Bradykinin B₂ receptor-induced and inositol tetrakisphosphate-evoked Ca²⁺ entry is sensitive to a protein tyrosine phosphorylation inhibitor in *ras*-transformed NIH/3T3 fibroblasts

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Signal transduction from mouse bradykinin B₂ receptors to calcium influx was studied in *ras*-transformed NIH/3T3 (DT) fibroblasts. DT cells were preloaded with fura-2 and whole-cell voltage-clamped. Activation of B₂ receptors resulted in a decrease of cellular fluorescence at the excitation wavelength of 340, or 360 nm after MnCl₂ application, in both the presence and absence of external Ca²⁺ in DT cells, at a holding potential of −40 mV. This Mn²⁺ entry through the Ca²⁺ influx pathway increased with membrane hyperpolarization. Internal application of inositol 1,3,4,5-tetrakisphosphate (InsP₄), but not of inositol 1,4,5-trisphosphate, mimicked membrane potential-dependent Mn²⁺ entry. Bradykinin- and InsP₄-induced Ca²⁺ influx was blocked by

10–100 μM genistein, a tyrosine kinase inhibitor. B₂ receptor activation induced time-dependent tyrosine phosphorylation of mitogen-activated protein kinase and 120 kDa protein, which was dose-dependently inhibited by genistein. Bradykinin was unable to induce Ca²⁺ oscillations in genistein-treated DT cells. Our results show that bradykinin-induced Ca²⁺ influx and oscillations depend upon protein tyrosine phosphorylation. The results suggest that two bradykinin B₂ receptor-activated signal pathways, protein tyrosine phosphorylation and formation of InsP₄, merge at the Ca²⁺ influx process in *ras*-transformed NIH/3T3 fibroblasts.

INTRODUCTION

Repetitive intracellular calcium (Ca²⁺) transients (cytosolic Ca²⁺ oscillations) are considered to play an important role in post-receptor signal transduction in non-excitatory cells. Ca²⁺ oscillations are maintained in part by Ca²⁺ influx [1,2], which is utilized to replenish intracellular Ca²⁺ stores. Ca²⁺ entry is triggered by the formation of the receptor–ligand complex or by emptying of the internal Ca²⁺ stores. The former is termed ‘receptor-operated Ca²⁺ entry’, which is triggered by GTP-binding protein (G-protein), InsP₃ and InsP₄ [2–6]. In the latter form of Ca²⁺ entry, depletion of the intracellular Ca²⁺ stores as a consequence of Ins(1,4,5)P₃-induced Ca²⁺ release appears to cause Ca²⁺ entry, termed ‘capacitative Ca²⁺ entry’ [7–9].

We have reported previously that application of bradykinin induces cytosolic Ca²⁺ oscillations and enhances membrane-potential-dependent increases in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) in Ki-*ras*-transformed NIH/3T3 (DT) fibroblasts [2]. The bradykinin-induced [Ca²⁺]_i increase is mimicked neither by intracellular injection of Ins(1,4,5)P₃ nor Ins(1,3,4,5,6)P₅, but by Ins(1,3,4,5)P₄, Ins(1,3,4,6)P₄ and Ins(3,4,5,6)P₄ [10]. Naturally occurring InsP₄-induced [Ca²⁺]_i rises are extracellular Ca²⁺ concentration ([Ca²⁺]_o)-dependent [2,10]. Stimulation of DT cells with bradykinin increased the levels of Ins(1,3,4,5)P₄ and Ins(3,4,5,6)P₄ [10], functioning as

second messengers, in the downstream of bradykinin receptor stimulation. Furthermore, bradykinin-induced and InsP₄-dependent [Ca²⁺]_i increases are significantly enhanced and prolonged in *ras*-transformed DT cells, when compared with non-transformed NIH/3T3 cells [10,11].

Recently, InsP₄-binding proteins and their cDNAs have been isolated and cloned [12–14]. It has been shown that InsP₄ interacts with conserved region 2 (C2)A and/or C2B domains of synaptotagmin II [13,14] or GTPase-activating protein (GAP) 1 [12,15]. GAP1, acting as an InsP₄-binding protein, specifically interacts with p21^{ras} (Ras) to activate GTP hydrolysis [12]. However, it has not yet been shown how these InsP₄-binding proteins can associate with Ca²⁺ entry pathways (channels). In this context, *ras*-transformed cells have advantages for analysing receptor-operated Ca²⁺ entry pathways in detail. The first question addressed here is to show directly whether bradykinin or InsP₄ causes Ca²⁺ influx, by measuring the quenching of the fura-2 signal by Mn²⁺ entering through Ca²⁺-permeable channels.

Signal transduction from receptors that couple to phospholipase C via heterotrimeric G-protein to the Ras mitogen-activated protein (MAP) kinase cascade was first shown for lysophosphatidic acid receptors [16,17]. Recently, it has been reported that stimulation of G_i-coupled α₂-adrenergic and M₂ muscarinic receptors activates Ras through the βγ subunit of G-protein [18,19]. On the other hand, bradykinin receptors are

Abbreviations used: [Ca²⁺]_i, cytoplasmic free Ca²⁺ concentration; C2, conserved region 2; GAP, GTPase-activating protein; Ras, p21^{ras}; MAP, mitogen-activated protein; FAK, focal adhesion kinase; DMEM, Dulbecco's modified Eagle's medium; fura-2 AM, fura-2 acetoxymethyl ester; p42, p44 and p120, 42, 44 and 120 kDa proteins; [Ca²⁺]_o, extracellular Ca²⁺; BCA, bicinchoninic acid; ECL, enhanced chemiluminescence.

