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Formyl peptides and ATP stimulate Ca^{2+} and Na^+ inward currents through non-selective cation channels via G-proteins in dibutyryl cyclic AMP-differentiated HL-60 cells

Involvement of Ca^{2+} and Na^+ in the activation of β-glucuronidase release and superoxide production

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In human neutrophils, the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induces increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) with subsequent activation of β-glucuronidase release and superoxide (O_2^-) production. Results from several laboratories suggest that the increase in $[Ca^{2+}]_i$ is due to activation of non-selective cation (NSC) channels. We studied the biophysical characteristics, pharmacological modulation and functional role of NSC channels in dibutyryl cyclic AMP (Bt_2cAMP)-differentiated HL-60 cells. fMLP increased $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores and influx of Ca^{2+} from the extracellular space. fMLP also induced Mn^{2+} influx. Ca^{2+} and Mn^{2+} influxes were inhibited by 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365). Under whole-cell voltage-clamp conditions, fMLP and ATP (a purinoceptor agonist) activated inward currents characterized by a linear current–voltage relationship and a reversal potential near 0 mV. NSC channels were substantially more permeable to Na^+ than to Ca^{2+}. SK&F 96365 inhibited fMLP- and ATP-stimulated currents with a half-maximal effect at about 3 μM. Pertussis toxin prevented stimulation by fMLP of NSC currents and reduced ATP-stimulated currents by about 80%. Intracellular application of the stable GDP analogue, guanosine 5′-*O*-[2-thio]diphosphate, completely blocked stimulation by agonists of NSC currents. In excised inside-out patches, single channel openings with an amplitude of 0.24 pA were observed in the presence of fMLP and the GTP analogue, guanosine 5′-*O*-[3-thio]triphosphate. The bath solution contained neither Ca^{2+} nor ATP. The current/voltage relationship was linear with a conductance of 4–5 pS and reversed at about 0 mV. fMLP-induced β-glucuronidase release and O_2^- production were substantially reduced by replacement of extracellular $CaCl_2$ or NaCl by ethylenebis(oxyethylenenitrilo)tetra-acetic acid and choline chloride respectively. In the absence of Ca^{2+} and Na^+, fMLP was ineffective. SK&F 96365 inhibited fMLP-induced β-glucuronidase release and O_2^- production in the presence of both Ca^{2+} and Na^+, and in the presence of Ca^{2+} or Na^+ alone. NaCl (25–50 mM) enhanced the basal and absolute extent of fMLP-stimulated GTP hydrolysis of heterotrimeric regulatory G-proteins in HL-60 membranes. The order of effectiveness of salts in enhancing GTP hydrolysis was LiCl > KCl > NaCl > choline chloride. Our results suggest that in Bt_2cAMP-differentiated HL-60 cells, (i) fMLP and ATP activate NSC channels permeable for Ca^{2+}, Mn^{2+} and Na^+; (ii) activation of NSC channels involves G-proteins and is independent of intracellular Ca^{2+} and protein kinases; (iii) Ca^{2+} and Na^+ influxes are involved in activation of β-glucuronidase release and O_2^- production; (iv) an increase in intracellular free Na^+ concentration may enhance activation of G-proteins, leading, among other possible mechanisms, to signal amplification.

INTRODUCTION

Neutrophils play a major role in host defence against bacterial infections and in the pathogenesis of various human diseases such as myocardial infarction, rheumatoid arthritis and nephritis (Malech & Gallin, 1987). Dibutyryl cyclic AMP (Bt_2cAMP)- and dimethylsulphoxide (Me_2SO)-differentiated HL-60 leukaemic cells are widely used as model systems for cell culture of neutrophils as they can be obtained in sufficiently large amounts for functional studies and for the purification of signal transduction components (Niedel *et al.*, 1980; Chaplinski & Niedel, 1982; Uhing *et al.*, 1987; Dubyak *et al.*, 1988; Gierschik *et al.*, 1989; Pittet *et al.*, 1989; Seifert *et al.*, 1989*a*; Cowen *et al.*, 1990; Wenzel-Seifert & Seifert, 1990; Tohkin *et al.*, 1991; Mizuno *et al.*, 1992). In addition, Bt_2cAMP-differentiated HL-60 cells are a particularly useful system for the identification of cDNAs of signal transduction components (Murphy *et al.*, 1987; Murphy & McDermott, 1991; Schultz *et al.*, 1992).

Neutrophils and HL-60 cells possess receptors for the chemotactic peptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), and for ATP both of which couple to pertussis-toxin-sensitive heterotrimeric regulatory G-proteins (Chaplinski & Niedel, 1982; Dubyak *et al.*, 1988; Gierschik *et al.*, 1989; Seifert *et al.*, 1989*a*,*b*, 1992*a*; Wenzel-Seifert & Seifert, 1990; Cowen *et al.*, 1990). In addition, ATP receptors couple to pertussis-toxin-insensitive G-proteins (Dubyak *et al.*, 1988; Seifert *et al.*, 1989*a*; Wenzel-Seifert & Seifert, 1990; Cowen *et al.*, 1990). Activation of neutrophilic cells results in the release of β-glucuronidase from azurophilic granules and NADPH oxidase-catalysed superoxide (O_2^-) production (Chaplinski & Niedel, 1982; Rossi, 1986; Sandborg & Smolen, 1988; Baggiolini & Wymann, 1990; Wenzel-Seifert & Seifert, 1990; Seifert & Schultz, 1991). It should be

Abbreviations used: $[Ca^{2+}]_i$, intracellular free-Ca^{2+} concentration; Bt_2cAMP, dibutyryl cyclic AMP; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GDPβS, guanosine 5′-*O*-[thio]diphosphate; GTPγS, guanosine 5′-*O*-[thio]triphosphate; Me_2SO, dimethylsulphoxide; $[Na^+]_i$, intracellular free-Na^+ concentration; NSC, non-selective cation; O_2^-, superoxide; SK&F 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride.

