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Dual Effects of SK&F 96365 in Human Leukemic HL-60 Cells

INHIBITION OF CALCIUM ENTRY AND ACTIVATION OF A NOVEL CATION INFLUX PATHWAY

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ABSTRACT. The effects of the receptor-mediated Ca^{2+} entry blocker, SK&F 96365 on thapsigargin (TSG)-induced Ca^{2+} entry in fura-2-loaded HL-60 cells were studied. After Ca^{2+} release induced by 30 nM TSG, readmission of Ca^{2+} resulted in a sustained Ca^{2+} entry, which could be partially inhibited by 1–3 μM SK&F 96365. Surprisingly, SK&F 96365 at 30–100 μM, instead of causing a stronger inhibition, actually promoted Ca^{2+} entry. Furthermore, at 16–100 μM, this drug released intracellular Ca^{2+} on its own and induced Ca^{2+} entry upon readmission of Ca^{2+}. This SK&F 96365-activated Ca^{2+} entry pathway was insensitive to nifedipine and, interestingly, accessible to Ni^{2+} and La^{3+}. However, SK&F 96365 (30 μM) almost completely blocked (basal) Mn^{2+} entry and only caused 4.4% of the cells to be stained with trypan blue, strongly suggesting that the SK&F 96365-activated cation entry was not due to damage nor to a very nonselective permeabilization of the plasma membrane. These data indicate that low concentrations of SK&F 96365 inhibited Ca^{2+} entry and higher concentrations activated a novel cation entry pathway. Because these 2 opposing effects overlapped at an intermediate concentration (16 μM), which is within the range commonly used to block Ca^{2+} entry, cautious use of this Ca^{2+} antagonist appears to be warranted. BIOCHEM PHARMACOL 51;5:605–612, 1996.

KEY WORDS. SK&F 96365; thapsigargin; Ca^{2+} entry; La^{3+}; Ni^{2+}; Mn^{2+}; HL-60 cells

AACE† is important in a number of physiological responses [1]. One of the means by which an agonist activates Ca^{2+} entry is the depletion of intracellular nonmitochondrial Ca^{2+} stores as a result of inositol 1,4,5-trisphosphate-induced Ca^{2+} release [1–3]. The notion of Ca^{2+} SOCE, also known as "capacitative Ca^{2+} entry" [2], has gained strong experimental support from observations that Ca^{2+} entry could be activated by TSG and CPA (selective inhibitors of microsomal Ca^{2+} pumps), which cause Ca^{2+} store depletion without raising the level of any known intracellular messengers [4–7]. Selective blockers of SOCE and AACE would be important in elucidating the physiological roles of these Ca^{2+} entry mechanisms. As SOCE [3] and certain mechanisms of AACE [8] are nonvoltage-operated, they are resistant to the classical Ca^{2+} antagonists such as nifedipine and verapamil. Until now only a limited number of organic compounds that could block nonvoltage-operated Ca^{2+} channels have been determined. SK&F 96365, claimed to be a novel inhibitor of "receptor-mediated Ca^{2+} entry," inhibits AACE in nonexcitable cells, such as human neutrophils, platelets, and endothelial cells [9], CPA-induced SOCE in thyroid FRTL-5 cells [10], and human leukemia HL-60 cells [11]. In this work, it was shown that SK&F 96365 partially inhibited TSG-induced SOCE at low concentrations (1–3 μM) and, unexpectedly, *activated* cation entry at higher concentrations. Here, we study the characteristics of this cation entry pathway and show that it prevents full inhibition of Ca^{2+} entry by SK&F 96365. The significance of the latter phenomenon with regard to the use of this pharmacological tool is also discussed.

MATERIALS AND METHODS

Materials

Ionomycin, Triton X-100, saponin, TSG, nifedipine and fura-2-acetoxymethyl ester were obtained from Sigma Chemical Co., St. Louis, MO. Fetal calf serum was purchased from Gibco BRL (Gaithersburg, MD). SK&F 96365 was purchased from BIOMOL Research lab. (Plymouth Meeting, PA) and dissolved in absolute DMSO to yield a stock solution of 30 mM.

Cell Culture

HL-60 and M12.4 cells obtained from the American Type Culture Collection, MD, were maintained in Roswell Park

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† *Abbreviations:* TSG, thapsigargin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; SOCE, store-operated Ca^{2+} entry; CPA, cyclopiazonic acid; AACE, agonist-activated Ca^{2+} entry; HBSS. Hank's buffered saline solution.

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Memorial Institute 1640 medium (Sigma) supplemented with 10% fetal calf serum, 5 μg gentamycin/mL and penicillin/streptomycin (100 U/mL, 100 μg/mL) in a humidified atmosphere with 5% CO_2 at 37°C. The cells were split in a 1:5 ratio every 2 or 3 days.

$[Ca^{2+}]_i$ Measurement

The method used to measure $[Ca^{2+}]_i$ was described previously [12]. HBSS, composed of 138 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5 mM glucose, and 25 mM HEPES buffered at pH 7.4, was used for cell washing. HL-60 and M12.4 cells grown to a density of approximately 1.5×10^6/mL were harvested, washed, and resuspended in a serum-free RPMI 1640 medium at a cell density of approx. 2×10^7/mL. Fura-2-acetoxymethyl ester was added to this cell suspension at a final concentration of 5 μM. The cell suspension was then incubated at 37°C for 45 min. Thereafter, the fura-2-loaded cells were washed twice in Ca^{2+}-containing HBSS and resuspended in the same buffer solution at a cell density of 2.8×10^6/mL. Before experimentation of each sample, 1 mL of this cell suspension was centrifuged and the cell pellet resuspended in 2 mL Ca^{2+}-free or Ca^{2+}-containing HBSS. This 2-mL cell suspension was then transferred to a quartz cuvette that contained a mini stirrer and warmed at 37°C for approx. 3 min before addition of drugs. Fura-2 fluorescence was detected with a Hitachi F-4000 fluorescence spectrophotometer. Excitation and emission wavelengths were set at 340 nm and 500 nm, respectively. For calibration, F_{max} was obtained by lysing the cells with 0.1% Triton X-100 and F_{min} by chelating Ca^{2+} with 20 mM EGTA. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* [13]. In Ca^{2+}-free medium, basal $[Ca^{2+}]_i$ ranged from 62–107 nM. However, in experiments where HL-60 cells were treated with 100 μM SK&F 96365 and Ca^{2+} was then readmitted, the fluorescence values were close to F_{max}, suggesting that calibration in these cases may not be reliable. Therefore, $[Ca^{2+}]_i$ is expressed instead as relative fluorescence intensity at 340 nm excitation wavelength.