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known to activate phospholipase C via $G_{q/11}$ [20], or to inhibit adenylate cyclase via G_i [21] and Ca^{2+} channels via G_{13} [22] types of G-protein. Furthermore tyrosine phosphorylation of focal adhesion kinase (FAK) is increased by bradykinin in Swiss 3T3 cells [23]. Therefore, in analogy to the observation above it seems possible that cross-talk of signal pathways could take place in bradykinin receptors. The second question addressed is to examine whether tyrosine phosphorylation plays a role in bradykinin-induced Ca^{2+} influx in DT cells, using a tyrosine kinase inhibitor, genistein, as a tool.

Finally, we have examined the question of which subtype of bradykinin receptor mediates signal transduction in DT cells. Recently, cDNA encoding a mouse B_2 receptor, which can activate phospholipase C, has been cloned from NG108-15 mouse neuroblastoma $\times$ rat glioma hybrid cells [24]. Using the cDNA as a probe, we examined whether or not the B_2 receptor is expressed in DT cells.

EXPERIMENTAL

Cell culture

v-Ki-ras-transformed NIH/3T3 fibroblasts (DT cells) [25] were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal-calf serum (Whittaker MA Bioproducts, Walkersville, MD, U.S.A.) as described previously [2].

RNA blot hybridization analysis

Total RNA was extracted from DT cells by the method of Chomczynski and Sacchi [26], and poly(A)⁺ RNA was selected on oligo(dT)-cellulose. A sample (15 μ g) of poly(A)⁺ RNA was separated on a 1.1% agarose-2.2 M formaldehyde gel and transferred on to a nylon membrane (Zetaprobe, Bio-Rad, Hercules, CA, U.S.A.). Hybridization was performed at 42 °C in a solution containing 50% formamide, 50 mM sodium phosphate buffer (pH 7.0), 5 $\times$ SSC, 250 μ g/ μ l denatured salmon sperm DNA, 0.5% SDS, 0.1% Ficoll, 0.1% poly(vinylpyrrolidone) and 0.1% BSA (1 $\times$ SSC: 0.15 M NaCl, 0.015 M sodium citrate). The probe used was the 1.7 kb *Bam*HI/*Eco*RI fragment from pSPMBR [24] labelled by the random primer method [27]. The filter was washed at 60 °C in 0.1 $\times$ SSC containing 0.1% SDS. Autoradiography was carried out at -80 °C with an intensifying screen for 18 h.

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} quenching

Cells were plated at a density of $(1-1.5) \times 10^2$ cells/mm² on a fluorescence-free glass coverslip attached to a silicon tube (1.5 cm diam. and 0.9 cm height) and cultured for 2 days at 37 °C. Microspectrofluorometric determination of $[Ca^{2+}]_i$ and Mn^{2+} quenching of fura-2 was performed in transformed cells as previously described [7,28]. Briefly, after washing with DMEM, the cells were loaded with 5 μ M fura-2 acetoxymethyl ester (fura-2 AM) for 45 min at 37 °C. De-esterification of the probe was facilitated by additional incubation for 15 min in fura-2-free medium. Fluorescence was measured at 35 °C at a determined site through a pin hole (10–20 μ m) with alternating excitation wavelengths of 340 and 380 nm [8], using a Ca^{2+} microspectrofluorometric system (OSP-3 model, Olympus, Tokyo, Japan). For measurement of Mn^{2+} quenching, an excitation wavelength of 360 nm and an emission wavelength of 510 nm were used and intensity was set at around 2 (arbitrary units) at the beginning of recording, according to the method described previously [29]. In three experiments (see Figures 1, 3 and 6)

Figure 1 Effects of three different concentrations of $MnCl_2$ on bradykinin-triggered Mn^{2+} quenching of fura-2 fluorescence in single DT cells

DT cells were preloaded with 5 μ M fura-2 AM and fluorescence changes were measured as described in the Experimental section. Upper trace in each set of records shows the time course of cellular fluorescence measured at the Ca^{2+} - and Mn^{2+} -sensitive excitation wavelength of 340 nm. Lower trace shows changes in fluorescence at an excitation wavelength of 360 nm to estimate Mn^{2+} influx. A, 10 μ l of 10 μ M bradykinin (BK, final concentration of 200 nM) was added prior to applications of 10 μ l of 2.5, 10 and 50 mM $MnCl_2$ (Mn, final concentration of 50, 200 and 1000 μ M, respectively) at arrows. Experiments were carried out in the presence (A) and absence (B) of 2 mM $[Ca^{2+}]_o$. Holding membrane potential of DT cells was -40 mV. Ordinate represents arbitrary units.

fluorescence intensity excited at 340 nm was also used for Mn^{2+} quenching to obtain information both on $[Ca^{2+}]_i$ and Ca^{2+} influx simultaneously [29]. Data obtained by excitation wavelengths of 340 and 360 nm provided almost identical Mn^{2+} quenching: $21 \pm 1\%$ ($n = 5$) by 340 nm and $22 \pm 2\%$ ($n = 5$) by 360 nm with 200 nM bradykinin respectively (see Figure 1).