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noted, however, that there are also differences in transmembrane signal transduction between neutrophils and Me_2SO-differentiated HL-60 cells on the one hand and Bt_2cAMP-differentiated HL-60 cells on the other (Seifert *et al.*, 1989*c*, 1992*a*; Wenzel-Seifert & Seifert, 1990; Tohkin *et al.*, 1991).

In neutrophilic cells, fMLP and ATP induce an increase in intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$) (von Tscharner *et al.*, 1986; Andersson *et al.*, 1986; Nasmith & Grinstein, 1987; Dubyak *et al.*, 1988; Pittet *et al.*, 1989; Wenzel-Seifert & Seifert, 1990; Seifert & Schultz, 1991). In neutrophils and Me_2SO-differentiated HL-60 cells, fMLP mobilizes Ca^{2+} from intracellular stores and induces a sustained influx of Ca^{2+} from the extracellular space through channels referred to as non-selective cation (NSC) channels (von Tscharner *et al.*, 1986; Andersson *et al.*, 1986; Nasmith & Grinstein, 1987; Simchowitz & Cragoe, 1988; Pittet *et al.*, 1989; Merritt *et al.*, 1989, 1990; Meldolesi *et al.*, 1991; Montero *et al.*, 1991; Demaurex *et al.*, 1992; Schumann *et al.*, 1992). 1-{β[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365) inhibits fMLP-induced Ca^{2+} and Mn^{2+} influxes in human neutrophils (Merritt *et al.*, 1990). There is a debate as to whether or not NSC-channel opening in human neutrophils and Me_2SO-differentiated HL-60 cells depends on a rise in $[Ca^{2+}]_i$ (von Tscharner *et al.*, 1986; Nasmith & Grinstein, 1987; Demaurex *et al.*, 1992; Schumann *et al.*, 1992). Preliminary results point to the presence of NSC channels in Bt_2cAMP-differentiated HL-60 cells as well (Aviram & Shaklai, 1990; Gusovsky *et al.*, 1990). However, the biophysical and pharmacological properties and the functional role of NSC channels in Bt_2cAMP-differentiated HL-60 cells are unknown.

In 1979 Simchowitz & Spilberg suggested that Na^+ influx is involved in the activation of O_2^- production in human neutrophils. More recently, Nordmann & Stuenkel (1991) put forward the hypothesis that an increase in intracellular free-Na^+ concentration ($[Na^+]_i$) plays a key role in the activation of exocytosis in neuronal cells. By contrast, the results of another study indicate that Na^+ plays an inhibitory role in the regulation of β-glucuronidase release and O_2^- production in human neutrophils (Della Bianca *et al.*, 1983). Na^+ (20–100 mM) inhibits basal GTPase activity of G-proteins in membranes from Me_2SO-differentiated HL-60 cells, but enhances the absolute extent of GTP hydrolysis caused by fMLP (Gierschik *et al.*, 1989). As $[Na^+]_i$ in stimulated human neutrophils and platelets may be as high as 60 mM (Simchowitz, 1985; Borin & Siffert, 1991; Sage *et al.*, 1991), the question arises of whether Na^+ could modulate the activity of G-proteins *in vivo*. Most intriguingly, SK&F 96365 inhibits the receptor-agonist-stimulated increase in $[Na^+]_i$ in platelets which suggests the involvement of NSC channels in Na^+ influx (Borin & Siffert, 1991; Sage *et al.*, 1991).

All these findings prompted us to study the properties of NSC channels in Bt_2cAMP-differentiated HL-60 cells with the fluorescent dye fura-2, and in whole-cell and single-channel voltage-clamp experiments, and to assess their functional role in the regulation of β-glucuronidase release and O_2^- production. We show that fMLP and ATP activate NSC channels permeable for Ca^{2+}, Mn^{2+} and Na^+ and that these channels are involved in the activation of β-glucuronidase release and O_2^- production.

MATERIALS AND METHODS

Materials

SK&F 96365 was kindly provided by Dr. J. E. Merritt, SmithKline Beecham (Welwyn, Herts., U.K.). Stock solutions of SK&F 96365 (30 mM) were prepared in Me_2SO and were stored at −20 °C. Dilutions of SK&F 96365 were made immediately before experiments were performed. Pertussis toxin was a gift from Dr. M. Yajima (Kyoto, Japan). Isradipine was from Sandoz (Basel, Switzerland). Sources of other materials have been described elsewhere (Seifert & Schultz, 1987; Seifert *et al.*, 1989*a–c*, 1992*a,b*; Wenzel-Seifert & Seifert, 1990).

Cell culture

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) horse serum as described (Seifert *et al.*, 1989*a*). To induce differentiation, cells were seeded at 1×10^6 cells/ml and were cultured for 48 h in the presence of 0.2 mM-Bt_2cAMP, or for 120 h in the presence of 160 mM-Me_2SO (Seifert & Schultz, 1987; Seifert *et al.*, 1989*a*). In some experiments pertussis toxin (100 ng/ml) or carrier was added to cells 24 h before experiments. Concentrations of SK&F 96365 up to 100 μM did not cause damage of Bt_2cAMP-differentiated HL-60 cells as assessed by Trypan Blue exclusion and lactate dehydrogenase release (results not shown). The release of lactate dehydrogenase from HL-60 cells generally amounted to < 5% of cellular content (results not shown). SK&F 96365 concentrations up to 30 μM did not stimulate β-glucuronidase release or O_2^- production (results not shown).

