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ORIGINAL ARTICLE

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Stimulation of cellular free Ca^{2+} elevation and inhibition of store-operated Ca^{2+} entry by kazinol B in neutrophils

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Abstract Kazinol B, a natural isoprenylated flavan, stimulated the $[Ca^{2+}]_i$ elevation in the presence or absence of Ca^{2+} in the medium. Treatment with chymotrypsin or phorbol 12-myristate 13-acetate to shedding of L-selectin had no effect on subsequent kazinol B-induced Ca^{2+} response. Upon initial cyclopiazonic acid (CPA) treatment in the absence of external Ca^{2+}, the subsequent $[Ca^{2+}]_i$ rise followed by challenge with kazinol B was greatly diminished. The ryanodine receptor blockers, 8-bromo-cyclic ADP-ribose and ruthenium red did not affect kazinol B-evoked Ca^{2+} release from internal stores. However, the inhibitors of sphingosine kinase, dimethyl-sphingosine, but not dihydrosphingosine, inhibited kazinol B-induced Ca^{2+} release. Kazinol B-induced $[Ca^{2+}]_i$ rise was not affected by two nitric oxidase inhibitors, *N*-(3-aminomethyl)benzylacetamidine (1400W) and 7-nitroindazole, cytochalasin B and Na^+-deprivation. This response was slightly attenuated by 2-aminoethyldiphenyl borate (2-APB), a D-*myo*-inositol 1,4,5-trisphosphate (IP_3) receptor blocker, and by genistein, a general tyrosine kinase inhibitor. However, the Ca^{2+} response was greatly diminished by two actin filament reorganizers, calyculin A and jasplakinolide, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), an inhibitor of phosphoinositide 3-kinase, *N*-(3-aminomethyl)benzylacetamidine (SB 203580), the p38 mitogen-activated protein kinase inhibitor, 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), the inhibitor of phospholipase C-coupled processes, and by 0.3 mM La^{3+} or Ni^{2+}. Kazinol B did not evoke any appreciable Ba^{2+} and Sr^{2+} entry into cells. The Ca^{2+} entry blockers, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), but not *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A), inhibited a kazinol B-induced $[Ca^{2+}]_i$ rise. Kazinol B had no effect on the pharmacologically isolated plasma membrane Ca^{2+}-ATPase activity. In a Ca^{2+}-free medium, kazinol B inhibited the subsequent Ca^{2+} addition, resulting in robust entry in CPA- and formyl peptide-activated cells. Kazinol B produced a concentration-dependent reduction in the mitochondrial membrane potential. These results indicate that kazinol B stimulates Ca^{2+} release from internal Ca^{2+} store, probably through the sphingosine 1-phosphate and IP_3 signaling, and activates external Ca^{2+} influx mainly through a non-store-operated Ca^{2+} entry (non-SOCE) pathway. Inhibition of SOCE by kazinol B is probably attributable to a break in the Ca^{2+} driven force of mitochondria.

Keywords Kazinol B · Cation entry · Intracellular-free Ca^{2+} · Non-store-operated Ca^{2+} entry · Mitochondria · Neutrophils

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Introduction

Neutrophils play a pivotal role in inflammatory reactions and constitute the first line of host defense. The increase in $[Ca^{2+}]_i$ is one of the early characteristic cellular reactions of neutrophil granulocytes and has been implicated in many cellular functions of neutrophils. The induction of

receptor-mediated cytosolic Ca^{2+} signals involves two closely coupled events: a rapid and transient release of Ca^{2+} stored, followed by slowly developing extracellular Ca^{2+} entry. It is well established that the initial Ca^{2+} spike is mediated by the activation of phosphoinositide-specific phospholipase C (PLC) that hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate to generate the second messenger, D-*myo*-inositol 1,4,5-trisphosphate (IP_3), which interacts with IP_3 receptor on the internal stores for the release of Ca^{2+} (Berridge and Irvine 1989). However, the mechanism regulating Ca^{2+} influx across the plasma membrane, which accounts for the sustained increase in $[Ca^{2+}]_i$, is still unclear. In non-excitable cells, including neutrophils, depletion of the intracellular Ca^{2+} stores induces entry of Ca^{2+} across the plasma membrane, referred to as store-operated Ca^{2+} entry (SOCE or capacitative Ca^{2+} entry; Putney 1990). It is far from certain that this mechanism is the only one involved in the increase in Ca^{2+} entry in non-excitable cells. A non-SOCE mechanism that involves protein kinase C has been reported in human platelet (Rosado and Sage 2000). The phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca^{2+} entry that is independent of the filling state of internal Ca^{2+} stores was observed in FcεRI-stimulated mast cells (Ching et al. 2001). In addition, arachidonic acid (AA) activates the non-SOCE in smooth muscle cells (Broad et al. 1999). The thiol modification of a number of important membrane proteins or channels induced Ca^{2+} entry in neutrophils through a non-SOCE mechanism (Wang 2003a, 2003b).

Kazinol B, a natural isoprenylated flavan isolated from the root bark of the Moraceous plant, *Broussonetia papyrifera*, has been shown to inhibit platelet aggregation and cyclooxygenase activity (Lin et al. 1996), 5-lipoxygenase activity (Chi et al. 2001), nitric oxide (NO) production in macrophages (Ryu et al. 2003), and to stimulate a Ca^{2+}-dependent superoxide anion generation in neutrophils (Wang et al. 1998). The aims of this study were to characterize the effect of kazinol B on Ca^{2+} signaling in rat neutrophils and to evaluate the underlying mechanism.

Materials and methods

Preparation of rat neutrophils Rat (Sprague–Dawley) blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of erythrocytes (Wang et al. 1998). Purified neutrophils containing >95% viable cell were normally resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice-bath before use. All experiments in the present study were performed under the guideline of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the principles and guideline of the U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals.

Measurement of intracellular-free Ca^{2+} Neutrophils (5×10^7 cells/ml) were incubated with 5 μM fluo-3/AM for 45 min at 37°C. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/ml. In some experiments, cells were suspended in Na^+-deprived HEPES buffer (124 mM *N*-methyl-D-glucamine, 4 mM KCl, 0.64 mM K_2HPO_4, 0.66 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 5.56 mM dextrose, and 15.2 mM $KHCO_3$). Fluorescence changes were monitored with a fluorescence spectrophotometer at 535 nm with excitation at 488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i=K_d[(F-F_{min})/(F_{max}-F)]$, where F is the observed fluorescence intensity. The values F_{max} and F_{min} were obtained at the end of experiments by the sequential addition of 0.33% Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM. In some experiments, neutrophils were loaded with 5 μM fura-2/AM at 37°C for 45 min (Wang et al. 1998). Fluorescence was monitored with a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation at 340 and 380 nm in the ratio mode, and calibration of the excitation ratio in terms of $[Ca^{2+}]_i$ was performed. The superimposed $[Ca^{2+}]_i$ response is obtained from a same batch of neutrophil preparation.

Measurement of Sr^{2+} and Ba^{2+} influx Fura-2-loaded neutrophils were activated in a Ca^{2+}-free medium, which were supplemented with 1 mM Sr^{2+} or Ba^{2+}. Fluorescence changes were monitored at 510 nm with excitation at 340 and 380 nm in a ratio mode.