Voltage-clamp

Patch voltage-clamp on fura-2-loaded or unloaded DT cells was performed in the whole-cell configuration using a patch electrode as described previously [2]. DT cells were superfused at 35 °C with Docherty's Ca^{2+} solution of the following composition (in mM), slightly modified from the original recipe [2]: $CaCl_2$, 2; tetraethylammonium chloride, 100; CsCl, 5; $MgCl_2$, 1; glucose, 25; Hepes, 25, pH 7.3 (buffered by about 7.5 mM CsOH). Ca^{2+} -free solution was prepared by omitting $CaCl_2$ in the solution. Patch electrodes contained solution of the following composition (in mM): CsCl, 150; $MgCl_2$, 1; Na_2ATP , 1; fura-2, 0.1; Hepes, 10, pH 7.2, buffered with CsOH. The electrode resistance was 8–16 M Ω . About 90 s after touching the cells, the electrode tip was sealed to the cell body by suction (seal resistance > 1 G Ω), and the membrane patch under the electrode was disrupted by further suction. After successfully establishing a whole-cell recording the cell was voltage-clamped, using a single-electrode voltage-clamp amplifier (Axoclamp 2A, Axon Instruments Inc.,

Foster City, CA, U.S.A.) in switching mode operation at 3–6 kHz, as described previously [30]. Inositol polyphosphates were added into the cytoplasm through patch pipettes containing 10 μM solutions of each chemical in the pipette solution (see above).

Measurement of protein tyrosine phosphorylation

DT cells (5×10^5) were seeded in 60-mm-diam. dishes and cultured for 2 days to near confluence. Cells were stimulated with 200 nM bradykinin for the indicated periods of time and immediately washed twice with ice-cold PBS containing 10 mM NaF, 1 mM Na_2VO_4 , 0.2 mM Na_2MoO_4 , 20 mM β -glycerophosphate and 1 mM PMSF. Cells were solubilized with 100 μl of solubilization buffer of the following composition: 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 25 mM Hepes (pH 7.4), 10 mM NaF, 1 mM Na_2VO_4 , 0.2 mM Na_2MoO_4 , 20 mM β -glycerophosphate, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF. Insoluble materials were removed by centrifugation at 15000 g for 20 min. Solubilized proteins (50 μg) were resolved by SDS/10%-PAGE and were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Membranes were probed with an anti-phosphotyrosine antibody (clone 1G4) or anti-(MAP kinase) antibody, and were visualized with enhanced chemiluminescence (ECL) reagents. Protein contents were measured by bicinchoninic acid (BCA) protein assay. The intensity of each protein band was analysed using a densitometer (Densitograph, Atto, Tokyo, Japan) in some experiments.

Sources of compounds

Tetraethylammonium chloride was purchased from Nacalai Tesque (Kyoto, Japan). $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$, fura-2 and fura-2 AM were purchased from Dojindo Laboratories (Kumamoto, Japan). Genistein and daidzein were purchased from Research Biomedical Incorporated (Natick, MA, U.S.A.) and Extrasynthese (Genay, France). Anti-phosphotyrosine and anti-(MAP kinase) antibodies were purchased from UBI (Lake Placid, NY, U.S.A.) and Transduction Laboratories (Lexington, KY, U.S.A.). ECL reagents and the BCA protein assay kit were obtained from Amersham International (Amersham, Bucks., U.K.) and Pierce (Rockford, IL, U.S.A.). All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise stated in the text.

RESULTS

First we examined which type of bradykinin receptor is expressed in *ras*-transformed NIH/3T3 (DT) fibroblasts originating from the mouse species. Blot hybridization analysis of poly(A)⁺ RNA from DT cells identified a major RNA species of 3400 bases which was hybridizable with mouse B_2 receptor cDNA (results not shown). We therefore judged that DT cells possess mouse B_2 bradykinin receptors, which could be partially or fully responsive to bradykinin.

In DT cells which had been preloaded with fura-2 AM and voltage-clamped at a holding potential of -40 mV, external application of 200 nM bradykinin produced an initial transient $[\text{Ca}^{2+}]_i$ increase followed by repetitive increases in $[\text{Ca}^{2+}]_i$, as measured by cellular fluorescence at the excitation wavelengths of 340 nm (Figure 1A) and of 380 nm (results not shown), or their ratio. These two types of $[\text{Ca}^{2+}]_i$ increase were essentially external Ca^{2+} -independent and -dependent, respectively (Figures 1A and 1B), as previously reported [2]. In order to examine the Ca^{2+} -dependent process in detail, Ca^{2+} influx was studied by quenching

Figure 2 Dependence of Mn^{2+} quenching on membrane potential in DT cells stimulated with or without bradykinin