In Ca^{2+}-free experiments, a small amount of EGTA was added to the cell suspension approximately 30 sec before drug stimulation. The purpose was to chelate any contaminating Ca^{2+} and maintain a stable baseline. The amount of EGTA (30–100 μM) added to each sample in a given experiment was the same, but might vary in separate experiments, depending on the level of the contaminating Ca^{2+}. In experiments where La^{3+} was used, phosphate salts were excluded from the HBSS and the concentration of HEPES was reduced to 8 mM to avoid precipitation upon adding La^{3+} [12]. Also, 1% fetal calf serum was included in the reaction medium to prevent cell aggregation induced by La^{3+} [12]. In experiments where we measured the entry of Ni^{2+} and Mn^{2+}, an excitation wavelength of 360 nm was used. (Emission wavelength at 500 nm.) At this excitation wavelength, fura-2 fluorescence is insensitive to $[Ca^{2+}]$ but quenchable by Ni^{2+} and Mn^{2+} [14, 15].

Statistical Analysis

Results are expressed as mean ± S.E. The Student's paired *t*-test was employed and differences were considered significant when $P < 0.05$.

RESULTS

SK&F 96365 at Low Concentrations Inhibited, But at High Concentrations Promoted, Ca^{2+} Entry

As shown in Fig. 1a, readmission of Ca^{2+} after TSG-induced Ca^{2+} release resulted in a substantial SOCE. This SOCE was manifested as an initial surge in $[Ca^{2+}]_i$ ("transient phase"), which then declined slightly to a stably elevated level ("plateau phase"). Treatment with 1–3 μM SK&F 96365 prior to Ca^{2+} readmission caused a partial suppression in both the transient and plateau phases of the SOCE. SK&F 96365 at 10 and 16 μM markedly inhibited the transient phase but not the plateau phase of the SOCE (see also Fig. 1b). Higher concentrations of SK&F 96365 (30–100 μM) actually promoted Ca^{2+} entry. The effect of SK&F 96365 on the plateau phase of the SOCE is summarized in Fig. 1b.

Experiments were also carried out to exclude the possibility that the substantial increase in fluorescence after treatment with high concentrations of SK&F 96365 (in the presence of Ca^{2+}) represented experimental artifacts. They showed that: (1) SK&F 96365 alone, even at 100 μM, was not fluorescent, (2) did not alter, qualitatively or quantitatively, the fluorescence excitation spectra of fura-2 free acid at any Ca^{2+} concentration, (3) there was no increase in autofluorescence (of unloaded cells) after adding SK&F 96365 in the presence of extracellular Ca^{2+} (results not shown) and (4) adding excess EGTA to SK&F 96365-treated cells (in the presence of extracellular Ca^{2+}) did not quench the SK&F 96365-elevated fluorescence (see Fig. 9b), suggesting that the latter was not due to fura-2 efflux.

SK&F 96365 (10–100 μM) on its own released Ca^{2+} from the intracellular stores in a concentration-dependent manner and, upon readmission of Ca^{2+}, promoted Ca^{2+} entry (Fig. 2). The relationship between the SK&F 96365- and TSG-releasable intracellular Ca^{2+} pool was next examined. After treatment with 300 μM SK&F 96365, the Ca^{2+} release by 30 nM TSG was totally abolished, and *vice versa* (results not shown), suggesting that SK&F 96365 and TSG released Ca^{2+} from the same Ca^{2+} store.

Pharmacological Properties of the SK&F 96365-Activated Ca^{2+} Entry Pathway

The Ca^{2+} entry pathway activated by 30 μM SK&F 96365 was unaffected by the L-type Ca^{2+} channel blocker nifedipine (Fig. 3c). In fact, depolarization of HL-60 cells with 100 mM KCl in Ca^{2+}-containing medium did not elevate $[Ca^{2+}]_i$ (not shown), indicating that these cells do not possess functional voltage-operated Ca^{2+} channels. Taken together, these data suggest that Ca^{2+} entry activated by SK&F 96365 did not pass through those voltage-operated Ca^{2+} channels. However, complete abolition of SK&F 96365-activated Ca^{2+} entry could be achieved by adding excess EGTA (2.4 mM) after SK&F 96365 treatment and before Ca^{2+} readmission (Fig. 3d).