Measurement of mitochondrial membrane potential Neutrophils (5×10^7 cells/ml) were incubated at room temperature with 5 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for 10 min. After being washed, the fluorescence changes were monitored with a double-wavelength fluorescence spectrophotometer alternatively at 528 nm with excitation at 485, and at 633 nm with excitation at 575 in a ratio mode (Thyagarajan et al. 2002). JC-1 fluorescence has two emission peaks, with red fluorescence of J-aggregates indicating hyperpolarized mitochondria and green fluorescence (JC-1 monomers) due to low mitochondria membrane potential.

Materials Kazinol B was isolated and purified (>99%) as described (Wang et al. 1998). Dextran T-500 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hanks' balanced salt solution was obtained from Invitrogen (Carlsbad, CA, USA). Fluo-3/AM and fura-2/AM were purchased from Molecular Probes (Invitrogen). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002) and *N*,*N*-dimethylsphingosine were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Cyclopiazonic acid (CPA), 2-piperazinyl-8-phenyl-4*H*-1-benzopyran-4-one (LY 303511), 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), 2-aminoethyldiphenyl borate (2-APB), ruthenium red, 1-[6-

[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U-73343), *N*-(3-aminomethyl)benzylacetamidine (1400W), 7-nitroindazole, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB 203580), JC-1, calyculin A and oligomycin A were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). Jasplakinolide and *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) were purchased from Alexis (San Diego, CA, USA). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of dimethyl sulfoxide (DMSO) in the reaction mixture was ≤0.5% (v/v).

Statistical analysis Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. Analyses were considered significantly different at $P<0.05$. Values are expressed as means ± SD.

Results and discussion

Kazinol B increases internal Ca^{2+} release and external Ca^{2+} entry through a L-selectin-independent pathway

Kazinol B showed a concentration-dependent increase in $[Ca^{2+}]_i$ preceded by a lag, reached a maximal level at 1–1.5 min then gradually declined in fluo-3-loaded neutrophils in a Ca^{2+}-containing medium (about 441±29 nM of maximal $[Ca^{2+}]_i$ at 30 μM kazinol B) (Fig. 1a) as well as in a Ca^{2+}-free medium (about 154±21 nM of maximal $[Ca^{2+}]_i$ at 30 μM kazinol B; Fig. 1b). Neutrophil surface adhesion molecular L-selectin plays a major role in neutrophil adherence and transmigration through endothelial cells. Activation of L-selectin induced significant changes in $[Ca^{2+}]_i$ (Crockett-Torabi and Fantone 1997) with a small delay. The ligation of L-selectin may activate PLC generating IP_3 through mechanisms independent of pertussis toxin-sensitive G protein (Laudanna et al. 1994). Our previous report demonstrated that kazinol B also activated IP_3 generation and $[Ca^{2+}]_i$ changes through a pertussis toxin-resistant pathway (Wang et al. 1998). Treatment of neutrophils with chymotrypsin and phorbol 12-myriatate 13-acetate (PMA) caused shedding of L-selectin and blunted the capability of neutrophils $[Ca^{2+}]_i$ changes to respond to sulfatide (Laudanna et al. 1994), which possess affinity towards L-selectin adhesion molecule. In the present study, neutrophils exposed to chymotrypsin and PMA under the conditions which abolished the sulfatide-induced $[Ca^{2+}]_i$ changes in rat neutrophils (data not shown) had no effect on kazinol B-induced response (Fig. 1c). In addition, chymotrypsin-treated neutrophils responded normally to formyl-Met-Leu-Phe (fMLP; data not shown), thus obviating the requirement for L-selectin in kazinol B-induced Ca^{2+} signal.

In the absence of external Ca^{2+}, application of CPA, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) blocker, and kazinol B induced the increase in $[Ca^{2+}]_i$ as a consequence of Ca^{2+} release from internal stores (Fig. 2a, c). Besides endoplasmic reticulum and mitochondria, various distinct cellular organelles, including Golgi apparatus, nucleus and lysosomes, can act as Ca^{2+} stores. Davies and Hallett (1996) have demonstrated the existence of two distinct Ca^{2+} storage locations in neutrophils, in which the Ca^{2+} storage site deep within the neutrophil released by formyl peptide and SERCA blocker. Pretreatment of cells with CPA depleted SERCA blocker-sensitive Ca^{2+} stores as evidenced by the complete abrogation of the subsequent $[Ca^{2+}]_i$ changes caused by CPA at the higher concentration (Fig. 2b). Under the same conditions, the $[Ca^{2+}]_i$ change which followed the addition of kazinol B was fully inhibited (Fig. 2d). Therefore, the emptying of SERCA blocker-sensitive Ca^{2+} stores may account for the major storage site for the Ca^{2+} release in response to kazinol B.

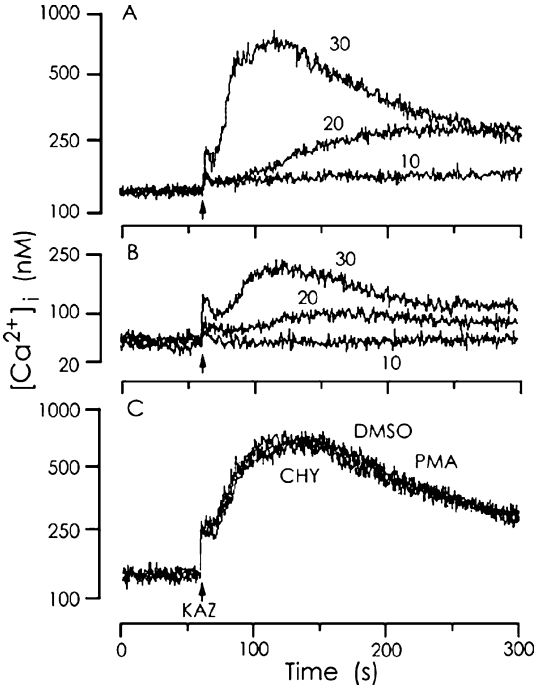

Fig. 1 Kazinol B-stimulated $[Ca^{2+}]_i$ elevation and the role of L-selectin in kazinol B-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were stimulated with the indicated concentrations (μM) of kazinol B (*KAZ*, *arrow*) in **a** a medium containing 1 mM Ca^{2+} or **b** a Ca^{2+}-free medium. In some experiments, cells were pretreated with **c** 0.1 μM phorbol 12-myriatate 13-acetate (*PMA*) or 10 U/ml of chymotrypsin (*CHY*) for 20 min at 37°C before stimulation with 30 μM KAZ in a medium containing 1 mM Ca^{2+}. Similar results were obtained from three to four independent experiments. *DMSO* dimethyl sulfoxide

Effect of Ca^{2+} signal blockers on kazinol B-induced internal Ca^{2+} release

Kazinol B has been demonstrated to elevate $[Ca^{2+}]_i$ through IP_3 generation in neutrophils (Wang et al. 1998). This finding is consistent with the result that U-73122, the inhibitor of PLC-coupled processes, blocked the kazinol B-induced Ca^{2+} signal in a Ca^{2+}-free medium (Fig. 3b),