(A) The average Mn^{2+} quench at various membrane potentials. Amount of change in cellular fluorescence measured at the excitation wavelength of 360 nm was determined as a net change between the levels at 5 min after and before application of 200 μM Mn^{2+} . DT cells were exposed to 200 nM bradykinin (●) or vehicle (○) in a solution with 2 mM $[\text{Ca}^{2+}]_o$. Then, Mn^{2+} (at a final concentration of 200 μM) was added to measure Mn^{2+} influx, as shown in (B). Ordinate indicates the mean $\pm$ S.E.M. in four or five experiments. * indicates significantly different from values at the indicated membrane potential in bradykinin-free DT cells, $P < 0.05$. Note that Mn^{2+} permeation was not evoked at the potentials tested in the absence of bradykinin. (B) Time courses of Mn^{2+} quenching in voltage-clamped DT cells at holding potentials of -15 , -65 and -90 mV, indicated at the left. 200 nM Bradykinin (BK) and 200 μM Mn^{2+} (Mn) were added to the medium at the times indicated by the arrows.

of fura-2 fluorescence with Mn^{2+} at the excitation wavelength of 360 nm, except for experiments shown in Figures 1, 3 and 6, where 340 nm, which is sensitive to both Ca^{2+} and Mn^{2+} , was used.

Bradykinin-induced Ca^{2+} influx measured by Mn^{2+} quenching

When 50, 200 and 1000 μM MnCl_2 were added 2.5–4.3 min after bradykinin application to DT cells clamped at -40 mV, the cellular fluorescence at 340 and 360 nm was decreased (Figure 1A). The levels and rates of the fluorescence decrease were nearly the same between two measurements at 340 and 360 nm and were dependent upon the Mn^{2+} concentrations used. The decrease was steeper and significantly larger in cells in the absence of 2 mM external Ca^{2+} (Figure 1A) than it was in the presence of Ca^{2+} (Figure 1B). The bradykinin-induced quenches (measured at 360 nm) at 50, 200 and 1000 μM Mn^{2+} were 6 ± 2 , 22 ± 2 and $42 \pm 4\%$ (mean $\pm$ S.E.M., $n = 5$) of the control level in the presence of external Ca^{2+} , and 14 ± 1 , 35 ± 5 and $62 \pm 2\%$ ($n = 5$) in the absence of external Ca^{2+} , respectively. The fluorescence intensity reached the steady basal level more quickly in the absence than in the presence of Ca^{2+} , at the same Mn^{2+}

Figure 3 Effects of membrane potential on Mn^{2+} quenching measured using 50 and 200 μ M Mn^{2+} in DT cells stimulated with bradykinin

Upper traces of each set of records show the holding potentials in DT cells preloaded with fura-2. Lower traces show cellular fluorescence at the excitation wavelengths of 340 nm (A and B) and 360 nm (C), respectively. A 10 μ l aliquot of 10 μ M bradykinin (BK, final concentration of 200 nM) was added before Mn^{2+} application in the absence ($-Ca$) and presence ($+Ca$) of 2 mM $[Ca^{2+}]_o$. A 10 μ l aliquot of 2.5 mM $MnCl_2$ (final concentration of 50 μ M, A and B) or of 10 mM $MnCl_2$ (final concentration of 200 μ M, C) was applied at the arrows indicated by Mn. Traces obtained in the presence (thick line) and absence (thin line) of $[Ca^{2+}]_o$ are superimposed in (C). The traces shown are typical findings from five experiments. Ordinate represents arbitrary units.

concentration. Taking the quench value at 5 min after 200 μ M Mn^{2+} application as the maximum, the half-maximal activation time was 50 ± 8 s in the absence of external Ca^{2+} , but 150 ± 13 s in the presence of Ca^{2+} , measured at 360 nm (Figure 1A).

In order to observe the effect of Mn^{2+} alone on fluorescence intensity, Mn^{2+} was first applied in the presence of 2 mM external Ca^{2+} (results not shown). While Mn^{2+} at 50 and 200 μ M had only a small effect on the 340 nm fluorescence change, 1000 μ M Mn^{2+} application markedly decreased it, suggesting that Mn^{2+} influx occurs even in the resting state. The bradykinin-evoked initial $[Ca^{2+}]_i$ peak due to intracellular Ca^{2+} mobilization appeared after a long delay (average 198 ± 45 s, $n = 11$) in cells pretreated with 50 and 200 μ M Mn^{2+} . Such $[Ca^{2+}]_i$ transients were completely suppressed in four cells pretreated with 1000 μ M Mn^{2+} , suggesting that Ca^{2+} influx occurs preceding Ca^{2+} mobilization, as observed in chromaffin cells stimulated with histamine [31].

Effect of membrane potential on bradykinin-stimulated Mn^{2+} quenching

The decrease in fluorescence intensity at the excitation wavelength of 360 nm after 200 μ M Mn^{2+} application was examined at different holding membrane potentials in both 200 nM

Figure 4 Average Mn^{2+} quench at various holding membrane potentials measured in the presence and absence of $[Ca^{2+}]_o$

Bradykinin (200 nM)-induced Mn^{2+} quenching was measured in DT cells under voltage-clamp conditions as shown in Figure 3(C) in the presence (A) and absence (B) of 2 mM $[Ca^{2+}]_o$. The decrease of fluorescence was determined over 100 s of holding at the given potentials with 200 μ M Mn^{2+} . Mn^{2+} quench at various membrane potentials is expressed as a percentage of the value at +10 mV ($n = 5$). The net Mn^{2+} quench was calculated by subtracting (B) from (A) ($\Delta A - B$) at each potential and is plotted in (C).

bradykinin-pretreated or untreated DT cells (Figure 2). Membrane hyperpolarization itself did not cause any Mn^{2+} quenching in bradykinin-untreated control cells (Figure 2A). In contrast, the bradykinin-stimulated Mn^{2+} quenching increased as the membrane potential was hyperpolarized (Figures 2A and 2B). Although quenching was not observed at holding potentials between +35 and -15 mV, the rate and level of quenching increased between -40 and -90 mV.