Further experiments were conducted to test whether or not La^{3+} and Ni^{2+}, 2 nonselective Ca^{2+} channel blockers, could

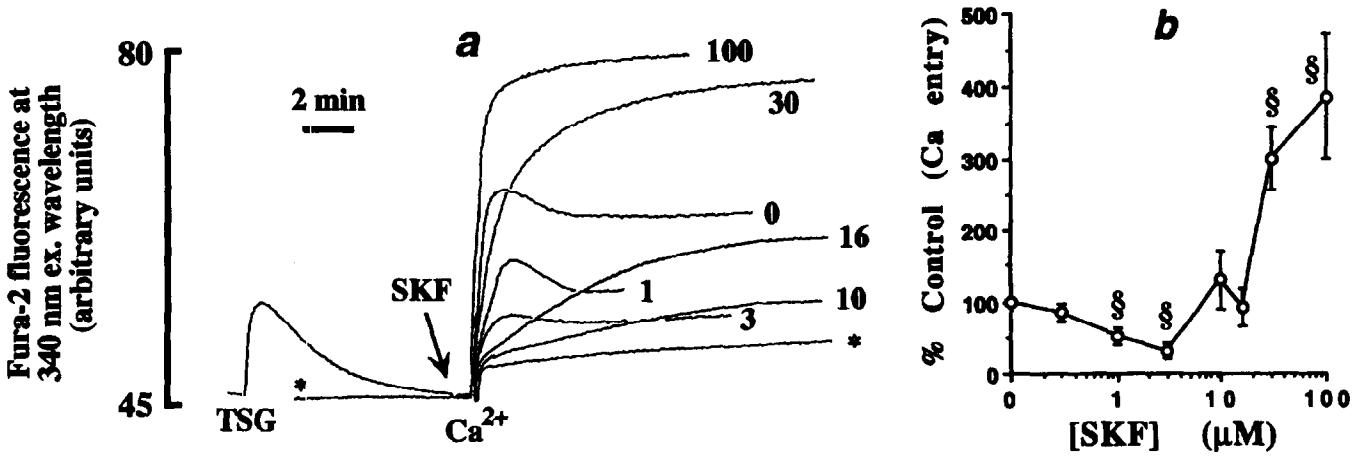


FIG. 1. Concentration-dependent effect of SK&F 96365 on Ca^{2+} entry activated by TSG. (a) HL-60 cells in EGTA-supplemented Ca^{2+}-free HBSS were challenged with 30 nM TSG and, before 1.2 mM Ca^{2+} readmission, various concentrations (μM) of SK&F 96365 (SKF) were added. The y-axis represents fura-2 fluorescence at 340 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in at least 2 other separate experiments. The lowest trace marked "*" was obtained in a sample that had not received drug treatment before Ca^{2+} readmission. The fluorescence values of these Ca^{2+}-readmitted untreated samples were subtracted from those of Ca^{2+}-readmitted drug-treated groups to quantify drug-activated Ca^{2+} entry (quantitative results shown in b). Such calculation was done at the time point when the fluorescence signals of the drug-treated and Ca^{2+}-readmitted samples reached plateau phases *relative* to the fluorescence signal of the Ca^{2+}-readmitted untreated group. (b) Concentration-inhibition curve of SK&F 96365. Results are expressed as % control, in which no SK&F 96365 was added to modulate TSG-activated Ca^{2+} entry. The fluorescence value of the control was 59.8 ± 4.4 (N = 7). Each point is the mean ± SE of 3–7 separate experiments. §Significantly different ($P < 0.05$) from the control.

block the SK&F 96365-induced Ca^{2+} entry (Fig. 4). Surprisingly, it was observed that after SK&F 96365-induced Ca^{2+} release, addition of La^{3+} increased fura-2 fluorescence whereas Ni^{2+} decreased it (Fig. 4c and d, as compared to the controls shown in Fig. 4a and b). The fluorescence excitation spectra of La^{3+}-fura-2 complex and Ca^{2+}-fura-2 complex have been shown to be qualitatively and quantitatively very similar, and Ni^{2+} is known to quench fura-2 fluorescence at all excitation wavelengths [15]. Hence, the above observations suggest that SK&F 96365 promoted the entry of La^{3+} and Ni^{2+}. As shown previously in Fig. 1, TSG activated a SOCE pathway, although this pathway was not accessible to La^{3+} nor Ni^{2+} (Fig. 4e and f). It is possible that the fluorescence increase after SK&F 96365 and La^{3+} treatment resulted from an increase in $[Ca^{2+}]_i$