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

$[Ca^{2+}]_i$ was determined with the dye, fura-2/acetoxymethyl ester, as described in detail (Grynkiewicz *et al.*, 1985; Seifert *et al.*, 1992*a*). In brief, HL-60 cells were suspended at 5×10^6 cells/ml in a buffer consisting of 138 mM-NaCl, 6.0 mM-KCl, 1.0 mM-$MgSO_4$, 1.0 mM-Na_2HPO_4, 5.0 mM-$NaHCO_3$, 5.5 mM-glucose, and 20 mM-Hepes/NaOH, pH 7.4, supplemented with 0.1% (w/v) BSA and fura-2/acetoxymethyl ester (2 μM). Cells were incubated for 1 h at 37 °C. Subsequently, cells were diluted with the above buffer to a cell density of 0.5×10^6 cells/ml and were centrifuged at 250 *g* for 10 min at 20 °C. Cells were resuspended at 1.0×10^6 cells/ml in the above buffer, and were kept at 20 °C until $[Ca^{2+}]_i$ had been measured. Cells were used up to 4 h after loading without substantial leakage of fura-2 from the cells, increase in basal $[Ca^{2+}]_i$ or decrease in responsiveness to fMLP (Dubyak *et al.*, 1988; Seifert *et al.*, 1992*a*). Fluorescence of HL-60 cells (1.0×10^6 cells) was determined at 37 °C with constant stirring, using a Ratio II spectrofluorimeter (Aminco, Silver Spring, MD, U.S.A.). Cells were incubated for 3 min at 37 °C in the presence of various substances before addition of fMLP; basal fluorescence was measured for 1 min. Excitation and emission wavelengths were 340 nm and 500 nm respectively. Mn^{2+} influx in HL-60 cells was measured by quenching of fura-2 fluorescence as described (Merritt *et al.*, 1989). Basal $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells was 105 ± 15 nM (mean ± S.D. of five different preparations of HL-60 cells). With each preparation, up to 40 separate experiments were performed.

Electrophysiology

HL-60 cells (8×10^6 cells) were centrifuged at 250 *g* for 5 min at 20 °C, and were resuspended in standard extracellular Tyrode's solution (E1) containing 140 mM-NaCl, 1.8 mM-$CaCl_2$, 1.0 mM-$MgCl_2$, 5.4 mM-KCl, 10 mM-glucose, 10 mM-Hepes/NaOH, pH 7.4, at 37 °C. Cells were transferred into a perfusion chamber (0.2 ml) mounted on an inverted microscope (Zeiss, Oberkochen, Germany). Cells settled to the bottom of the chamber within 3 min. Patch pipettes were prepared from glass capillaries (Jencons, Leighton Buzzard, U.K.) according to Hamill *et al.* (1981). With an open diameter of 1–2 μm, the average resistance of the electrodes was 4–6 MΩ.

Whole-cell recordings. Pipettes were filled with a solution (I1) containing 90 mM-potassium aspartate, 50 mM-KCl, 1.0 mM-$MgCl_2$, 3.0 mM-sodium ATP, 0.1 mM-EGTA, 10 mM-Hepes/

KOH, pH 7.4, at 37 °C. Electrical approach to HL-60 cells was gained by suction for access resistance below 50 MΩ. Membrane potentials and whole-cell membrane currents were measured according to Hamill *et al.* (1981), using a List LM/EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Resting potentials in HL-60 cells ranged between −60 and −30 mV and cell capacitance between 4 and 8 pF. Currents and conductances were referred to membrane capacitance. HL-60 cells were superfused at 2 ml/min with E1. In some experiments E1 was modified as follows. For Ca^{2+} depletion, $CaCl_2$ (1.8 mM) was replaced by choline chloride (1.8 mM), and the solution in addition contained EGTA (1 mM) (E2). In some experiments all permeant cations (i.e. Na^+, K^+ and Mg^{2+}) with the exception of Ca^{2+} (1.8 mM, E3, or 100 mM, E4) were replaced by choline. For Na^+ depletion, NaCl (140 mM) was replaced by choline chloride (140 mM) (E5). In solution E6, all permeant cations were replaced by choline.

Single-channel recordings. Excised inside-out patches were obtained from the on-cell configuration with GΩ-seal resistance. Single-channel currents were recorded at 25 °C. The pipette solution (I2) contained 135 mM-NaCl, 2.0 mM-$MgCl_2$, 100 nM-fMLP and 20 mM-Hepes/NaOH, pH 7.4, at 25 °C. Inside-out patches were faced to a bath solution (E7) containing 65 mM-caesium aspartate, 13 mM-CsCl, 50 mM-potassium aspartate, 2.0 mM-$MgCl_2$, 1.0 mM-EGTA, 0.01 mM-guanosine 5′-*O*-[thio]triphosphate (GTPγS) and 20 mM-Hepes/CsOH, pH 7.4, at 25 °C. Cs^+ was added to block K^+ currents. The unfiltered currents at various potentials were continuously recorded on a Sony DTR 1200 DAT-recorder (Biologic, Echirolles, France). For off-line analysis, data were filtered at a frequency of 800 Hz using a three-pole Bessel filter (Frequency Devices, Haverhill, MA, U.S.A.) and were sampled at 3 kHz using the Axon AD/DA converter (Axon, Foster City, CA, U.S.A.).

β-Glucuronidase release

Enzyme release was assessed as described (Seifert *et al.*, 1989*a*,*b*; Wenzel-Seifert & Seifert, 1990). HL-60 cells were centrifuged at 250 ***g*** for 10 min at 20 °C. Cells were suspended in buffer consisting of 138 mM-NaCl, 3.0 mM-KCl, 1.0 mM-$MgCl_2$, 5.5 mM-glucose, 20 mM-Hepes/NaOH, pH 7.4, or in buffer consisting of 138 mM-choline chloride, 3.0 mM-KCl, 1.0 mM-$MgCl_2$, 5.5 mM-glucose, 20 mM-Hepes/NaOH, pH 7.4. Cells were recentrifuged and were suspended in the above buffers. Prior to the addition of stimuli, HL-60 cells (5.0×10^6 cells in 0.5 ml) were incubated for 5 min at 37 °C in the presence of cytochalasin B (5 μg/ml) with or without SK&F 96365. Reaction mixtures contained either 1.0 mM-$CaCl_2$ or 1.0 mM-EGTA. Reactions were conducted for 10 min and were terminated by placing the tubes on to melting ice. Reaction mixtures were centrifuged at 1000 ***g*** for 10 min at 4 °C. The determinations of the activities of lactate dehydrogenase and β-glucuronidase in supernatant fluids of reaction mixtures and of cell lysates were performed as described (Seifert *et al.*, 1989*b*; Wenzel-Seifert & Seifert, 1990). Basal β-glucuronidase release in HL-60 cells generally amounted to < 5% of cellular content (results not shown).