At 50 μ M Mn^{2+} , bradykinin-evoked fluorescence intensity measured at 340 nm decreased in a graded fashion when the membrane potential of the cell was hyperpolarized in steps from -40 mV to -100 mV at 2 mM external Ca^{2+} (Figure 3A). Similar voltage-dependency of the Mn^{2+} quenching was not observed in the absence of external Ca^{2+} (Figure 3B). Interestingly, cytosolic Ca^{2+} oscillations (upper deflections in Figure 3A) were evoked after bradykinin application even in the presence of 50 μ M Mn^{2+} at holding potentials of -60 to -80 mV. These oscillations were, however, suppressed at -80 and -100 mV, potentials at which Mn^{2+} influx was prominent.

At 200 μ M Mn^{2+} , the fluorescence measured at 360 nm decrease was apparent at membrane potentials more hyperpolarized than -30 mV in the presence of 2 mM $[Ca^{2+}]_o$ (Figure 3C, $+Ca$). The intensity decrease was less clear and showed no dependence on membrane potential in the absence of external Ca^{2+} (Figure 3C, $-Ca$). These observations are summarized in Figure 4, which plots the amount of the Mn^{2+} quench as a function of membrane potential. The apparent Mn^{2+} quench increased with membrane hyperpolarization below -20 mV for 200 nM Mn^{2+} with 2 mM external Ca^{2+} (Figure 4A). Apparent increase of fluorescence intensity, rather than quenching, was observed in the absence of Ca^{2+} (Figure 4B). The voltage-dependence of net Mn^{2+} influx obtained by subtracting the values in Figure 4(B) from those in Figure 4(A) is shown in Figure 4(C). The net Ca^{2+} influx measured as the Mn^{2+} quench was enhanced at membrane potentials more negative than -20 mV.

Figure 5 Relationship between Mn^{2+} quenching and holding membrane potential in DT cells injected with $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$

(A) Upper traces of each set of records show membrane potential. Lower traces show the time course of Mn^{2+} quenching measured at the excitation wavelength of 360 nm in arbitrary units. Polyphosphate isomers were infused into two cells after patch rupture (indicated by open arrows) through patch pipettes filled with solutions containing each isomer at a concentration of 10 μM . Mn^{2+} was applied to the cells at the time indicated by the arrows marked Mn. (B) Four similar experiments for $\text{Ins}(1,3,4,5)\text{P}_4$ (●) and for $\text{Ins}(1,4,5)\text{P}_3$ (○) yielded the membrane potential dependency curves. Mn^{2+} quenching is represented as percentage of the value at -40 mV. Each point shows the mean. Bars indicate S.E.M. * indicates significantly different from values at each membrane potential in $\text{Ins}(1,4,5)\text{P}_3$ -injected DT cells at $P < 0.05$. Star indicates significantly different from the value at -40 mV in $\text{Ins}(1,3,4,5)\text{P}_4$ -injected DT cells at $P < 0.05$.

Effects of inositol polyphosphate isomers on Ca^{2+} influx measured by Mn^{2+} quenching

We have previously shown the long-lasting $[\text{Ca}^{2+}]_i$ increases are preceded by an initial transient increase triggered by intracellular injections of $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$, but not by $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5,6)\text{P}_5$, which is external Ca^{2+} -dependent and is enhanced by membrane hyperpolarization in *ras*-transformed DT cells [2,10,11]. We therefore examined whether these increases are due to Ca^{2+} influx or not, by measuring Mn^{2+} quenching. The fluorescence intensity at the excitation wavelength of 360 nm decreased following 50 μM Mn^{2+} application in a cell injected with $\text{Ins}(1,3,4,5)\text{P}_4$ at a holding potential of -40 mV (Figure 5A, upper trace). This $\text{Ins}(1,3,4,5)\text{P}_4$ -evoked Mn^{2+} quench was enhanced by holding at -60 mV or more negative potentials ($P < 0.05$) (Figure 5B). In contrast, although an initial rapid quench by 50 μM Mn^{2+} was observed at a holding membrane potential of -40 mV in $\text{Ins}(1,4,5)\text{P}_3$ -injected cells (Figure 5A, lower trace), quenching was not increased with membrane hyperpolarization to -100 mV (Figure 5B). The Mn^{2+} quenches at -80 and -100 mV were 2 ± 3 and $6 \pm 5\%$ of the value at -40 mV ($n = 4$). The Mn^{2+}

Figure 6 Effects of genistein on bradykinin-evoked Mn^{2+} quenching and $[\text{Ca}^{2+}]_i$ elevation in DT cells