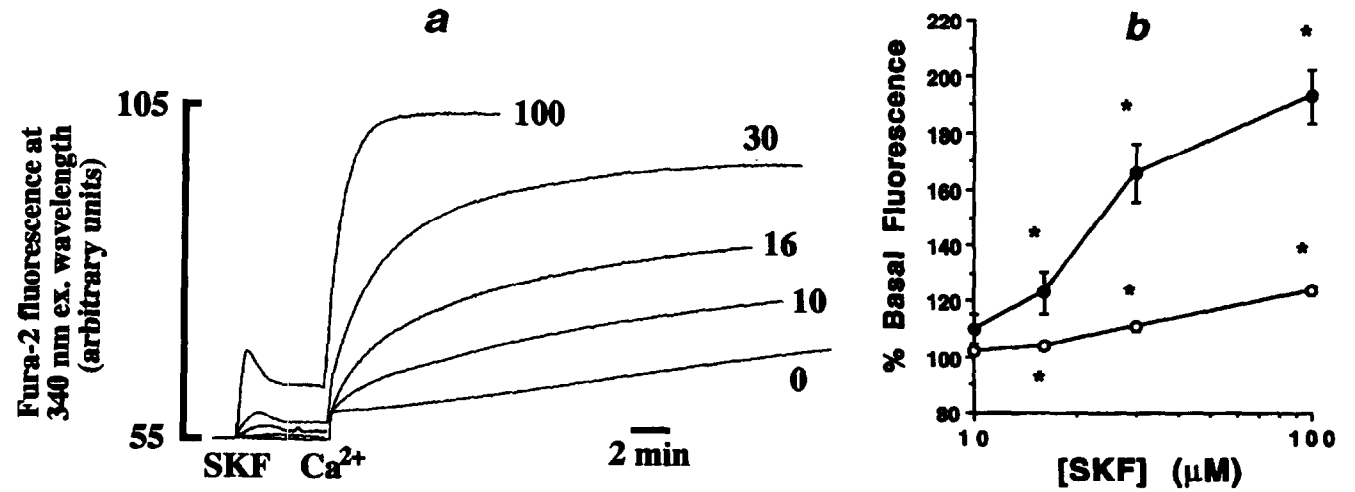


FIG. 2. Concentration-dependent effect of SK&F 96365 on Ca^{2+} release and Ca^{2+} entry. (a) HL-60 cells in EGTA-supplemented Ca^{2+}-free HBSS were stimulated with various concentrations of SK&F 96365 (SKF) before 1.2 mM Ca^{2+} readmission. 0 (DMSO as vehicle control), 10, 16, 30, and 100 μM SKF were added (from bottom trace to top). The y-axis represents fura-2 fluorescence at 340 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in at least 2 other separate experiments. The fluorescence values of the Ca^{2+}-readmitted untreated samples were subtracted from those of Ca^{2+}-readmitted drug-treated groups to quantify drug-activated Ca^{2+} entry (quantitative results shown in b). Such calculation was done at the time point when the fluorescence signals of the drug-treated and Ca^{2+}-readmitted samples reached plateau phases relative to the fluorescence signal of the Ca^{2+}-readmitted untreated group. (b) $[Ca^{2+}]_i$ elevation due to intracellular Ca^{2+} release (○) and Ca^{2+} entry (●) stimulated by SK&F 96365 is shown. Results are expressed as % basal fluorescence (fluorescence values before drug addition, which ranged from 39.2 to 56.7). Each point is the mean ± SE of 3–4 separate experiments. *Significantly different ($P < 0.05$) from the basal $[Ca^{2+}]_i$.

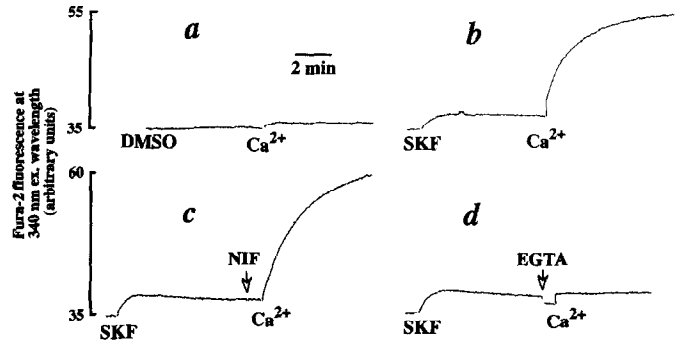


FIG. 3. Inhibition of SK&F 96365-activated Ca^{2+} entry. HL-60 cells in EGTA-supplemented Ca^{2+}-free HBSS were treated with (a) DMSO or (b–d) SK&F 96365 (SKF, 30 μM) and 1.2 mM Ca^{2+} was subsequently readmitted. In (c) and (d), nifedipine (NIF, 1 μM) and EGTA (2.4 mM) were added, respectively, before Ca^{2+} readmission. The y-axis represents fura-2 fluorescence at 340 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in 2 other separate experiments.

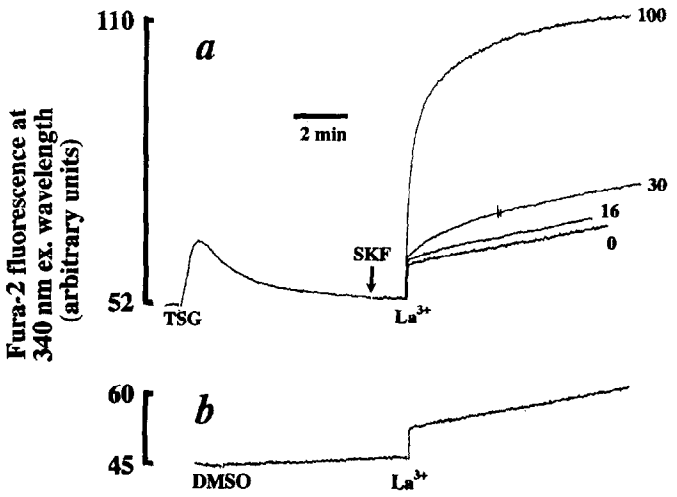


FIG. 5. Concentration-dependent effect of SK&F 96365 on promotion of La^{3+} entry after TSG treatment. (a) HL-60 cells in EGTA-supplemented Ca^{2+}-free HBSS were stimulated with 30 nM TSG and, shortly before addition of 1.2 mM La^{3+}, various concentrations of SK&F 96365 (SKF) were added. (b) A control sample that only received DMSO (vehicle) before La^{3+} addition. The y-axis represents fura-2 fluorescence at 340 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in 2 other separate experiments.

which was, in turn, a result of the inhibitory effect of La^{3+} on the efflux of Ca^{2+} released (by SK&F 96365), as was suggested previously in rat lacrimal acinar cells [16]. The experimental protocol shown in Fig. 5 was designed to rule out this possibility. After the Ca^{2+} store has been depleted by TSG, addition of SK&F 96365 did not cause further Ca^{2+} release but, in a concentration-dependent manner, caused a prominent increase in fluorescence upon La^{3+} addition. This rise in fluorescence was unrelated to intracellular Ca^{2+} release (because the Ca^{2+} pool had already been emptied), but reflected La^{3+} entry. The experiment shown in Fig. 6 confirmed that SK&F 96365 could promote Ni^{2+} entry. In this experiment, an excitation wavelength of 360 nm was used so that fura-2 fluorescence was independent of $[Ca^{2+}]_i$ but quenchable by heavy metals such as Ni^{2+} and Mn^{2+} [14, 15]. SK&F 96365 on its own did not affect fluorescence but caused, in a concentration-dependent manner, quenching upon Ni^{2+} addition. The concentration-dependent effects of SK&F 96365 on promotion of La^{3+} and Ni^{2+} entry were qualitatively similar to those on Ca^{2+} entry (Fig. 2a), suggesting that the SK&F 96365-activated entry of Ca^{2+}, La^{3+}, and Ni^{2+} might utilize the same pathway.