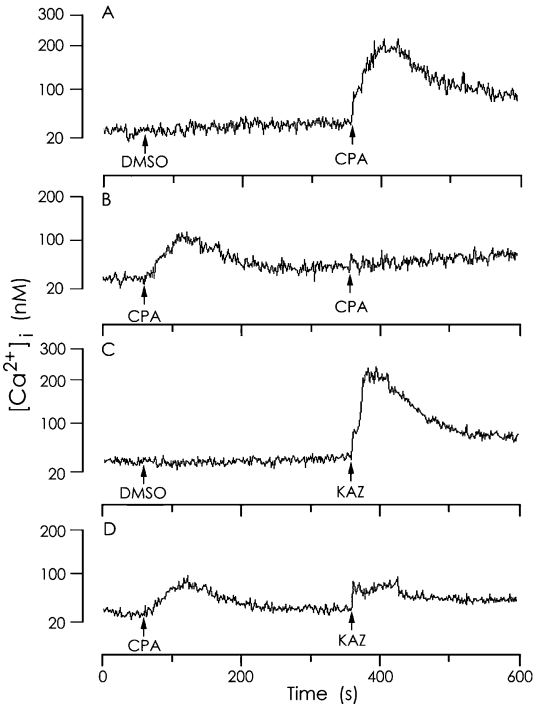

Fig. 2 Effect of cyclopiazonic acid on kazinol B-induced internal Ca^{2+} release. Fluo-3-loaded cells were incubated (*first arrow*) with **a**, **c** DMSO or **b**, **d** 10 μM cyclopiazonic acid (*CPA*), followed by the addition (*second arrow*) of 30 μM CPA or 30 μM kazinol B (*KAZ*) in a Ca^{2+}-free medium. Similar results were obtained from three to four independent experiments.

while the inactive analog U-73343 had no effect. However, tenfold higher concentration of U-73122 was required to diminish kazinol B- and CPA-induced Ca^{2+} release than the fMLP-induced response (Wang 1996). At present, we cannot explain the different potencies of the U-73122 effect between the fMLP and CPA. Lipid mediators derived from membrane sphingolipids, such as sphingosine 1-phosphate (S1P), have been characterized as important intracellular messengers. The Ca^{2+}-mobilizing effect of S1P is often mediated through endothelial differentiation gene receptor (edg), which couples G proteins to PLC activation and IP_3 formation (Ishii et al. 2001). Dimethylsphingosine and dihydrosphingosine have been used to competitively inhibit the phosphorylation of sphingosine to S1P via sphingosine kinase (Yatomi et al. 1996). However, both dimethylsphingosine and dihydrosphingosine cause a sustained Ca^{2+} influx through the non-SOCE pathway in neutrophil-like HL60 cells (Shin et al. 2000). In this study, these pharmacological tools were used to determine the role of S1P in kazinol B-induced Ca^{2+} signal in a Ca^{2+}-free medium to minimize interference with Ca^{2+} influx. At the same concentration, dimethylsphingosine, but not dihydrosphingosine, inhibited (53.2±6.3% inhibition of maximal $[Ca^{2+}]_i$; $P<0.01$) the kazinol B-induced response (Fig. 3a). The discrepancy may be because dimethylsphingosine is more effective than dihydrosphingosine in the blockade of sphingosine kinase (Yatomi et al. 1996). Furthermore, dihydrosphingosine is likely converted to dihydro-S1P, which triggers release of intracellular Ca^{2+}, while dimethylsphingosine is a poor substrate for sphingosine kinase (Shin et al. 2000).

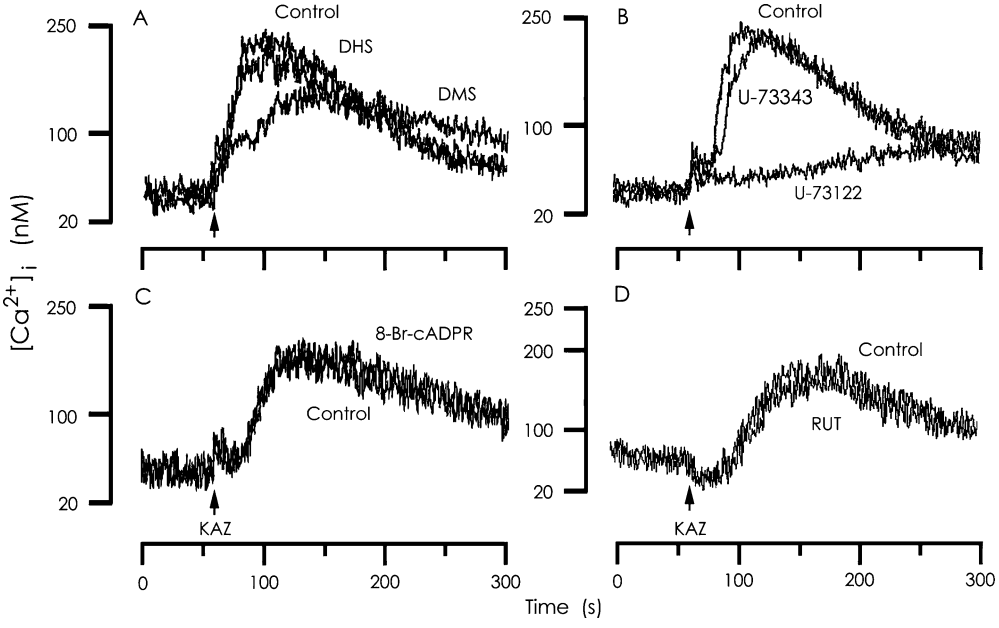

Fig. 3 Effect of dimethylsphingosine, dihydrosphingosine, U-73122, 8-Br-cADPR and ruthenium red on kazinol B-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with vehicle (as control), **a** 30 μM dimethylsphingosine (*DMS*) or 30 μM dihydrosphingosine (*DHS*) for 3 min, **b** 10 μM U-73122 or 10 μM U-73343 for 1 min, **c** 100 μM 8-Br-cADPR for 10 min at 37°C before stimulation with 30 μM kazinol B (*KAZ*, *arrow*) in a Ca^{2+}-free medium. **d** Fura-2-loaded cells were incubated with 30 μM ruthenium red (*RUT*) for 1 min before stimulation with KAZ in a Ca^{2+}-free medium. The fluorescence changes were monitored by fura-2 ratio-fluorimetry. Similar results were obtained from three to four independent experiments.

Thus, it is plausible that the S1P signal is associated with kazinol B-induced $[Ca^{2+}]_i$ changes.

Besides the IP_3, the other pathway for Ca^{2+} release from internal stores is operated by cyclic ADP-ribose (cADPR) through the ryanodine receptor (Galione et al. 1991). The transmembrane glycoprotein CD38 (ADP-ribosyl cyclase) catalyzes the production of cADPR from its substrate NAD^+ (Howard et al. 1993). Pretreatment of cells with 8-Br-cADPR, an inactive analog of cADPR that competitively antagonizes cADPR binding to ryanodine receptor (Guse 1999), had no appreciable effect on kazinol B-induced Ca^{2+} response (Fig. 3c), suggesting that the $[Ca^{2+}]_i$ rise is not mediated by cADPR signaling. It has been reported that ruthenium red inhibited the increase in $[Ca^{2+}]_i$ in ryanodine-stimulated neutrophils (Partida-Sánchez et al. 2001). Thus, the lack of inhibition by ruthenium red (Fig. 3d) further obviates the requirement for the activation of ryanodine receptor in kazinol B-induced Ca^{2+} release.