O_2^- production in intact HL-60 cells

O_2^- production was monitored by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase, using a Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) (Seifert *et al.*, 1989*a*). Reaction mixtures (0.5 ml) contained 2.5×10^6 Bt_2cAMP-differentiated HL-60 cells and 100 μM-ferricytochrome *c* in the buffers described above supplemented with 1.0 mM-$CaCl_2$ or 1.0 mM-EGTA. Reaction mixtures were incubated for 3 min at 37 °C with or without SK&F 96365 before addition of stimuli.

O_2^- production in cell-free systems

Reaction mixtures (0.5 ml) contained 50 μg of membrane protein from Me_2SO-differentiated HL-60 cells, 150 μg of cytosolic protein from Me_2SO-differentiated HL-60 cells, 10 μM-FAD, 500 μM-NADPH, 100 μM-ferricytochrome *c*, 2 mM-$MgCl_2$, 20 mM-KH_2PO_4, 40 mM-KCl, and 20 mM-triethanolamine/HCl, pH 7.0. Reaction mixtures were incubated for 2 min at 25 °C with or without SK&F 96365. O_2^- production was initiated by the addition of arachidonic acid (0.2 mM) (Seifert & Schultz, 1987).

Measurement of GTPase activity

GTP hydrolysis was measured as described previously (Seifert *et al.*, 1992*b*). Reaction mixtures (100 μl) contained HL-60 membranes (7.5 μg of protein/tube), 0.5 μM-[γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM-$MgCl_2$, 0.1 mM-EGTA, 0.1 mM-ATP, 1 mM-adenosine 5′-[β,γ-imido]triphosphate, 5 mM-creatine phosphate, 40 μg of creatine kinase, 1 mM-dithiothreitol and 0.2% (w/v) BSA in 50 mM-triethanolamine/HCl, pH 7.4. Reaction mixtures additionally contained NaCl, KCl, LiCl or choline chloride at various concentrations with or without fMLP. Reactions were initiated by the addition of [γ-^{32}P]GTP and were conducted for 15 min at 25 °C. Low-affinity GTPase activity was determined in the presence of 50 μM-GTP and amounted to < 5% of GTP hydrolysis in the presence of 0.5 μM-GTP.

Miscellaneous

Membranes from Bt_2cAMP- or Me_2SO-differentiated HL-60 cells and cytosol from Me_2SO-differentiated HL-60 cells were prepared as described (Seifert & Schultz, 1987). [γ-^{32}P]GTP was prepared according to Johnson & Walseth (1979). Protein was determined according to Lowry *et al.* (1951), using BSA as standard.

RESULTS

The effects of fMLP on the release of Ca^{2+} from intracellular stores and on Ca^{2+} influx in Bt_2cAMP-differentiated HL-60 cells are shown in Fig. 1. In the presence of extracellular Ca^{2+}, fMLP induced a rapid and large increase in $[Ca^{2+}]_i$ which slowly declined to a sustained plateau above basal values. SK&F 96365 decreased peak $[Ca^{2+}]_i$ values as well as the magnitude and duration of the plateau phase. SK&F 96365 (10 μM and 30 μM) inhibited fMLP-stimulated peak $[Ca^{2+}]_i$ values by about 40% and 55% respectively (Table 1). Isradipine (1 μM), a blocker of voltage-dependent Ca^{2+} channels, did not inhibit fMLP-induced increases in $[Ca^{2+}]_i$ (results not shown). In the absence of extracellular Ca^{2+}, the magnitude and duration of the rise in $[Ca^{2+}]_i$ induced by fMLP were greatly reduced (see Fig. 1*b* and Table 1). Under these conditions neither SK&F 96365 (see Fig. 1*b* and Table 1) nor isradipine (1 μM) (results not shown) affected the increase in $[Ca^{2+}]_i$. These results suggest that the fMLP-induced increase in $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells depended largely on Ca^{2+} influx.

To answer the question whether fMLP stimulated the influx of bivalent cations, quenching of fura-2 fluorescence by Mn^{2+} was studied. At an excitation wavelength of 340 nm, fluorescence is increased by Ca^{2+} and is reduced by Mn^{2+}; at an excitation wavelength of 360 nm, fluorescence is quenched by Mn^{2+} as well but is insensitive to changes in $[Ca^{2+}]_i$ (Merritt *et al.*, 1989). At both excitation wavelengths Mn^{2+} induced a small decrease in fluorescence, indicating basal Mn^{2+} influx (Fig. 2). At excitation wavelengths of 340 nm, but not of 360 nm, fMLP transiently increased fluorescence, reflecting a release of Ca^{2+} from intracellular stores. At excitation wavelengths of 340 nm and 360 nm fMLP substantially enhanced fluorescence quenching,

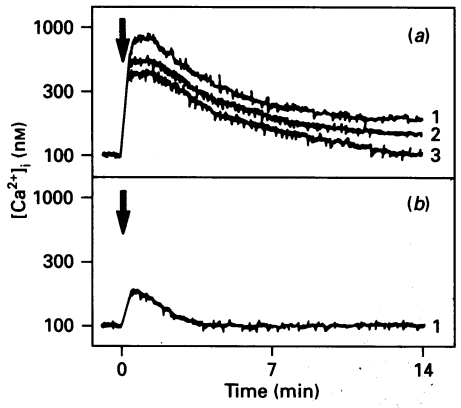

Fig. 1. Effect of extracellular Ca^{2+} and SK&F 96365 on the time course of the fMLP-induced increase in $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells

HL-60 cells were loaded with fura-2/acetoxymethyl ester, and the increases in $[Ca^{2+}]_i$ induced by fMLP (1 μM) were assessed. Arrows indicate the addition of stimulus. Various substances were added to cells 3 min before fMLP. (*a*) Experiments performed in the presence of Ca^{2+} (1 mM). Trace 1, solvent (control); trace 2, SK&F 96365 (10 μM); trace 3, SK&F 96365 (30 μM). Superimposed original fluorescence tracings are shown. (*b*) Experiments performed in presence of EGTA (1 mM). Trace 1, solvent (control). Traces of fMLP-induced increases in $[Ca^{2+}]_i$ in the presence of SK&F 96365 (10 μM or 30 μM) were virtually superimposable on trace 1. Similar results were obtained four times with each preparation of HL-60 cells and in three experiments with different preparations of HL-60 cells.

Table 1. Effect of SK&F 96365 on fMLP-induced increases in $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells in the presence and absence of extracellular Ca^{2+}

HL-60 cells were loaded with fura-2/acetoxymethyl ester. Basal and peak $[Ca^{2+}]_i$ values stimulated by fMLP (1 μM) were determined. Basal $[Ca^{2+}]_i$ values were subtracted from the corresponding peak $[Ca^{2+}]_i$ values to calculate the increase in $[Ca^{2+}]_i$ induced by fMLP. Experiments were performed in the presence of Ca^{2+} or EGTA (1 mM each). SK&F 96365 or solvent (control) was added to cells 3 min before fMLP treatment. Results shown are the means ± S.D. of four experiments performed with one preparation of HL-60 cells. Similar results were obtained in three experiments with different preparations of HL-60 cells.

	Increase in $[Ca^{2+}]_i$ (nM)	
Addition	Ca^{2+} (1 mM)	EGTA (1 mM)
Solvent	852±63	192±23
SK&F 96365 (10 μM)	517±52	204±13
SK&F 96365 (30 μM)	395±36	186±15

reflecting Mn^{2+} influx. SK&F 96365 (30 μM) abolished fMLP-induced Mn^{2+} influx without affecting release of Ca^{2+} from intracellular stores (see Fig. 2*b*). Isradipine (1 μM) did not alter fMLP-induced changes in fluorescence quenching (results not shown).

The results shown in Figs. 1 and 2 and Table 1 suggest that fMLP induced Ca^{2+} and Mn^{2+} influxes in Bt_2cAMP-differentiated HL-60 cells via NSC channels. Therefore, the properties of NSC channels were studied in more detail in whole-cell voltage-clamp experiments under physiological ionic conditions (E1). HL-60 cells showed membrane potentials ranging from −60 to −30 mV. Within this voltage range, the input conductance was linear and

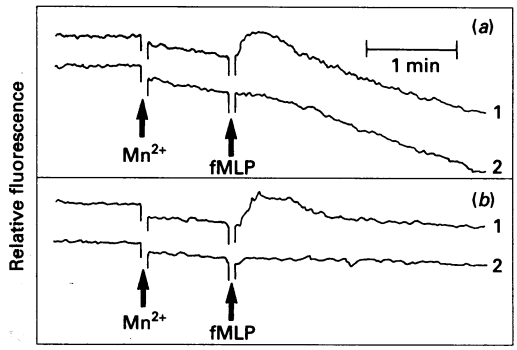

Fig. 2. Effect of fMLP on Mn^{2+} quenching of fura-2 fluorescence in Bt_2cAMP-differentiated HL-60 cells

HL-60 cells were loaded with fura-2/acetoxymethyl ester, and fura-2 fluorescence was monitored at excitation wavelengths of 340 nm (traces 1) or 360 nm (traces 2). The emission wavelength was 500 nm. Experiments were performed in the absence of Ca^{2+}. Arrows indicate the addition of $MnCl_2$ (100 μM) and fMLP (1 μM). (*a*) Experiments performed in the absence of SK&F 96365 (control). (*b*) Experiments performed in the presence of SK&F 96365 (30 μM). SK&F 96365 or solvent (control) were added to cells 3 min before Mn^{2+}. Superimposed original fluorescence tracings are shown. Similar results were obtained four times with each preparation of HL-60 cells and in three experiments with different preparations of HL-60 cells.

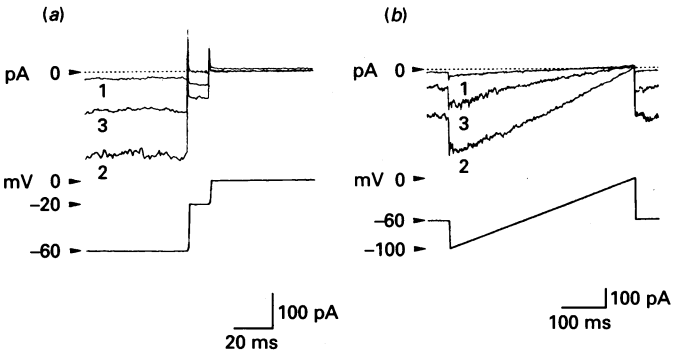

Fig. 3. Current–voltage relationship of fMLP-induced inward currents in Bt_2cAMP-differentiated HL-60 cells

In whole-cell voltage-clamp experiments currents were measured in the same cell. (*a*) Depolarizing pulses. (*b*) Ramp pulses. Traces 1, currents in the absence of fMLP; traces 2, currents after superfusion of cell with fMLP (100 nM) for 6 s; traces 3, currents after superfusion of fMLP-stimulated cell with SK&F 96365 (10 μM) for 6 s. Broken lines represent the zero-current level. Similar currents were observed in 16 experiments.

amounted to 45±9 pS/pF (mean±S.D., $n = 25$). In a manner similar to human neutrophils, at potentials more positive than −20 mV approx. 40% of the examined cells additionally showed an outward-rectifying K^+ current (results not shown) (von Tscharner *et al.*, 1986; Krause & Welsh, 1990). The properties of this K^+ current were not further investigated.

