In the basal state; *b*, cardiac myocyte after insulin application (100 nM) for 10 min; *c*, cardiac myocyte then following PMA application (10 nM) for 10 s; *d*, cardiac myocyte then following PMA application (10 nM) for 1 min. Panels on *bottom* in *A* and *B* indicate quantitative assessment of fluorescence. Scale bar indicates 10  $\mu$ m.

*Inhibitory effect of ANG II on insulin-mediated glucose uptake was reversed through a Ca<sup>2+</sup>-dependent pathway, but not through a PKC-dependent pathway.* We confirmed whether the inhibitory effects of ANG II on insulin induced PI(3,4,5)P<sub>3</sub> production related to glucose uptake in cardiomyocytes. Insulin-mediated uptake of glucose in a clonal line of rat cardiomyocytes was measured using a fluorescent D-glucose derivative, 2-NBDG. ANG II inhibited insulin-mediated glucose uptake, which was reversed with BAPTA-AM, an intracellular Ca<sup>2+</sup> chelator, but not with the PKC inhibitor K-2526 (Fig. 7). Changes in glucose uptake corresponded to changes in PI(3,4,5)P<sub>3</sub> production with these reagents.

#### DISCUSSION

ANG II inhibition of insulin signaling has been taken as one of the important mechanisms for the genesis of IR in various cells, including adipocytes (4, 26) and VSMC (22). Activation of PKC and an increase in intracellular Ca<sup>2+</sup> are reported to be involved in ANG II-induced IR in adipocytes (2, 40) and VSMC (22). Although it was also reported that ANG II inhibits insulin signaling in cardiac myocytes (7, 36), its intracellular mechanisms remain unknown.

Using real-time imaging techniques with a fluorophore conjugated domain of Akt/PKB we analyzed the time course of changes in PI(3,4,5)P<sub>3</sub> concentration in the cardiac cell mem-

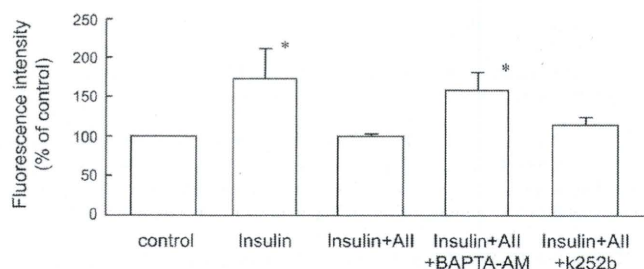


Fig. 7. Inhibitory effect of ANG II (All) on insulin-mediated glucose uptake in rat cardiomyocyte was reversed through a  $Ca^{2+}$ -dependent pathway, but not through a PKC-independent pathway. Glucose uptake in H9c2 cells was measured using a fluorescent D-glucose derivative, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose. Data are expressed as means  $\pm$  SE of 3 experiments. BAPTA-AM, intracellular  $Ca^{2+}$  chelator; K-252b, PKC inhibitor, \* $P < 0.05$  vs. control.

brane under various conditions. Both ANG II and ionomycin caused sustained inhibition of insulin-mediated  $PI(3,4,5)P_3$  production. However, phorbol ester caused transient inhibition of insulin-mediated  $PI(3,4,5)P_3$  production even though PKC activation was sustained under blocking the elevation of intracellular  $Ca^{2+}$  by BAPTA. These pharmacological investigations strongly suggest that ANG II-mediated inhibition of insulin signaling in cardiac myocytes is caused mainly by elevation of intracellular  $Ca^{2+}$  but not by activation of PKC. We have confirmed that this inhibitory effect of ANG II on insulin-mediated  $PI(3,4,5)P_3$  production and the intracellular pathways corresponded with those on insulin-mediated glucose uptake.

On the contrary, in VSMC, the dominant signal to inhibit insulin-mediated Akt activation by ANG II is PKC- $\alpha$  but not intracellular  $Ca^{2+}$  (22). Therefore, the signal mechanism underlying ANG II inhibition of insulin signaling may vary according to cell types. Transient inhibition of insulin-mediated Akt activation by PMA in cardiomyocytes suggests activated PKC near the sarcolemma interfered with insulin signaling. However, this inhibitory effect of PMA was not sustained, and ANG II inhibition of insulin signaling was not suppressed by the PKC inhibitor in heart muscle cells. Taken together, we speculate that translocation of activated PKC to the sarcolemma is functional in VSMC but not in heart muscle cells.

Although we showed that the elevation of intracellular  $Ca^{2+}$  was involved in ANG II inhibition of insulin-mediated production of  $PI(3,4,5)P_3$ , we could not identify the signaling cascade following elevation of intracellular  $Ca^{2+}$ : W-7, a specific blocker for calmodulin, and cyclosporine, a blocker of calcineurin, did not influence inhibition of the %SI of Venus-PH domain by ionomycin (Fig. 4, A and B), suggesting that neither calmodulin nor calcineurin may be involved in the ANG II effect in cardiac myocytes.