Characteristics of kazinol B-mediated cation permeability

The effectiveness of specific Ca^{2+} channel blockers, La^{3+} and Ni^{2+}, in blocking passage of Ca^{2+} has been widely used as criteria for defining differences between putative entry mechanisms. Varying concentrations of La^{3+} or Ni^{2+} were added with kazinol B. Application of 0.1 mM Ni^{2+} attenuated $[Ca^{2+}]_i$ to 56.3±4.7% of kazinol B-induced maximal response ($P<0.01$). Ni^{2+} up to 0.3 mM nearly abolished the Ca^{2+} signal (Fig. 4a). La^{3+} significantly reduced (42.6±5.2% inhibition at 0.1 mM La^{3+}; $P<0.01$) the kazinol B-induced maximal $[Ca^{2+}]_i$ elevation and eliminated the kazinol B-induced response at 0.3 mM La^{3+} (Fig. 4b). The inhibitory activities of La^{3+} and Ni^{2+} were within the same range of potency as that in the CPA-induced SOCE pathway (Wang 2003a), suggesting a similarity exists between these two modes of Ca^{2+} entry.

In neutrophils, store emptying stimulated entry of Ca^{2+}, Ba^{2+} and Sr^{2+} has been reported (Wenzel-Seifert et al. 1996; Wang 2003a). Ba^{2+} is not pumped by Ca^{2+}-ATPase either into internal stores or out of the cell (Kwan and Putney 1990) and hence can be used as a surrogate for Ca^{2+} to trace channel activity. Entry of these divalent cations in SOCE pathway was concentration-dependent with permeability in the following order: $Ca^{2+}>Ba^{2+}\geq Sr^{2+}$. Kazinol B induced Ca^{2+} entry, however, no appreciable entry of Ba^{2+} and Sr^{2+} occurred by fura-2 ratio-fluorimetry (Fig. 4c). The results with Ba^{2+} and Sr^{2+} revealed a difference in the apparent selectivity for passage of cations activated by SOCE as opposed to kazinol B-induced Ca^{2+} entry pathway in neutrophils.

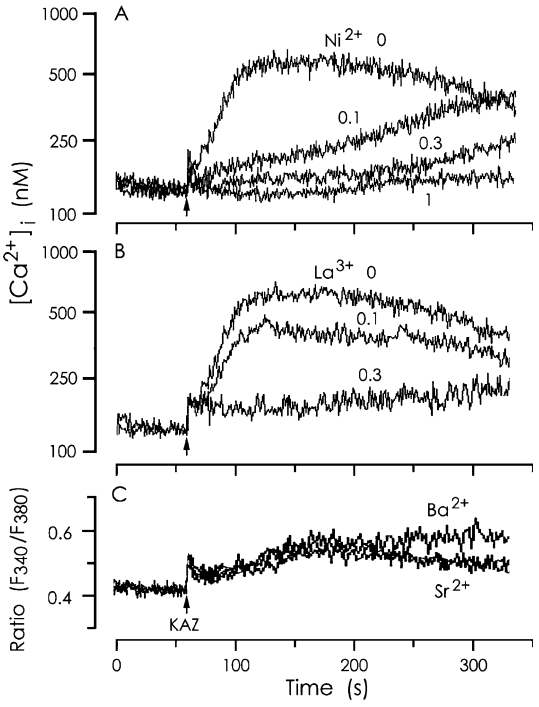

Fig. 4 Effect of Ni^{2+} and La^{3+} on kazinol B-induced $[Ca^{2+}]_i$ changes and the Ba^{2+} and Sr^{2+} permeability in response to kazinol B. Fluo-3-loaded cells were stimulated with 30 μM kazinol B (*KAZ*, *arrow*) plus the indicated concentrations (mM) of **a** Ni^{2+} or **b** La^{3+} in a medium containing 1 mM Ca^{2+}. **c** Fura-2-loaded neutrophils were stimulated with KAZ in a Ca^{2+}-free medium supplemented with 1 mM Sr^{2+} or 1 mM Ba^{2+}. Fluorescence changes were measured in a ratio mode. Similar results were obtained from three to four independent experiments.

Effects of Ca^{2+} signal blockers on kazinol B-mediated $[Ca^{2+}]$i changes

A useful tool in elucidating the coupling mechanism for SOC channel activation has been the cell-permeant IP_3 receptor blocker, 2-APB (Maruyama et al. 1997). 2-APB abolished the CPA-induced $[Ca^{2+}]_i$ changes (Wang 2003a), but only partially reduced the kazinol B-induced response (34.9±6.2% inhibition of maximal $[Ca^{2+}]_i$; $P<0.05$; Fig. 5a). It seems likely that IP_3 receptor activation in kazinol B-induced Ca^{2+} response plays a minor role of. Recent evidence indicates that the principal antagonistic effect of 2-APB is on Ca^{2+} entry rather than Ca^{2+} release (Bootman et al. 2002) and that 2-APB has no inhibitory effect on Ca^{2+} influx caused by activation of non-SOCE channels (Wang 2003a, 2003b; Luo et al. 2001). It is likely that kazinol B-activates, probably, via non-SOCE pathway in neutrophils. Genistein, the general tyrosine kinase inhibitor, inhibited the SOCE in neutrophils (Waddell et al. 1995; Wang 2003a) and also affected the kazinol B-induced response (38.3±5.4% inhibition of maximal $[Ca^{2+}]_i$; $P<0.05$; Fig. 5a), thus protein tyrosine phosphorylation might play a role in kazinol B-induced $[Ca^{2+}]_i$ elevation.

MDL-12,330A blocked the Ca^{2+} entry, independent of adenylyl cyclase, following store emptying (van Rossum et al. 2000). In addition, SKF-96365, the receptor-operated