The effect of SK&F 96365 on the entry of Mn^{2+}, a conventional Ca^{2+} ion surrogate [14], was next investigated (again, the 360 nm excitation wavelength was used). As shown in Fig. 7a, addition of Mn^{2+} caused a quenching, which reflected the basal Mn^{2+} entry. Challenge with TSG or ionomycin accelerated Mn^{2+} entry. However, 30 μM SK&F 96365 completely

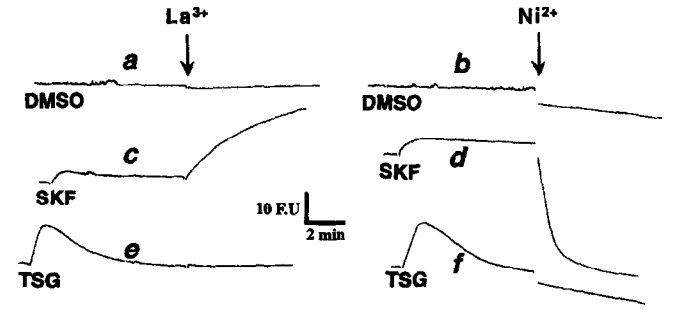


FIG. 4. Ni^{2+} and La^{3+} entry could be stimulated by SK&F 96365, but not TSG. HL-60 cells in EGTA-supplemented CA^{2+}-free HBSS were challenged with DMSO (vehicle control), SK&F 96365 (SKF, 30 μM) or TSG (30 nM) before 1.2 mM La^{3+} (left column) or 1.2 mM Ni^{2+} (right column) was added. The vertical bar represents 10 fura-2 fluorescence units at 340 nm excitation wavelength. Similar traces were obtained in 2 other separate experiments.

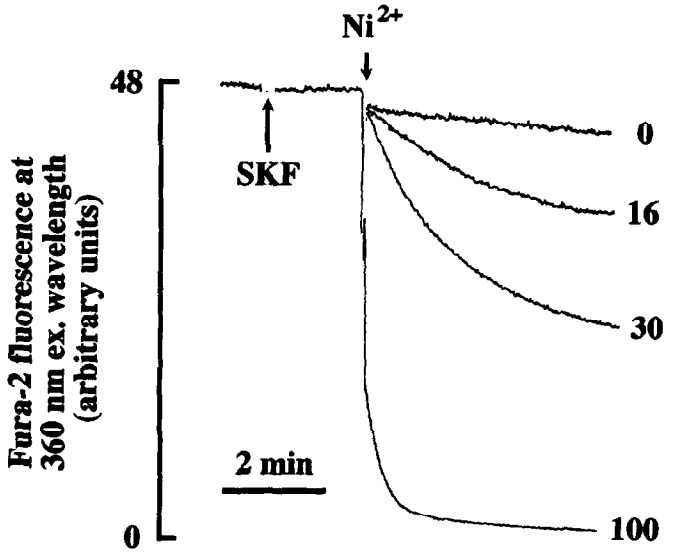


FIG. 6. Concentration-dependent effect of SK&F 96365 on promotion of Ni^{2+} entry measured at 360 nm excitation wavelength. HL-60 cells in Ca^{2+}-free HBSS (no EGTA supplementation) were stimulated with various concentrations of SK&F 96365 (SKF) before addition of 1.2 mM Ni^{2+}. The y-axis represents fura-2 fluorescence at 360 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in 3 other separate experiments.

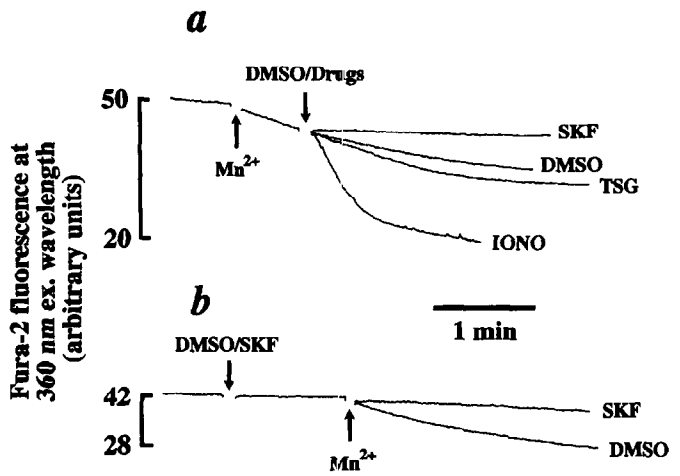


FIG. 7. SK&F 96365 inhibited the entry of Mn^{2+}. (a) HL-60 cells in Ca^{2+}-free HBSS (no EGTA supplementation) were exposed to 0.2 mM Mn^{2+} and subsequently challenged with DMSO (vehicle control), 30 nM TSG, 30 μM SK&F 96365 (SKF), or 2.7 μM ionomycin (IONO). (b) Cells were treated with DMSO or 30 μM SK&F 96365 (SKF) before addition of 0.2 mM Mn^{2+}. The y-axis represents fura-2 fluorescence at 360 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in 2 other separate experiments.

inhibited Mn^{2+} entry. Similarly, SK&F 96365 treatment markedly suppressed the subsequent Mn^{2+} entry (Fig. 7b).

In murine B lymphoma M12.4 cells, SK&F 96365 also caused Ca^{2+} release and stimulated entry of Ca^{2+}, La^{3+}, and Ni^{2+} (Fig. 8, lower row), indicating that the nonselective cation entry activated by SK&F 96365 was not cell-specific. Although TSG also activated SOCE in M12.4 cells, it did not promote La^{3+} and Ni^{2+} entry (Fig. 8, middle row).