(A) Traces show cellular fluorescence measured at the excitation wavelength of 340 nm in DT cells treated with various conditions. From top to bottom, cells treated for 30 min with 100 μM genistein (Gen), 10 μM genistein and 100 μM daidzein (Dz) or without inhibitor (Cont). The cells were then voltage-clamped at -40 mV in the presence of external Ca^{2+} (2 mM). Bradykinin (BK; 200 nM) was added subsequent to Mn^{2+} (Mn; 200 μM) at the times indicated by the arrows. Transient upward deflections in fluorescence traces just after the arrows indicated by BK represent $[\text{Ca}^{2+}]_i$ rises. Continuous decline of fluorescence after the arrows indicated by Mn represents Mn^{2+} quenching. (B) Dose-response curves for inhibition of bradykinin-evoked $[\text{Ca}^{2+}]_i$ rise (○, BK response) and Mn^{2+} influx (●, Mn^{2+} quenching) measured as shown in (A). Values are means $\pm$ S.E.M. of four experiments. *, ** indicate significantly different from the genistein-free control value at $P < 0.05$ and $P < 0.01$. Inset, the two responses on a normalized scale.

quench evoked by $\text{Ins}(1,3,4,5)\text{P}_4$ was significantly larger than that produced by $\text{Ins}(1,4,5)\text{P}_3$ at -60, -80 and -100 mV ($P < 0.05$). In the absence of external Ca^{2+} , a hyperpolarization-dependent Mn^{2+} quench was not observed in $\text{Ins}(1,3,4,5)\text{P}_4$ -injected cells ($n = 4$ cells, results not shown). Thus, the results clearly show that InsP_4 elicits Ca^{2+} influx.

Effect of genistein on bradykinin-stimulated Mn^{2+} quenching and $[\text{Ca}^{2+}]_i$ rise

To characterize the molecular mechanism underlying bradykinin-induced Ca^{2+} influx, we examined the effect on Mn^{2+} quenching (measured at 340 nm) of a tyrosine kinase inhibitor, genistein [32]. The bradykinin (200 nM)-stimulated Mn^{2+} quench measured in DT cells with 200 μM MnCl_2 at a holding potential of -40 mV was suppressed in a dose-dependent way by preincubation for 30 min with 0.1–100 μM genistein (Figure 6). Mn^{2+} quenches decreased to 4 ± 1 and $3 \pm 1\%$ ($n = 4$) of the control value with 10 and 100 μM of the inhibitor respectively (Figure 6B). The dose of genistein causing 50% inhibition of the Mn^{2+} quench (IC_{50}) was about 2.8 μM (Figure 6B, inset). Furthermore, in cells preincubated with 100 μM daidzein, an inactive analogue of genistein, the bradykinin-induced Mn^{2+} quench occurred at $17 \pm 3\%$ ($n = 4$) of the control value, which is the same as in the non-treated control cells ($18 \pm 8\%$, $n = 10$) (Figure 6A).

It was also possible simultaneously to observe the effect of genistein on bradykinin-evoked $[\text{Ca}^{2+}]_i$ increases, by measuring the fluorescence at 340 nm (Figure 6A). The bradykinin-induced initial $[\text{Ca}^{2+}]_i$ rise was also suppressed by pretreatment with genistein in a dose-dependent fashion, with an IC_{50} value of about 25 μM (Figure 6B, inset). The $[\text{Ca}^{2+}]_i$ level evoked by 200 nM bradykinin decreased significantly from a level of $135 \pm 5\%$ ($n = 5$) in the absence of the inhibitor to $121 \pm 2\%$ ($n = 4$) and $111 \pm 4\%$ ($n = 4$) of the pre-stimulated (control) level

Figure 7 Effects of genistein on Mn^{2+} quench in DT cells injected with $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,4,5)\text{P}_3$

DT cells were treated with $10\ \mu\text{M}$ genistein for 30 min before and during measurement. (A) Upper traces of each set of records show membrane potential. Lower traces show the time course of Mn^{2+} quenching measured at the excitation wavelength of 360 nm in arbitrary units. $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,4,5)\text{P}_3$ was infused into two cells after patch rupture (indicated by open arrows) through patch pipettes filled with solutions containing each isomer at a concentration of $10\ \mu\text{M}$. Mn^{2+} (final concentration of $50\ \mu\text{M}$) was applied to the cells at the time indicated by the filled arrows (Mn). (B) Performing five similar experiments for $\text{Ins}(1,3,4,5)\text{P}_4$ (●) or $\text{Ins}(1,4,5)\text{P}_3$ (○) yielded the membrane potential dependence curves. Mn^{2+} quench is represented as percentage of values at $-40\ \text{mV}$. Each point shows the mean. Bars indicate $\pm$ S.E.M. Note that there is no significant difference in the values at each membrane potential from the values at $-40\ \text{mV}$ and also between values for $\text{Ins}(1,4,5)\text{P}_3$ - and $\text{Ins}(1,3,4,5)\text{P}_4$ -injected cells.

in the presence of 10 and $100\ \mu\text{M}$ genistein respectively (Figure 6B).