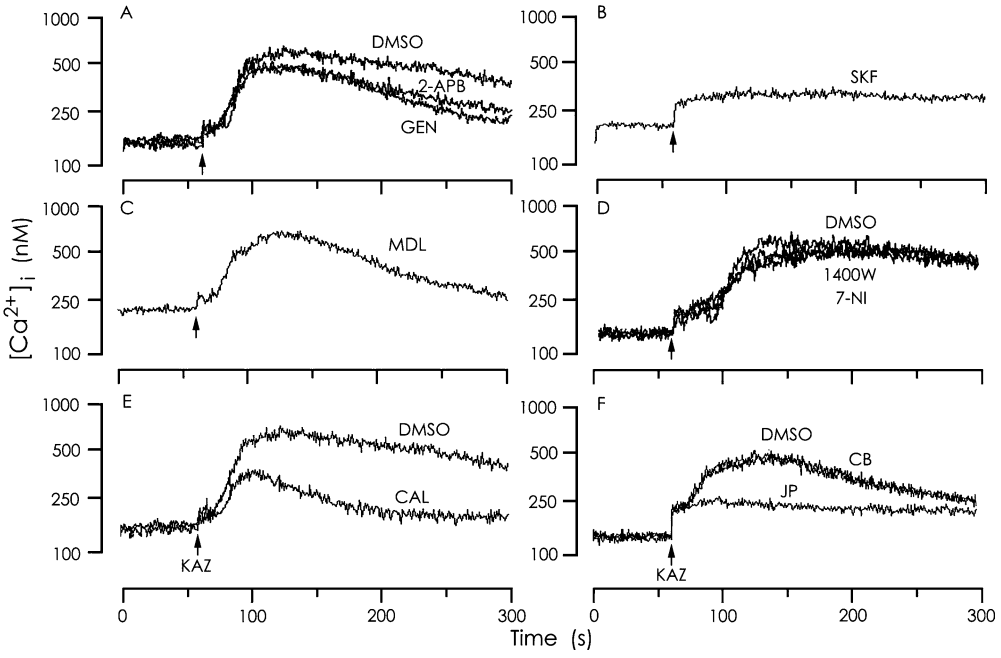

Fig. 5 Effects of 2-APB, genistein, SKF-96365, MDL-12,330A, calyculin A, 1400W, 7-nitroindazole, cytochalasin B, and jasplakinolide on kazinol B-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with DMSO (as control), **a** 100 μM 2-APB, 100 μM genistein (*GEN*), **b** 50 μM SKF-96365 (*SKF*) for 1 min, or with **c** 100 μM MDL-12,330A (*MDL*), **d** 100 μM 1400 W or 150 μM 7-nitroindazole (*7-NI*), **e** 0.1 μM calyculin A (*CAL*) for 10 min, or with **f** 5 μg/ml of cytochalasin B (*CB*) or 10 μM jasplakinolide (*JP*) for 30 min at 37°C before stimulation with 30 μM kazinol B (*KAZ*, *arrow*) in a medium containing 1 mM Ca^{2+}. Similar results were obtained from three to four independent experiments.

and voltage-gated Ca^{2+} channel inhibitor, blocked the fMLP-activated Ca^{2+} entry in neutrophils (Wenzel-Seifert et al. 1996; Wang et al. 2001). Our recent reports indicated that MDL-12,330A and SKF-96365 greatly reduced the $[Ca^{2+}]_i$ elevation by CPA in neutrophils (Wang 2003a). Under the same conditions, SKF-96365 nearly abolished kazinol B-induced Ca^{2+} signal, while MDL-12,330A had no significant inhibitory effect (Fig. 5b, c). Thus, the notion that different mechanisms exist in the operations of SOCE and kazinol B-induced Ca^{2+} entry is further strengthened by comparison of the effects of these two Ca^{2+} signal blockers. It has been reported that SKF-96365 causes the release of intracellular Ca^{2+} and in some cases influx of Ca^{2+} (Harper and Daly 2000), which could account for a significant rise in $[Ca^{2+}]_i$ by SKF-96365 alone. Interestingly, pretreatment with MDL-12,330A also increased the basal levels of $[Ca^{2+}]_i$.

NO has been proposed to control Ca^{2+} transport via cGMP-dependent and -independent pathways. Our recent report demonstrated that the NO donors stimulated Ca^{2+} influx through the non-SOCE pathway independent of cGMP (Wang 2003b). A functional neuronal nitric oxide synthase (nNOS) system was found, while there was no evidence for inducible nitric oxide synthase (iNOS) in normal rat neutrophils (Greenberg et al. 1998). However, iNOS expression is upregulated in neutrophils after administration of endotoxin. The finding that 7-nitroindazole, an inhibitor of nNOS, and 1400W, an inhibitor of iNOS, failed to attenuate the kazinol B-induced Ca^{2+} response (Fig. 5d), obviates the involvement of NO.

Several hypotheses have been considered for the mechanism of SOCE. Recently, a secretion-like coupling model based on a physical and reversible trafficking of portions of the endoplasmic reticulum toward the plasma membrane has been proposed (Patterson et al. 1999), which is supported by the dynamic cytoskeletal structure (Rosado and Sage 2001). Calyculin A, an inhibitor of protein serine/threonine phosphatases 1/2, redistributed actin filaments to the cell peripheral, which prevents SOCE activation by acting as a barrier to block the coupling process. Calyculin A greatly attenuated the CPA- and fMLP-induced Ca^{2+} entry in neutrophils (Wang et al. 2001; Wang 2003a), and also diminished the action of kazinol B (65.2±7.2% inhibition of maximal $[Ca^{2+}]_i$; $P<0.01$) in the present study (Fig. 5e). Jasplakinolide has been used as a tool for inducing polymerization and stabilization of actin filaments (Bubb et al. 1994), which reorganized into a tight cortical layer adjacent to the plasma membrane and prevented activation of SOCE (Rosado et al. 2000). Like calyculin A, cells exposed to jasplakinolide substantially reduced the kazinol B-induced $[Ca^{2+}]_i$ rise (Fig. 5f). In contrast, cytochalasin B, the widely employed pharmacological tool to assess the role of actin filament disruption in cell activation, failed to exert any appreciable inhibitory effect on kazinol B-induced response. The findings suggest that the stabilization of the cortical F-actin network might also prevent $[Ca^{2+}]_i$ rise in the kazinol B-mediated process. So far, we don't know how the signaling pathways of tyrosine kinase and actin filament implicated in the kazinol B-induced $[Ca^{2+}]_i$ changes. SOCE must have some characteristics similar to non-SOCE because several Ca^{2+} signal inhibitors suppress SOCE as well as non-SOCE. Our previous report demonstrated that calyculin A, MDL-12,330A and SKF-96365 reduced both SOCE and non-SOCE caused by CPA and *N*-ethylmaleimide, respectively (Wang 2003a).

Effects of PI3K pathway blockers on kazinol B-mediated $[Ca^{2+}]i$ changes

Phosphatoinositide 3-kinase (PI3 K) and its product phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) are involved in the recruitment and activation of PLC-γ in many different cell types including neutrophils (Vossebeld et al. 1997). In addition, $PI(3,4,5)P_3$ directly stimulates a Ca^{2+} transport system in plasma membrane independent of PLC activity in RBL-2H3 mast cells (Ching et al. 2001). We used two distinct inhibitors of PI3 K, wortmannin and LY 294002, to examine the implication of PI3 K pathway in kazinol B-induced $[Ca^{2+}]_i$ rise. These inhibitors blocked PLC-γ activation and/or Ca^{2+} signals in neutrophils (Vossebeld et al. 1997). LY 294002 produced a concentration-dependent reduction in kazinol B-induced $[Ca^{2+}]_i$ elevation in the presence or absence of Ca^{2+} in medium (nearly abolished the Ca^{2+} response at 30 μM LY 294002; Fig. 6a, c), while the inactive analog LY 303511 had no effect. However, wortmannin failed to inhibit the kazinol B-induced response (data not shown). The reason for this apparent discrepancy is not clear, which makes it difficult to interpret the role of PI3 K in kazinol B-mediated process in the absence of additional experiments, although LY 294002 has been reported to be a more selective PI3K inhibitor (Vlahos et al. 1994). The activation of Akt, the downstream protein kinase of PI3 K, requires phosphorylation by p38 mitogen-activated protein kinase (MAPK) in neutrophils (Rane et al. 2001). Interestingly, the p38 MAPK inhibitor SB 203580 alone produced a rapid and transient $[Ca^{2+}]_i$ rise, while reducing the subsequent $[Ca^{2+}]_i$ elevation following the stimulation with kazinol B in a concentration-dependent manner (59.2±7.5% inhibition of maximal $[Ca^{2+}]_i$ at 20 μM SB 203580; $P<0.01$) in a Ca^{2+}-containing medium (Fig. 6b). Because SB 203580 at 10 μM nearly abolished the kazinol B-induced Ca^{2+} release from internal stores in a Ca^{2+}-free medium (Fig. 6d), the further inhibition of $[Ca^{2+}]_i$ at concentrations >10 μM may arise from the blockade of external Ca^{2+} entry in a Ca^{2+}-containing medium. SB 203580 has also been reported to block the phosphorylation and activation of Akt by inhibiting the 3-phosphoinositide-dependent kinase-1 (Lali et al. 2000). It is unclear at present whether or not the PI3 K/Akt and/or p38 MAPK pathways are attributable to the kazinol-induced Ca^{2+} signal. Nevertheless, the substantial inhibition by LY 294002 of kazinol B-induced Ca^{2+} response is inconsistent with the finding in our recent report that the inhibition of CPA-induced $[Ca^{2+}]_i$ rise was slight (about 25% inhibition) by 50 μM LY 294002 (Wang 2003a), which clearly confirms the hypothesis that the kazinol B-mediated process utilizes different mechanisms.