There may be three possibilities for the downstream mechanism. First, we speculate the existence of unspecified molecules that cross talk with intracellular  $Ca^{2+}$  mobilization specifically in cardiac myocytes. The second possibility is that the target protein of PKC- $\alpha$  in VSMC is expressed at very low levels or absent in cardiac myocytes. The third possibility is that the levels of PKC activation by ANG II in our study might be insufficient to inhibit insulin signaling like as previously reported in PKC-transfected L6 muscle cells (8). Regarding the first possible mechanism, Jang et al. (13) speculated the exist-

tence of  $Ca^{2+}$ -dependent phosphatase because chelation of intracellular  $Ca^{2+}$  improved insulin sensitivity in high-fat-fed rats (13). Further studies are required to clarify the molecules that cross talk with intracellular  $Ca^{2+}$  mobilization in cardiac myocytes.

Insulin has close relationships with nutrition and energy metabolism. Between heart and vasculature, the importance of insulin signaling seems to be different because of different energy sources (11), i.e., free fatty acids for cardiac myocytes but glucose for VSMC. Although our studies were performed using nonfailing cardiomyocytes, the significance of insulin signaling in cardiac myocytes increases in the patients with heart failure because the development of heart failure induces crucial changes in energy source from fatty acid to glucose. It is reported that cardiac and plasma levels of ANG II increased in patients with heart failure (29). Taken together, clinical findings of usefulness of the renin-angiotensin blockers in patients with heart failure (18) may be explained in part through improvement of ANG II-mediated IR in cardiomyocytes of which energy source has changed to glucose. Exploration of the target molecules that cross talk with intracellular  $Ca^{2+}$  mobilization may lead to the further progress in the treatment of heart failure.

**Study limitations.** The first limitation of our study is that the analysis of the intensity of fluorophore is weak in quantification. We believe that %SI, which indicates the percent distribution of fluorophore in the edge site of whole fluorophore, is very useful to assess semiquantitatively the time course of changes in the distribution of fluorophore in response to pharmacological stimulation. Furthermore, the result of the quantitative analysis of glucose uptake was consistent with the semiquantitative result of the changes in  $PI(3,4,5)P_3$  production.

The second limitation is that we could not make any conclusion about signaling pathways other than the ANG II-mediated pathway, such as the  $\alpha$ -adrenergic receptor pathway that cross talks or overlaps with the insulin receptor signaling. Our previous experiment, however, indicates that the ANG II-mediated pathway plays a more important role than the  $\alpha$ -adrenergic receptor pathway in hyperinsulinemia-induced cardiac hypertrophy of fructose-fed rats (14).

In summary, using the real-time imaging techniques, we have shown that ANG II may negatively regulate insulin-mediated  $PI(3,4,5)P_3$  production via an increase of intracellular  $Ca^{2+}$  rather than via PKC activation through the stimulation of AT<sub>1</sub> receptor in the cardiac myocytes. The real-time imaging techniques may hold enormous potential to explore new cell signaling. The present finding indicates that the cardiomyocyte-specific interaction between insulin signaling and ANG II could be a new target for improving the metabolism of cardiomyocytes.

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#### DISCLOSURES

H. Rakugi reports receiving lecture fees from Daiichi Sankyo.

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## PERSPECTIVES

# Control of Osteoclast Precursor Migration: A Novel Point of Control for Osteoclastogenesis and Bone Homeostasis

Taeko Ishii, Junichi Kikuta, Atsuko Kubo and Masaru Ishii

*Laboratory of Biological Imaging, WPI-Immunology Frontier Research Center, Osaka University, Suita, Japan*

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### Abstract

Osteoclasts are bone-resorbing, multinucleated giant cells that differentiate from mononuclear macrophage/monocyte-lineage hematopoietic precursors. They have critical roles not only in normal bone remodeling but also during pathogenesis of destructive bone disorders such as osteoporosis, rheumatoid arthritis, and cancers metastatic to bone. Many molecules, especially macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), make significant contributions to osteoclast differentiation. However, the process of osteoclast precursor trafficking to and from the bone surface, where cell fusion occurs to form the fully differentiated multinucleated cells that mediate bone resorption, is less well-documented. Recent studies have shed light on the mechanisms involved and have demonstrated the vital participation of various chemokines such as CCL2, CCL5, CXCL12, and CX<sub>3</sub>CL1, and lipid mediators such as sphingosine-1-phosphate (S1P). In addition, advances in imaging technologies, such as the development of intravital multiphoton microscopy, have enabled the *in situ* visualization of the behavior of osteoclasts and their precursors within intact bone tissue. This capability will be extremely useful for dissecting the mechanisms controlling the migration of these cells *in vivo*. In this *Perspective*, we review the latest knowledge in this new field of bone biology, with a focus on novel imaging methodology and its applications in this field. *IBMS BoneKEy*. 2010 August;7(8):279-286.  
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### Introduction

Bone is a dynamically regulated tissue that continuously undergoes remodeling to maintain mineral homeostasis and structural robustness. The balance between bone resorption by osteoclasts and bone formation by osteoblasts is finely regulated (1-3), and several complex mechanisms maintain this equilibrium. The mechanism that has been investigated most extensively is the control of both osteoclast and osteoblast differentiation (1;2;4). Recently, the regulation of precursor cell recruitment has attracted attention (5-14). We have also investigated extensively the highly organized migration of osteoclast precursors between the bone marrow and blood vessels, in real-time using intravital multiphoton microscopy (14;15). In this review, we summarize recent findings regarding the recruitment of osteoclast precursor cells to the bone

surface and briefly introduce *in vivo* imaging of bone.