Upon step depolarizations from −60 to −20 mV and 0 mV, fMLP stimulated inward currents (Fig. 3). Under these conditions, currents showed no time-dependent activation or inactivation. Currents at −60 mV were approx. 3-fold greater in amplitude than currents at −20 mV, and at 0 mV no net currents were apparent. When ramp pulses (400 ms from −100 to 0 mV) were used as voltage-clamp protocol, currents were linear and showed a reversal potential close to 0 mV (see Fig. 3*b*). fMLP-stimulated currents resulted in membrane depolarization as revealed under current-clamp conditions (results not shown).

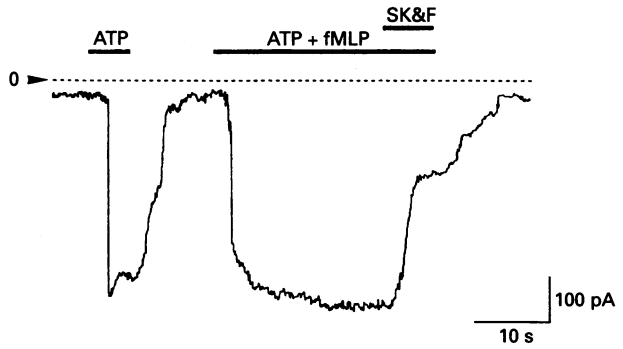

Fig. 4. Time course of agonist-stimulated inward currents in Bt_2cAMP-differentiated HL-60 cells

Currents were measured in a cell continuously voltage-clamped to a holding potential of −60 mV. Application periods of ATP (30 μM) and fMLP (100 nM) and of SK&F 96365 (10 μM) are indicated by the horizontal bars. The broken line represents the zero-current level. Similar results were obtained in three experiments.

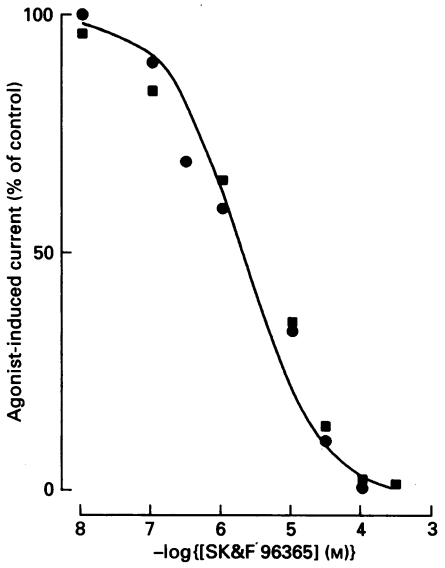

Fig. 5. Concentration–inhibition curves for SK&F 96365 on inward currents in Bt_2cAMP-differentiated HL-60 cells

Currents were stimulated with fMLP (100 nM) (●) or ATP (30 μM) (■) in the presence of various concentrations of SK&F 96365. Currents were measured at a holding potential of −60 mV. Each point represents the mean of 3–8 experiments; the S.D. of results generally amounted to less than 15% of the means.

fMLP-stimulated currents were present in about 60% of the examined cells (results not shown). This differential responsiveness to fMLP may be due to the fact that differentiated HL-60 cells are not a homogeneous population and may show differences in expression of formyl peptide receptors (Niedel *et al.*, 1980).

ATP stimulated inward currents as well (Fig. 4). fMLP did not further enhance ATP-stimulated currents, indicating that both agonists activated the same current. The effects of ATP and fMLP on inward currents were rapid in onset, i.e. within a few seconds, and were reversible upon washing out agonists.

The effects of SK&F 96365 on inward currents in HL-60 cells were studied. SK&F 96365 (10 μM) reduced the effect of fMLP by about 60%, independently of the membrane potential (see Fig. 3). No change in current linearity was observed. Blockade of currents by SK&F 96365 occurred within a few seconds (see Fig.

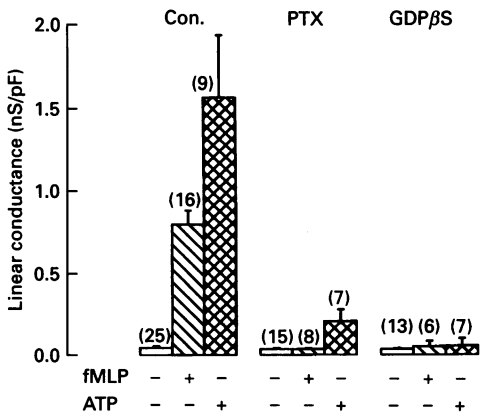

Fig. 6. Effects of pertussis toxin and GDPβS on fMLP- and ATP-stimulated inward currents in Bt_2cAMP-differentiated HL-60 cells

Currents stimulated by fMLP (100 nM) and ATP (30 μM) were measured in control cells (Con.), in pertussis-toxin-pretreated cells (PTX) and in GDPβS-infused cells (GDPβS). These cells were infused with GDPβS (0.5 mM) for 5 min. Results shown represent the means ± S.D. of linear conductances measured at −60 mV referred to the membrane capacitance. The number of experiments is given in parentheses.

4). On washing, the effect of SK&F 96365 was reversible. The concentration–inhibition curve for SK&F 96365 on inward currents is shown in Fig. 5. SK&F 96365 inhibited both fMLP- and ATP-stimulated currents with IC_{50} values of about 3 μM, again supporting the view that both agonists activated the same current (see Fig. 4). Isradipine (1 μM) did not inhibit fMLP- and ATP-stimulated inward currents (results not shown).