Effect of kazinol B on membrane Ca^{2+}-ATPase activity

After cell activation, removal of Ca^{2+} from the cytosol occurs via extrusion by the plasma membrane Ca^{2+}-ATPase (PMCA) and the plasmalemmal Na^+/Ca^{2+} exchanger as well as sequestration into intracellular stores by the SERCA. It is unlikely that kazinol B stimulation of $[Ca^{2+}]_i$ rise occurs through the blockade of SERCA, because this resulted in activation of the SOCE pathway. To address the effect of kazinol B on PMCA, we attempted to isolate the PMCA activity pharmacologically. Neutrophils were stimulated with fMLP/CPA to maximally empty the internal Ca^{2+} store, inhibit SERCA, and to induce Ca^{2+} entry in a Na^+-deprived medium containing 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of mitochondria function, to prevent mitochondrial Ca^{2+} uptake, and 1 μM oligomycin A to inhibit the mitochondrial ATP synthase and prevent ATP consumption. This treatment caused a large increase

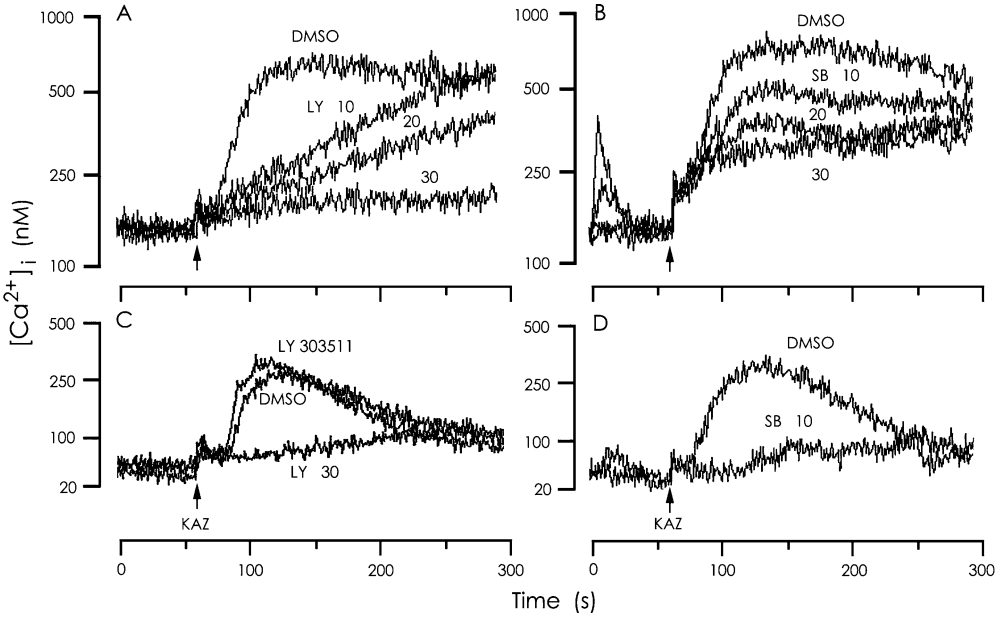

Fig. 6 Effects of LY 294002 and SB 203580 on kazinol B-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with DMSO (as control), the indicated concentrations (μM) of **a** LY 294002 (*LY*) or **b** SB 203580 (*SB*) for 1 min at 37°C in a medium containing 1 mM Ca^{2+}, or incubated with **c** 30 μM LY, 30 μM LY 303511 or **d** 10 μM SB for 1 min at 37°C in a Ca^{2+}-free medium before stimulation with 30 μM kazinol B (*KAZ*, *arrow*). Similar results were obtained from three to four independent experiments.

in $[Ca^{2+}]_i$. Subsequent removal of external Ca^{2+} with 1 mM EDTA evoked a decline in $[Ca^{2+}]_i$ possibly due to Ca^{2+} clearance by PMCA. Under these conditions, the Ca^{2+} clearance rate was inhibited by La^{3+} and calmidazolium (data not shown) but was not inhibited by kazinol B (Fig. 7a), suggesting that PMCA is not the site of action of kazinol B. In addition, there was no indication that kazinol B affected Na^+–Ca^{2+} exchange activity on the plasma membrane because similar responses of kazinol B-activated Ca^{2+} signals were obtained in normal as well as in Na^+-deprived HEPES buffer (Fig. 7b).

Effect of kazinol B on SOCE pathway and mitochondrial membrane potential

Our recent report revealed that *N*-ethylmaleimide, a thiol-alkylating agent, stimulated the non-SOCE and augmented the CPA-induced Ca^{2+} response in neutrophils (Wang 2003a). In contrast, AA stimulated the non-SOCE, but inhibited the SOCE in A7r5 vascular smooth muscle cells (Moneer et al. 2003). We were interested in the influence of Ca^{2+} signals by kazinol B-induced non-SOCE process on the store emptying operation in neutrophils. In a Ca^{2+}-free medium, CPA selectively inhibits the Ca^{2+} pump of internal stores, allowing the stores to be emptied independently of receptor activation and thereby activating the SOCE mechanism, in which robust Ca^{2+} entry commences immediately upon addition of Ca^{2+} into the medium. Application of 10 μM kazinol B, which alone was ineffective in Ca^{2+} signal, with CPA augmented the internal Ca^{2+} release in a Ca^{2+}-free medium, however, inhibited the subsequent Ca^{2+} entry upon supplementing with Ca^{2+} (51.6±5.7% inhibition of maximal $[Ca^{2+}]_i$; $P<0.01$; Fig. 8a). A similar level of inhibition of Ca^{2+} entry was also observed by addition of kazinol B with fMLP, which emptied internal stores via receptor activation (Fig. 8b).