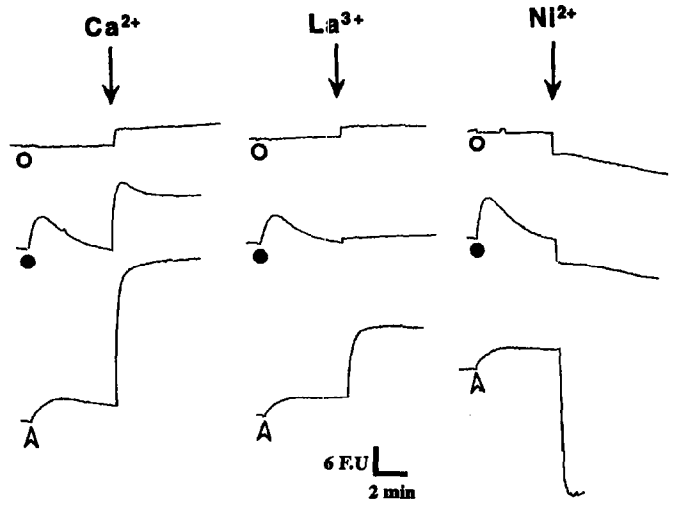


FIG. 8. SK&F 96365 promoted entry of Ca^{2+}, La^{3+}, and Ni^{2+} in M12.4 cells. M12.4 cells in EGTA-supplemented Ca^{2+}-free HBSS were stimulated with DMSO (○, vehicle control), TSG (●, 30 nM), or SK&F 96365 (Δ, 50 μM) before addition of 1.2 mM Ca^{2+}, 1.2 mM La^{3+}, and 0.3 mM Ni^{2+}. The vertical bar represents 6 fura-2 fluorescence units at 340 nm excitation wavelength. Similar traces were obtained in 2 other separate experiments.

Does SK&F 96365 Cause Cation Entry Simply by Damaging the Plasma Membrane?

Treatment of HL-60 cells with SK&F 96365 (30 μM), saponin (100 μg/mL) or Triton X-100 (0.02%) substantially increased $[Ca^{2+}]_i$ in Ca^{2+}-containing medium (Fig. 9b, e and f). However, although all the cells treated with the permeabilizing agents (saponin and Triton X-100) were stained with trypan blue, only 4.4% of the SK&F 96365-treated cells were stained. These results show that SK&F 96365 increased cation entry but not by damaging the plasma membrane. Furthermore, if there had been plasma membrane damage, as in the cases of saponin- and Triton X-100-treated cells, there would have been an immediate and drastic fall in fluorescence after EGTA addition due to the rapid movement of Ca^{2+} into the extracellular space from the permeabilized cells (Fig. 9e and f). This was certainly not true in the SK&F 96365-treated cells (Fig. 9b).

The sustained $[Ca^{2+}]_i$ elevation induced by TSG or ionomycin was gradually abolished by adding an excess of EGTA (Fig. 9c and d), which is indicative of Ca^{2+} extrusion into the extracellular space. The failure of EGTA to abolish the SK&F 96365-induced $[Ca^{2+}]_i$ elevation (Fig. 9b) suggests that the

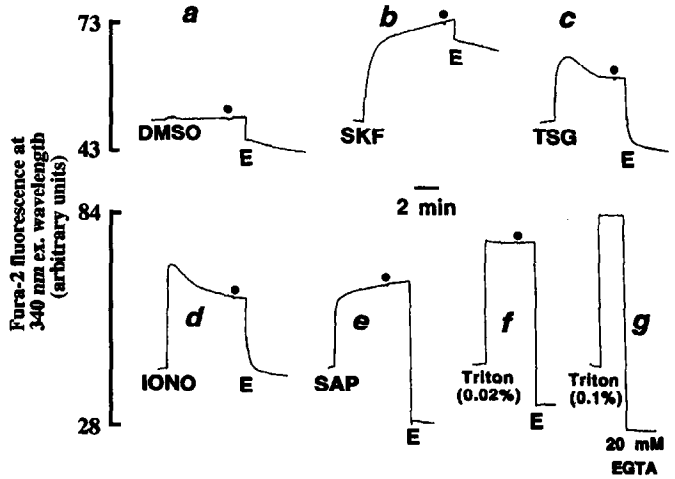


FIG. 9. Effects of SK&F 96365, TSG, ionomycin, saponin, and triton X-100 on plasma membrane permeability in HL-60 cells. HL-60 cells in Ca^{2+}-containing HBSS were challenged with DMSO (vehicle control), SK&F 96365 (SKF, 30 μM), TSG (30 nM), ionomycin (IONO, 1.34 μM), saponin (SAP, 100 μg/mL), and Triton X-100 (0.02%) (a–f, respectively) before addition of EGTA (E, 10 mM). The aliquots of EGTA added were from a 0.5 M EGTA stock solution of pH 7.8, and the pH of the cell suspension after EGTA addition decreased only slightly (from 7.41 to 7.34). In (g), fluorescence changes during the calibration procedures [adding 0.1% Triton X-100 and then 20 mM EGTA (from a 1 M stock solution of pH 8.5)] are shown for comparison with those obtained in other samples. The y-axis represents fura-2 fluorescence at 340 nm excitation wavelength (arbitrary units in a linear scale). Similar traces were obtained in at least 2 other separate experiments. ● indicates the time at which a small aliquot of cells was taken out of the cuvette and checked for trypan blue staining: DMSO (control), 5.2 ± 2.0%; SK&F 96365, 4.4 ± 0.6%; TSG, 3.9 ± 1.1%; ionomycin, 10.1 ± 1.1%; saponin, 100%; Triton X-100, 100% (data from 3 separate experiments).