The involvement of G-proteins in fMLP- and ATP-stimulated inward currents was studied. ATP was substantially more effective than fMLP at stimulating currents (Fig. 6). Similarly, UTP and a stable analogue of ATP were more effective than fMLP at inducing phosphoinositide turnover and an increase in $[Ca^{2+}]_i$ (Wenzel-Seifert & Seifert, 1990; Cowen *et al.*, 1990). Pertussis toxin differentially inhibited inward currents. In pertussis-toxin-pretreated cells, fMLP-stimulated currents were abolished, and ATP-stimulated currents were reduced by approx. 80%. The stable GDP analogue, guanosine 5′-*O*-[thio]diphosphate (GDPβS), which stabilizes α-subunits of G-proteins in the inactive form (Gilman, 1987), was applied intracellularly. GDPβS prevented stimulation of inward currents by ATP and fMLP.

For determination of ion selectivity of NSC channels, fMLP-stimulated inward currents were assessed under various extracellular ionic conditions (Fig. 7). Choline was employed as a cation which does not permeate NSC channels (Schumann *et al.*, 1992). Withdrawal of Ca^{2+} (E2) resulted in only slightly diminished inward-current amplitudes [cf. Figs. 7(*a*) and 7(*b*)]. The reversal potential was unaffected. However, when Na^+ was removed (E5), the reversal potential shifted by approx. −20 mV and the amplitude was diminished by approx. 90% [cf. Figs. 7(*a*) and 7(*d*)]. Omission of all permeant cations (E6) resulted in outward-rectifying currents (see Fig. 7*f*), presumably carried by K^+. fMLP did not stimulate inward currents under these conditions, and the reversal potential was strongly shifted to negative values (−75 mV).

Permeation of Ca^{2+} through NSC channels was studied in the presence of Ca^{2+} as the only permeant cation. In the presence of Ca^{2+} (1.8 mM) (E3) fMLP stimulated a small but significant inward current with an amplitude of about −50 pA at −60 mV (see Fig. 7*c*). Elevation of Ca^{2+} concentration up to 100 mM (E4) resulted in a small increase in currents (see Fig. 7*e*). Under all

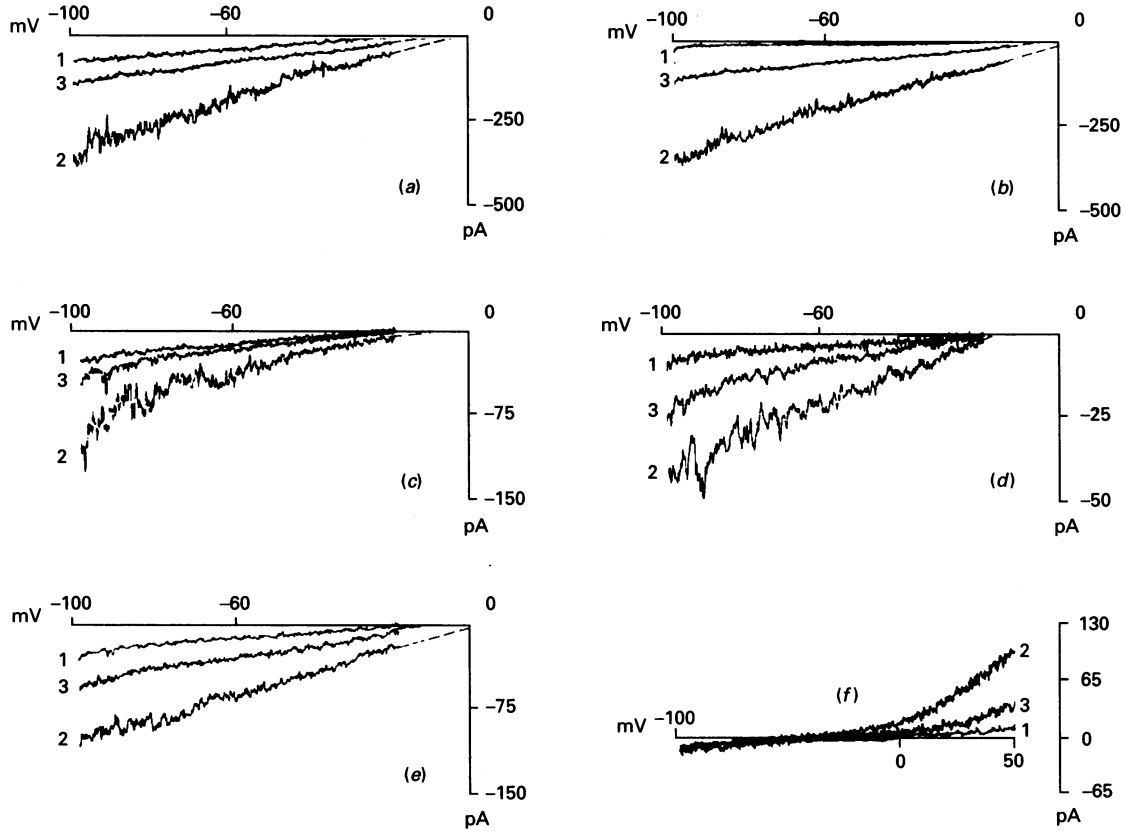

Fig. 7. Effects of various extracellular ionic conditions on fMLP-induced inward currents in Bt_2cAMP-differentiated HL-60 cells

Current voltage relations were measured during linear-voltage ramp pulses from −100 to −20 mV (0.4 V/s). Traces 1, currents in the absence of fMLP; traces 2, currents after superfusion with fMLP (100 nM) for 6 s; traces 3, currents after superfusion of fMLP-stimulated cells with SK&F 96365 (10 μM) for 6 s. Broken lines are extrapolations to the reversal potential. Cells were superfused with buffers (E1–E6) described in more detail in the Materials and methods section. (*a*) E1 (1.8 mM-Ca^{2+} and 140 mM-Na^+). (*b*) E2 (140 mM-Na^+ without Ca^{2+}). (*c*) E3 (1.8 mM-Ca^{2+} in the absence of other permeant cations). (*d*) E5 (1.8 mM-Ca^{2+} in the absence of Na^+). (*e*) E4 (100 mM-Ca^{2+} in the absence of other permeant cations). (*f*) E6 (no permeant cations). Similar currents were observed in 6–16 experiments.