Mitochondria are well known participants in the regulation of $[Ca^{2+}]_i$ homeostasis, capable of modulating cytosolic Ca^{2+} signals. Functional mitochondria appear to maintain SOCE by effective sequestration of subplasmalemmal Ca^{2+} and by the consequent attenuation of Ca^{2+}-induced inactivation of SOC channels (Hoth et al. 1997). Mitochondrial Ca^{2+} uptake is driven by the membrane potential that is maintained by extrusion of H^+. Thus, hyperpolarization increases the driving force for mitochondrial Ca^{2+} uptake. CCCP dissipates mitochondrial H^+ gradients and inactivates SOC channels (Makowska et al. 2000). JC-1 fluorescence was reduced by 1 μM CCCP and by kazinol B in a concentration-dependent manner (vehicle control value vs. ≥10 μM kazinol B, $P<0.05$; Fig. 9). It is plausible that kazinol B can mimic the CCCP effect to suppression of mitochondrial function and thus the inhibition of SOCE.

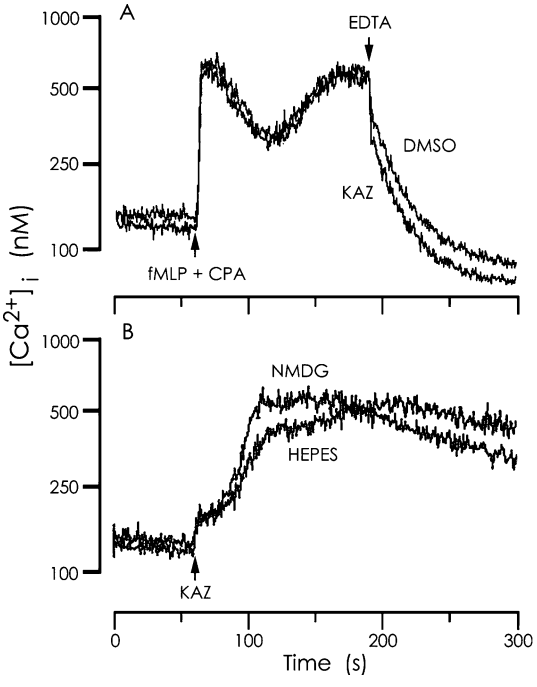

Fig. 7 Effects of kazinol B on plasma membrane Ca^{2+}-ATPase activity and the effect of Na^+ deprivation on kazinol B-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were **a** stimulated with 0.3 μM fMLP plus 10 μM cyclopiazonic acid (*CPA*) followed by the addition of DMSO or 30 μM kazinol B (*KAZ*) together with 1 mM EDTA (*downward arrow*) in an Na^+-deprived medium containing 0.3 mM Ca^{2+} supplemented with 1 μM oligomycin A and 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (*CCCP*), or were **b** stimulated with KAZ in a normal HEPES medium or a Na^+-deprived medium (*NMDG*). Similar results were obtained from three to four independent experiments.

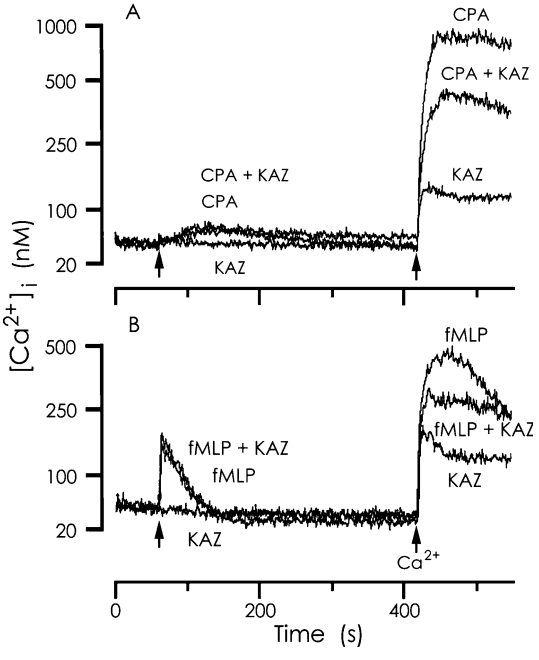

Fig. 8 Effect of kazinol B on fMLP- and cyclopiazonic acid-induced Ca^{2+} entry. Fluo-3-loaded cells were **a** exposed (*first arrow*) to 10 μM cyclopiazonic acid (*CPA*), 10 μM kazinol B (*KAZ*) or CPA plus KAZ in a Ca^{2+}-free medium followed by the addition (*second arrow*) of 1 mM Ca^{2+} into the medium, or were **b** exposed (*first arrow*) to 0.3 μM fMLP, KAZ or fMLP plus KAZ in a Ca^{2+}-free medium followed by the addition of Ca^{2+} into the medium. Similar results were obtained from three to four independent experiments.

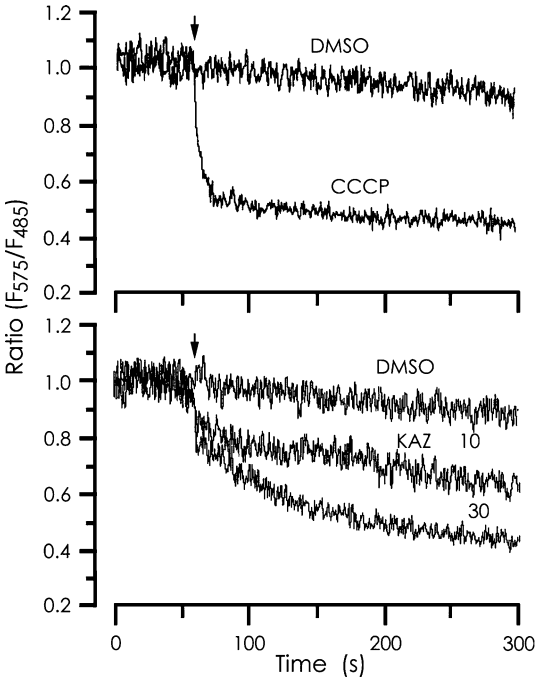

Fig. 9 Effect of kazinol B on mitochondrial membrane potential. DMSO, 1 μM CCCP or the indicated concentrations (μM) of kazinol B (*KAZ*) were added (*arrow*) to JC-1-loaded neutrophils at 37°C in a medium containing 1 mM Ca^{2+}. Fluorescence changes were measured in a ratio mode. Similar results were obtained from three to four independent experiments.

In conclusion, kazinol B, a natural plant product, stimulates $[Ca^{2+}]_i$ rise in rat neutrophils through the release of internal store Ca^{2+} and the activation of Ca^{2+} entry. It is unlikely that kazinol B evokes Ca^{2+} signal via L-selectin ligation, or by influence on PMCA, SERCA and plasmalemmal Na^+/Ca^{2+} exchanger. Analysis of the cation permeability and the actions of Ca^{2+} signal blockers suggests a fundamental difference between the function of SOCE and the kazinol B-mediated mechanism. It is plausible that kazinol B induced Ca^{2+} release through IP_3 and S1P signaling, and Ca^{2+} entry mainly via non-SOCE pathway. In addition, kazinol B probably inhibits SOCE by breaking the Ca^{2+} driving force of mitochondria.