Ca^{2+} efflux was somehow suppressed by SK&F 96365. The SK&F 96365-induced $[Ca^{2+}]_i$ elevation (in the presence of extracellular Ca^{2+}) did not return to the basal level even after extensive washing of the cells; also, there was no Ca^{2+} release in response to TSG in the washed cells (not shown), suggesting that the effects of SK&F 96365 were not readily reversible.

DISCUSSION

Mechanism of SK&F 96365-Activated Cation Entry

During the past few years, SK&F 96365 has been widely used to block non-voltage-operated Ca^{2+} channels in a number of cell types [9–11]. In this study, using HL-60 cells, 1–3 μM SK&F 96365 caused a partial inhibition of TSG-induced SOCE. However, this compound at 30–100 μM, instead of causing a stronger inhibition, actually promoted Ca^{2+} entry in a concentration-dependent manner. SK&F 96365 at 10 and 16 μM strongly suppressed the initial transient phase of TSG-induced SOCE (Fig. 1a). These concentrations could possibly have substantially inhibited the plateau phase of SOCE, but might simultaneously have activated another Ca^{2+} entry mechanism, which might account for the slow elevation of $[Ca^{2+}]_i$ (Fig. 1a and b). This postulation is supported by our findings that SK&F 96365 on its own (that is, without TSG pretreatment) activated a Ca^{2+} entry pathway (Fig. 2) accessible to La^{3+} and Ni^{2+} (Fig. 4–6). This pathway appears to be pharmacologically distinct from the TSG-induced SOCE pathway, which was inaccessible to La^{3+} and Ni^{2+} (Fig. 4). Further, while the TSG-induced SOCE pathway was accessible to Mn^{2+}, SK&F 96365 did not accelerate Mn^{2+} entry (indeed, it inhibited basal Mn^{2+} entry) (Fig. 7). Therefore, although SK&F 96365 released intracellular Ca^{2+}, the nonspecific SK&F 96365-activated cation entry pathway appeared to be unrelated to the depletion of intracellular Ca^{2+} stores.

How does SK&F 96365 activate cation entry? Results in Fig. 9, together with the observation that SK&F 96365 did not accelerate Mn^{2+} entry, suggest that SK&F 96365 did not cause damage to the plasma membrane (ie., SK&F 96365 is not a detergent). Also, it is unlikely that SK&F 96365 acted as a Ca^{2+} ionophore because ionomycin accelerated Mn^{2+} entry but SK&F 96365 did not (Fig. 7). The promotion of the entry of 3 ionic species (Ca^{2+}, Ni^{2+}, and La^{3+}) by SK&F 96365 suggests that this drug may cause a generalized perturbation in plasma membrane permeability. In line with this contention, SK&F 96365 (30 μM) causes a depolarization in neurosecretory PC12 cells [17]. However, the inability of SK&F 96365 to promote Mn^{2+} entry in HL-60 cells reveals that the effect of SK&F 96365 on plasma membrane permeability did exhibit a certain degree of selectivity. Two recent studies using other cell types demonstrate that SK&F 96365 activates certain cation channels. SK&F 96365 (25 μM) stimulates a small, but significant, nonselective cation current in MDCK renal epithelial cells (whole cell patch clamp technique) [18]. Schwarz *et al.* [19], using the same technique, demonstrated that 100 μM or higher concentrations of SK&F 96365 activated a nonselective cation channel in human endothelial cells. Electrophysiological studies of the effects of SK&F 96365 on plasmalemmal cation fluxes in HL-60 cells will certainly be of future research interest.

Schwarz *et al.* [19] also reported that Ni^{2+} could permeate SK&F 96365-treated cells (fura-2 fluorescence technique). However, the concentration of SK&F 96365 required was 200 μM, which is beyond the range used to block Ca^{2+} entry. Our present results show that Ni^{2+} entry could be activated by a much lower concentration of SK&F 96365 (16 μM) (see below). The ability of SK&F 96365 to activate the entry of the divalent cation Ni^{2+}, and even the trivalent cation La^{3+}, is considered unusual because Ni^{2+} and La^{3+} are generally impermeable in either unstimulated or stimulated cells; this report represents the first description of a Ca^{2+}-, Ni^{2+}-, and La^{3+}-permeable pathway activated by SK&F 96365.

The Use of SK&F 96365 Requires Caution

Our work shows that the 2 opposing effects of SK&F 96365, inhibition of Ca^{2+} entry at low concentrations and activation of cation entry at high concentrations, overlapped at an intermediate concentration of 16 μM. This finding is of particular significance because this concentration of SK&F 96365 is well within the range commonly used to block Ca^{2+} entry. The work on MDCK renal epithelial cells by Dietl and Volkl [18] also illustrates this point: at 25 μM SK&F 96365 partially inhibited maitotoxin-induced Ca^{2+} entry but it also increased slightly $[Ca^{2+}]_i$ and activated a nonselective cation current. Consequently, substantial or full inhibition of Ca^{2+} entry with the simultaneous activation of another cation (including Ca^{2+}) entry pathway may offset each other and result in partial Ca^{2+} entry blockade. This situation may cause misleading interpretation if the effect of SK&F 96365 itself on $[Ca^{2+}]_i$ or Ca^{2+} entry is not carefully investigated. (It is noteworthy that SK&F 96365 itself at intermediate concentrations (approximately 25 μM) has highly variable effects on $[Ca^{2+}]_i$ or Ca^{2+} entry among different cell types [18–21]). Indeed, in a number of studies in which SK&F 96365 was shown to cause a partial inhibition of AACE and SOCE, the effect of SK&F 96365 itself was not examined or reported. Therefore, the use of SK&F 96365 and the interpretation of data would require more caution.