Effect of genistein on inositol polyphosphate-induced $[\text{Ca}^{2+}]_i$ rise and Ca^{2+} influx

The same experimental procedure as described above for Figure 5 was applied to examine the role of tyrosine phosphorylation in inositol polyphosphate-induced responses. The $[\text{Ca}^{2+}]_i$ increase soon after infusion of $\text{Ins}(1,3,4,5)\text{P}_4$ at $-40\ \text{mV}$ in $10\ \mu\text{M}$ genistein-treated cells was smaller than that in untreated control cells (results not shown). $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Mn^{2+} quenching was also abolished in the genistein-treated cells at $-40\ \text{mV}$. Increase in the $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Mn^{2+} quench with membrane hyperpolarization was not observed in the treated cells. At -60 , -80 and $-100\ \text{mV}$, the quenches were only 3 ± 3 , 6 ± 4 and $11 \pm 4\%$ respectively of the value at $-40\ \text{mV}$ ($n = 5$) (Figure 7B).

The effect of $\text{Ins}(1,4,5)\text{P}_3$ on hyperpolarization-enhanced Mn^{2+}

quenching was negligibly small in genistein-treated DT cells (Figure 7A, lower trace), just as seen in untreated control cells (Figure 5A). The Mn^{2+} quenches at -80 and $-100\ \text{mV}$ were only 5 ± 2 and $10 \pm 3\%$ ($n = 5$) of the value at $-40\ \text{mV}$, respectively. The sole apparent effect of genistein was seen in the lack of a rapid quench immediately after introduction of MnCl_2 .

Inhibition of bradykinin-stimulated protein tyrosine phosphorylation by genistein

We next examined the nature of the biochemical subsequence in bradykinin-treated DT cells, especially with respect to protein tyrosine phosphorylation, again by using genistein as a tool. A $200\ \text{nM}$ concentration of bradykinin stimulated tyrosine phosphorylation of several proteins of around 42, 44, 60, 120 and 150–180 kDa (results not shown), as previously described in bradykinin-stimulated Swiss 3T3 cells [33,34] and endothelial cells [35]. Tyrosine phosphorylation of these proteins was detectable as early as 1 min after bradykinin stimulation and reached a nearly maximal level at around 2–3 min. Both 42 and 44 kDa proteins (p42 and p44) were recognized by a specific anti-(MAP kinase) antibody. Since the activated form of MAP kinase displays an electrophoretic mobility shift, both 42 and 44 kDa bands were seen as doublets in bradykinin-stimulated samples (results not shown). The upper bands (pp42 and pp44) corresponded to tyrosine phosphorylated forms. Genistein inhibited bradykinin-stimulated protein tyrosine phosphorylation in a concentration-dependent manner. The IC_{50} values of genistein for phosphorylation of both 120 kDa protein (p120) and p42 were around 2.6 and $3.4\ \mu\text{M}$, respectively. However, $100\ \mu\text{M}$ daidzein showed weak inhibitory effects.

Effect of genistein on cytosolic Ca^{2+} oscillations

Previously, we postulated that Ca^{2+} influx is essential for maintenance of bradykinin-induced repetitive $[\text{Ca}^{2+}]_i$ rises in DT cells [2]. Since genistein inhibited Ca^{2+} influx and the phosphorylation of two proteins, we examined whether or not genistein inhibits Ca^{2+} oscillations as a consequence of these events. At holding potentials between -40 and $-80\ \text{mV}$, at which bradykinin application usually evokes Ca^{2+} oscillations, Ca^{2+} oscillation was not evoked by $200\ \text{nM}$ bradykinin after pretreatment with $10\ \mu\text{M}$ genistein for 30 min in five cells (results not shown). In addition, the basal $[\text{Ca}^{2+}]_i$ was not increased as the membrane potential was hyperpolarized from $-20\ \text{mV}$ to $-80\ \text{mV}$ in genistein-treated cells, unlike the untreated control cells [2].

DISCUSSION

The results show that stimulation of mouse B_2 bradykinin receptors expressed in DT *ras*-transformed NIH/3T3 fibroblasts induces Ca^{2+} influx, measured as Mn^{2+} quench, which is membrane-potential-dependent. Intracellular injection of $\text{Ins}(1,3,4,5)\text{P}_4$ through the patch pipette elicited a Mn^{2+} quench in a membrane-potential-dependent manner, but $\text{Ins}(1,4,5)\text{P}_3$ did not have the same effect. These processes, as well as bradykinin-induced Ca^{2+} oscillations, were effectively inhibited by $\geq 10\ \mu\text{M}$ genistein. Genistein also reduced bradykinin-induced tyrosine phosphorylation of p42 (MAP kinase) and p120 in DT cells, while daidzein had a weak effect on these responses at high concentrations. The results suggest that bradykinin-induced Ca^{2+} influx is controlled by tyrosine phosphorylation in *ras*-transformed fibroblasts.