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References

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signaling. Nature 1341:197–205

Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM (2002) 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. FASEB J 16:1145–1150

Broad LM, Cannon TR, Taylor CW (1999) A non-capacitative pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentration of vasopressin. J Physiol 517:121–134

Bubb MR, Senderowicz AM, Sausville EA, Duncan KL, Korn ED (1994) Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J Biol Chem 269:14869–14871

Chi YS, Jong HG, Son KH, Chang HW, Kang SS, Kim HP (2001) Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: cyclooxygenase and lipoxygenase. Biochem Pharmacol 62:1185–1191

Ching TT, Su AL, Johnson AJ, Chen CS (2001) Phosphoinositide 3-kinase facilitates antigen-stimulated Ca^{2+} influx in RBL-2H3 mast cells via a phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca^{2+} entry mechanism. J Biol Chem 276:14814–14820

Crockett-Torabi E, Fantone JC (1997) L-Selectin stimulation of canine neutrophil initiates calcium signal secondary to tyrosine kinase activation. Am J Physiol 272:H1302–H1308

Davies EV, Hallett MB (1996) Near membrane Ca^{2+} changes resulting from store release in neutrophils: detection by FFP-18. Cell Calcium 19:355–362

Galione A, Lee HC, Busa WB (1991) Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. Science 253:1143–1146

Greenberg SS, Ouyang J, Zhao X, Giles TD (1998) Human and rat neutrophils constitutively express neural nitric oxide synthase mRNA. Nitric Oxide 2:203–212

Guse AH (1999) Cyclic ADP-ribose: a novel Ca^{2+} mobilizing second messenger. Cell Signal 11:309–316

Harper JL, Daly JW (2000) Effect of calmidazolium analogs on calcium influx in HL-60 cells. Biochem Pharmacol 60:317–324

Hoth M, Fanger CM, Lewis RS (1997) Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol 137:633–648

Howard M, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RM, Walseth TF, Lee HC (1993) Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. Science 262:1056–1059

Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. J Biol Chem 276:33697–33704

Kwan CY, Putney JW (1990) Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells: dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. J Biol Chem 265:678–684

Lali FV, Hunt AE, Turner SJ, Foxwell BM (2000) The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independent of p38 mitogen-activated protein kinase. J Biol Chem 275:7395–7402

Laudanna C, Constantin G, Baron P, Scarpini E, Scarlato G, Cabrini G, Dechecchi C, Rossi F, Cassatella MA, Berton G (1994) Sulfatides trigger increase of cytosolic free calcium and enhanced expression of tumor necrosis factor-α and interleukin-8 mRNA in human neutrophils: evidence for a role of L-selectin as a signaling molecule. J Biol Chem 269:4021–4026

Lin CN, Lu CM, Lin HC, Fang SC, Shieh BJ, Hsu MF, Wang JP, Ko FN, Teng CM (1996) Novel antiplatelet constituents from Formosan Moraceous plants. J Nat Prod 59:834–838

Luo D, Broad LM, Bird GS, Putney JW (2001) Signaling pathways underlying muscarinic receptor-induced $[Ca^{2+}]_i$ oscillations in HEK293 cells. J Biol Chem 276:5613–5621

Makowska A, Zablocki K, Duszynski J (2000) The role of mitochondria in the regulation of calcium influx into Jurkat cells. Eur J Biochem 267:877–884

Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P_3-induced Ca^{2+} release. J Biochem (Tokyo) 122:498–505

Moneer Z, Dyer J, Taylor CW (2003) Nitric oxide co-ordinates the activities of the capacitative and non-capacitative Ca^{2+}-entry pathways regulated by vasopressin. Biochem J 370:439–448

Partida-Sánchez S, Cockayne DA, Monard S, Jacobson EL, Oppenheimer N, Garvy B, Kusser K, Goodrich S, Howard M, Harmsen A, Randall TD, Lund FE (2001) Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. Nat Med 7:1209–1216

Patterson RL, van Rossum DB, Gill DL (1999) Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. Cell 98:487–499

Putney JW (1990) Capacitative calcium entry revisited. Cell Calcium 11:611–624

Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P, McLeish KR (2001) p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. J Biol Chem 276:3517–3523

Rosado JA, Sage SO (2000) Protein kinase C activates non-capacitative calcium entry in human platelet. J Physiol 529:159–169

Rosado JA, Sage SO (2001) Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. Biochem J 356:191–198

Rosado JA, Jenner S, Sage SO (2000) A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets: evidence for conformational coupling. J Biol Chem 275:7527–7533

Ryu JH, Ahn H, Jin LH (2003) Inhibition of nitric oxide production in LPS-activated macrophages by kazinol B from *Broussonetia kazinoki*. Fitoterapia 74:350–354

Shin Y, Daly JW, Choi OH (2000) Diverse effects of sphingosine on calcium mobilization and influx in differentiated HL-60 cells. Cell Calcium 27:269–280

Thyagarajan B, Malli R, Schmidt K, Graier WF, Groschner K (2002) Nitric oxide inhibits capacitative Ca^{2+} entry by suppression of mitochondria Ca^{2+} handling. Br J Pharmacol 137:821–830

Van Rossum DB, Patterson RL, Ma HT, Gill DL (2000) Ca^{2+} entry mediated by store depletion, *S*-nitrosylation, and TRP3 channels: comparison of coupling and function. J Biol Chem 275:28562–28568

Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase: 2-(4-morpholinyl)-8-phenyl-4*H*-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248

Vossebeld PJ, Homburg CH, Schweizer RC, Ibarrola I, Kessler J, Koenderman L, Roos D, Verhoeven AJ (1997) Tyrosine phosphorylation-dependent activation of phosphatidylinositide 3-kinase occurs upstream of Ca^{2+}-signalling induced by Fcγ receptor cross-linking in human neutrophils. Biochem J 323:87–94

Waddell TK, Fialkow L, Chan CK, Kishimoto TK, Downey GP (1995) Signaling functions of L-selectin: enhancement of tyrosine phosphorylation and activation of MAP kinase. J Biol Chem 270:15403–15411

Wang JP (1996) U-73122, an aminosteroid phospholipase C inhibitor, may also block Ca^{2+} influx through phospholipase C-independent mechanism in neutrophil activation. Naunyn-Schmiedebergs Arch Pharmacol 353:599–605

Wang JP (2003a) Characterization of maleimide-activated Ca^{2+} entry in neutrophils. Biochem Pharmacol 65:1923–1929

Wang JP (2003b) GEA3162 stimulates Ca^{2+} entry in neutrophils. Eur J Pharmacol 458:243–249

Wang JP, Tsao LT, Raung SL, Lin CN (1998) The signal transduction mechanism involved in kazinol B-stimulated superoxide anion generation in rat neutrophils. Br J Pharmacol 125:517–525

Wang JP, Chang LC, Huang LJ, Kuo SC (2001) Inhibition of extracellular Ca^{2+} entry by YC-1, an activator of soluble guanylyl cyclase, through a cyclic GMP-independent pathway in rat neutrophils. Biochem Pharmacol 62:679–684

Wenzel-Seifert K, Krautwurst D, Musgrave I, Seifert R (1996) Thapsigargin activates univalent- and bivalent-cation entry in human neutrophils by a SK&F 96365- and Gd^{3+}-sensitive pathway and is a partial secretagogue: involvement of pertussis-toxin-sensitive G-proteins and protein phosphatases 1/2A and 2B in the signal-transduction pathway. Biochem J 314:679–686

Yatomi Y, Ruan F, Megidish T, Toyokuni T, Kakomori S. Igarashi Y (1996) *N*,*N*-Dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. Biochemistry 35:626–633