### What Are Osteoclasts? Where Do They Come From, and Where Are They Going?

Even in adults who have completed their growth, osseous tissue is continuously remodeled via bone resorption by osteoclasts and bone formation by osteoblasts, to maintain bone strength and electrolyte balance. In several pathological states, including osteoporosis, tumor-induced osteolysis, and rheumatoid arthritis, osteoclasts are activated excessively, and the balance between bone formation and resorption is disrupted. Consequently, the inhibition of osteoclast function is a major therapeutic target in these diseases (3).

The osteoclast is a unique multinucleated giant cell formed by the fusion of mononuclear precursors of the macrophage-

monocyte lineage. The differentiation of precursor cells into mature osteoclasts requires two important molecules: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) (16-18). In addition, although this is still controversial, it has been reported that many hormones, cytokines, and growth factors such as parathyroid hormone, estrogen, 1,25-dihydroxyvitamin D<sub>3</sub>, and interleukin-6 can affect osteoclast differentiation by regulating the expression of M-CSF, RANKL, and osteoprotegerin, a non-signaling decoy receptor for RANKL (1-3). The major source of M-CSF, RANKL, and osteoprotegerin in bone tissue is osteoblastic lineage cells, or stromal cells, and the interaction between osteoclast and osteoblast lineages is critical in bone homeostasis (1-3). Signaling also occurs in the opposite direction. Stimulatory factors such as transforming growth factor- $\beta$ , insulin-like growth factor-1, and cardiotrophin-1 are released from osteoclasts during bone resorption and stimulate bone formation by osteoblasts (4). In addition, EphrinB2 expressed by osteoclasts and EphB4 expressed by osteoblasts participate in bidirectional communication between osteoclast- and osteoblast-lineage cells (19).

The mechanism by which osteoclast precursors are recruited at the proper time to appropriate sites for differentiation is unclear. The bone marrow cavity and bloodstream contain monocyte-lineage precursor cells, which can differentiate into osteoclast precursor cells. These precursors are recruited to the surface of bone, where they differentiate into mature osteoclasts. Moreover, cell cycle-arrested quiescent osteoclast precursors on the bone surface can differentiate into mature osteoclasts upon exposure to several stimuli (22). Although the differentiation stage at which osteoclast precursor cells migrate to the bone surface is controversial, several cytokines were shown recently to be involved in their recruitment (5-13). In addition to *in vitro* migration assays, intravital two-photon imaging permits the observation of cell behavior *in vivo* in real-time and is a powerful tool for determining

spatiotemporal control mechanisms (15;23;24). Below, we review recent findings from many groups, including ours, regarding the recruitment and release of osteoclast precursors.

### Attractants and Repellents

Although the mechanism of osteoclast precursor recruitment remains elusive, several chemoattractants and chemorepellants have been shown to play critical roles in controlling the migration of monocyte-lineage precursor cells from blood vessels into the bone marrow cavity. As with leukocytes, the migration of osteoclast precursors is regulated mainly by short peptides (approximately 70-90 amino acids) known as chemokines. Chemokines have been classified into C, CC, CXC, and CX3C subfamilies, according to their structural cysteine motif. Chemokine receptors are G protein-coupled receptors (GPCRs, also known as seven-transmembrane receptors), and act specifically through pertussis toxin (PTx)-sensitive G $\alpha$ i components. Although some chemokine-receptor pairs are exclusive, most receptors interact with multiple ligands, and most ligands interact with more than one receptor. This redundancy makes their regulation complex (25). The best-known chemoattractant is CXCL12 (or stromal derived factor-1, SDF-1), a CXCR4 ligand (5;6). CXCL12 is expressed constitutively at high levels within bone by osteoblastic stromal cells and vascular endothelial cells, while CCR4 is expressed on a wide variety of cells, including circulating monocytes and osteoclast precursors. CXCL12 has chemotactic effects on osteoclast precursors, which express high levels of CCR4 (5). Recently, another chemokine, CX<sub>3</sub>CL1 (or fractalkine), which works as an adhesion molecule as a membrane-bound chemokine, and as a chemoattractant after being cleaved by ADAM10 and ADAM17. CX<sub>3</sub>CL1, which is the only known member of the CX3C subfamily and expressed by osteoblastic stromal cells, was reported to be involved in both the recruitment and attachment of osteoclast precursors (7). These cytokines are engaged mainly in the interaction between osteoclasts and