In the present experiments, we demonstrated that the bradykinin- and InsP_4 -induced sustained $[\text{Ca}^{2+}]_i$ increases in DT

cells described in our previous papers [2,10] are indeed due to sustained Ca^{2+} influx. Bradykinin elicited a Mn^{2+} influx in the presence of external Ca^{2+} , which became more prominent with higher concentrations of Mn^{2+} . Therefore, Ca^{2+} and Mn^{2+} seem to share this influx pathway in DT cells, as reported in hepatocytes [9]. Membrane hyperpolarization also enhanced this Ca^{2+} influx. Since the pattern of membrane potential dependence obtained for the $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Mn^{2+} quench (Figure 5) was quite similar to that of the bradykinin-evoked quench (Figure 6), it is likely that $\text{Ins}(1,3,4,5)\text{P}_4$ mimics bradykinin-induced Ca^{2+} influx. According to Clapham [36], InsP_4 -gated Ca^{2+} channels with a unit conductance of 2 pS have equal selectivity for Ca^{2+} , Ba^{2+} and Mn^{2+} in endothelial cells. To determine whether Ca^{2+} influx in DT cells is due to this type of Ca^{2+} channel will require single-channel recording.

Our results also showed that bradykinin-induced Mn^{2+} quenching was markedly enhanced by removing external Ca^{2+} . With careful inspection of the time course, the Mn^{2+} quench (200 μM) can be seen to reach more rapidly the plateau level at which no further Ca^{2+} influx occurs, in the $[\text{Ca}^{2+}]_o$ -free condition than in the presence of Ca^{2+} . Long exposure to Ca^{2+} -free solution may cause depletion of intracellular Ca^{2+} stores. Thus, the larger Mn^{2+} quench under the Ca^{2+} -free condition may be explained in part by Ca^{2+} -depletion-mediated Ca^{2+} entry, or 'capacitative entry' [8]. It has been reported that the behaviour of Mn^{2+} in capacitative Ca^{2+} influx is tissue-specific: Mn^{2+} enters more rapidly in platelets and neutrophils in the absence of extracellular Ca^{2+} [29,37], but shows no entry in hepatocytes and mast cells [9,38,39]. In addition, $\text{Ins}(1,4,5)\text{P}_3$ injection resulted in a rapid fluorescence decrease after MnCl_2 application (Figure 5A). Altogether, the results suggest that the capacitative Ca^{2+} entry mechanism also functions in bradykinin-treated DT cells.

In DT cells, bradykinin B_2 receptor stimulation caused phosphorylation of p42 MAP kinase and p120, and their phosphorylation was inhibited by genistein, as just shown by bradykinin in Swiss 3T3 cells [33,34] and endothelial cells [35]. This suggests that stimulation of bradykinin receptors that couple with $\text{G}_{q/11}$ [20] and G_i [21] can activate the Ras-MAP kinase cascade. This might occur via the Shc-Grb2-Sos complex, the $\beta\gamma$ subunit of G_i protein or new members of the tyrosine kinase family, such as proline-rich tyrosine kinase 2 (PYK2) and FAK [40–42]. Although we do not know that such intermediary kinases function in DT cells, it is necessary to identify whether p120 is FAK, because of the close molecular mass between FAK (120–125 kDa) and p120.

The inhibitor of tyrosine kinase, genistein, blocked both bradykinin- and $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} influx (Figures 6 and 7). Our observation belongs to the same line of evidence showing that a rise in $[\text{Ca}^{2+}]_i$ or depletion of Ca^{2+} stores increases tyrosine kinase activity [43,44], and that, conversely, tyrosine kinase activity also controls Ca^{2+} influx [29,45,46]. Bradykinin-evoked Ca^{2+} influx was more sensitive to genistein than $[\text{Ca}^{2+}]_i$ increases (Figure 6). Mn^{2+} quenching and MAP kinase phosphorylation had similar IC_{50} values (2.8 μM and 3.4 μM , respectively), indicating that MAP kinase activation and Mn^{2+} influx are located downstream of tyrosine phosphorylation of Ras or some other proteins. This leads us to predict that signals cause Ca^{2+} influx and internal Ca^{2+} mobilization, a signal pathway with two branches, both of which may converge at the site of interaction with InsP_4 . Ca^{2+} release results from the direct action of InsP_3 formed by phospholipase C [1], while Ca^{2+} influx probably requires InsP_4 formation and protein tyrosine phosphorylation of Ras-GAP [15] or some other proteins closely related to Ca^{2+} influx. The question of whether MAP kinase

activation is a result of Ras activation or the cause of Ca^{2+} influx [47] remains to be answered in DT cells.

It has been reported that GAP contains a region homologous to the C2 domain of protein kinase C, which is a Ca^{2+} -dependent phospholipid-binding domain [48]. Recently, a specific $\text{Ins}(1,3,4,5)\text{P}_4$ -binding GAP protein has been characterized in non-excitable cells. This protein is a member of the GAP1 family which is constituted of the C2, highly conserved Ras-GAP and pleckstrin homology domains [12]. Semenchuk and Di Salvo reported that a 5-hydroxytryptamine-induced $[\text{Ca}^{2+}]_i$ increase, which is associated with tyrosine phosphorylation of Ras-GAP in vascular smooth muscle cells, is suppressed by genistein [49]. It will be interesting to know if Ras-GAP functions as the InsP_4 -binding site in bradykinin- and InsP_4 -activated Ca^{2+} influx in DT cells.

In conclusion, our results show that signals from B_2 receptors may be transduced both to the phospholipase C pathway, which produces InsP_3 and InsP_4 , and to the Ras-MAP kinase cascade. The results suggest that the tyrosine kinase cascade interacts with InsP_4 -activated Ca^{2+} influx in *ras*-transformed fibroblast cells.

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