Other Effects of SK&F 96365 on $[Ca^{2+}]_i$ Homeostasis

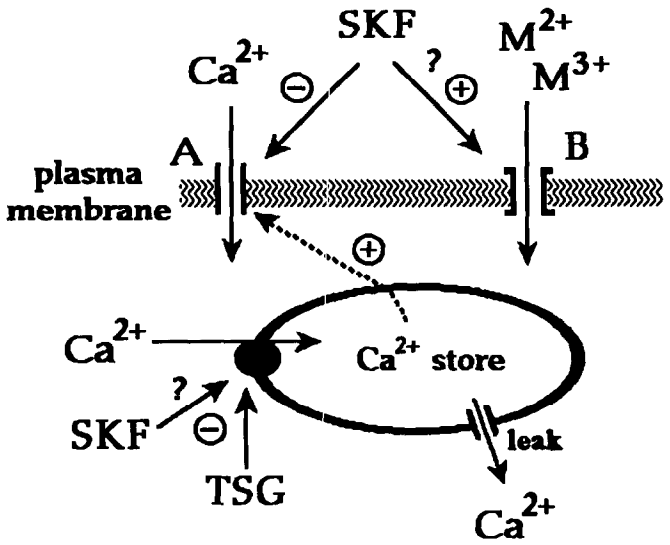
High concentrations of SK&F 96365 also released intracellular Ca^{2+} in HL-60 cells (Fig. 2). SK&F 96365 (10–50 μM) has previously been shown to release intracellular Ca^{2+} in several cell types [9, 21, 22] but, at a concentration as high as 50 μM, this drug does not release intracellular Ca^{2+} in human endothelial cells [19]. It is unclear why SK&F 96365 releases Ca^{2+} in some cells but not in others, and it is also uncertain how this drug could cause Ca^{2+} release. In view of the relatively slow Ca^{2+} mobilization SK&F 96365 induced, this agent possibly caused Ca^{2+} release by inhibiting Ca^{2+} reuptake into intracellular stores. Indeed, it has recently been shown that SK&F 96365 (30–100 μM) inhibited Ca^{2+} pump activity in isolated sarcoplasmic reticulum of rabbit skeletal muscles [22].

It is remarkable that, unlike the transient nature of TSG-induced Ca^{2+} release, the SK&F 96365-induced Ca^{2+} release had a sustained phase, suggesting that somehow Ca^{2+} was prevented from being extruded into the extracellular space (see below).

While addition of excess EGTA following SK&F 96365-induced Ca^{2+} release and prior to Ca^{2+} readmission abolished subsequent Ca^{2+} entry (Fig. 3d), EGTA addition during the SK&F 96365-induced $[Ca^{2+}]_i$ elevation in a Ca^{2+}-containing medium did not return $[Ca^{2+}]_i$ to its basal level (Fig. 9b). These observations indicate that, once Ca^{2+} ions entered the cell after SK&F 96365 treatment, they were prevented from being extruded into the extracellular space. A plausible explanation is that SK&F 96365 also inhibited Ca^{2+} efflux. The observation that SC 38249, an analogue of SK&F 96365, could inhibit Ca^{2+} efflux in rat cerebellar granule cells [23] is consistent with this interpretation. Direct evidence using the $^{45}Ca^{2+}$ flux technique will be needed to verify the inhibition of Ca^{2+} efflux by SK&F 96365.

Summary and Conclusion

The diagram in Fig. 10 summarizes the multiple effects of SK&F 96365 on $[Ca^{2+}]_i$-handling machineries of HL-60 cells.



	Ca^{2+}	Mn^{2+}	Ni^{2+}	La^{3+}
TSG	+	+	0	0
SKF	±	−	+	+

FIG. 10. A diagram summarizing the effects of SK&F 96365 on $[Ca^{2+}]_i$-handling machineries in HL-60 cells. See text for explanation. Note that the way in which pathways A and B are drawn does not imply that these pathways are necessarily channels, but only indicates that they are cation-transporting machineries. M^{2+} and M^{3+} represent divalent and trivalent metallic cations, respectively. The inset summarizes the effects of TSG and SK&F 96365 (SKF) on the entry of various cations. +, stimulates; 0, no effect; −, inhibits; ±, stimulates or inhibits, depending on the concentration of SK&F 96365 used.

Ca^{2+} store depletion due to impairment of Ca^{2+} recycling by TSG stimulates the opening of a Ca^{2+} entry pathway at the plasma membrane (pathway A) which was also accessible to Mn^{2+}, but not to Ni^{2+} and La^{3+} (see inset). This pathway was suppressed by low concentrations of SK&F 96365. At high concentrations, SK&F 96365 also caused Ca^{2+} store depletion (possibly due to inhibition of microsomal Ca^{2+} pumps) and promotion of Ca^{2+} entry, but inhibited Mn^{2+} entry and activated Ni^{2+} and La^{3+} influx (see inset). The differences in ionic permeability affected by TSG and SK&F 96365 lead to the speculation that SK&F 96365 activated a Ca^{2+}-permeable nonselective cation entry pathway (pathway B) distinct from that triggered by TSG-induced Ca^{2+} store depletion. Indirect evidence in this work suggests that SK&F 96365 could also inhibit Ca^{2+} efflux (not indicated in the diagram). Such an action of SK&F 96365 might play a role in sustaining SK&F 96365-induced $[Ca^{2+}]_i$ elevation which, in turn, resulted primarily from intracellular Ca^{2+} release and entry of extracellular Ca^{2+}.

In conclusion, in HL-60 cells, SK&F 96365 causes partial Ca^{2+} entry blockade at low concentrations and promotion of cation entry at high concentrations. These opposing effects overlap at some intermediate concentrations and more validation is, therefore, warranted in using this drug as an antagonist of nonvoltage-operated Ca^{2+} channels. On the other hand, the very unique pharmacological properties of the SK&F 96365-activated nonselective cation entry pathway in HL-60 cells await further investigation.

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