Table 2. Effect of various extracellular ionic conditions on fMLP-stimulated inward-current conductances and reversal potentials in Bt_2cAMP-differentiated HL-60 cells

Conductances and reversal potentials ($E_{rev.}$) of fMLP-stimulated inward currents in Bt_2cAMP-differentiated HL-60 cells under various ionic conditions (E1–E6, see the Materials and methods section) were calculated. E1 (1.8 mM-Ca^{2+} and 140 mM-Na^+), E2 (140 mM-Na^+ without Ca^{2+}), E3 (1.8 mM-Ca^{2+} in the absence of other permeant cations), E4 (100 mM-Ca^{2+} in the absence of other permeant cations), E5 (1.8 mM-Ca^{2+} in the absence of Na^+), E6 (no permeant cations). Results shown are the means ± S.D. (n = 6–16) of linear conductances measured at −60 mV referred to membrane capacitance and the approx. reversal potentials.

Extracellular solution	Conductance (pS/pF)	$E_{rev.}$ (mV)
E1	785±72	2±5
E2	730±50	−2±3
E3	40±15	−10±5
E4	65±20	−7±6
E5	42±10	−19±2
E6	-	−75±4

conditions employed (E1–E6), fMLP-stimulated NSC currents were inhibited by SK&F 96365. Table 2 summarizes conductances and reversal potentials of currents under these conditions.

Single NSC channels were characterized further in inside-out patches under voltage-clamp conditions. The experiments were performed in the presence of fMLP (100 nM) in the patch pipette and GTPγS (10 μM) in the bath medium. At −60 mV, inward currents with an amplitude of 0.24±0.01 pA (mean±S.D., n = 6, sampling interval of 4–5 min) were observed (Fig. 8*a*). The open probability was 0.025±0.005 (mean±S.D., n = 6). Currents reversed at about 0 mV, indicating similar permeabilities for the cations present on both sides of the channel. At a holding potential of +60 mV, outward currents with similar amplitudes and open probabilities as at −60 mV were evident (see Fig. 8*a*). The current–voltage relationship was linear with a conductance of 4–5 pS (see Fig. 8*b*). When SK&F 96365 (10 μM) was present in the bath solution, no channel openings were observed (results not shown, n = 6).

The above results show that both Ca^{2+} and Na^+ permeate NSC channels. Therefore the roles of extracellular Ca^{2+} and Na^+ in the regulation of β-glucuronidase release and O_2^- production were studied. In the presence of both cations, fMLP stimulated the release of approx. 20% of the cellular content of β-glucuronidase (Table 3). SK&F 96365 (30 μM) inhibited β-glucuronidase release by more than 80%. By contrast, isradipine (1 μM) was without effect (results not shown). When Ca^{2+} was replaced by EGTA, and when Na^+ was replaced by choline, fMLP-stimulated β-glucuronidase release was reduced by 80% and 65% respectively. Under these conditions, SK&F 96365 (30 μM) reduced the respective stimulatory effects of fMLP by 85% and 65%. In the

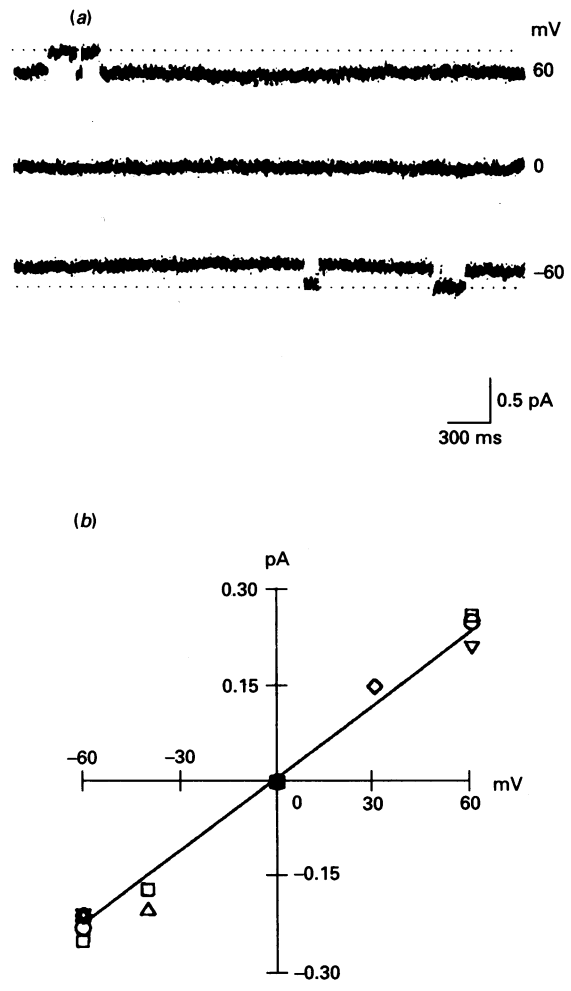

Fig. 8. Single-channel recordings in inside-out patches of Bt_2cAMP-differentiated HL-70 cells

Single-channel currents in the presence of fMLP (100 nM) were measured in inside-out patches. (*a*) Original current recordings at various holding potentials. Broken lines represent open-state levels of channels. Currents were filtered at 800 Hz. Representative segments of recordings from a single patch are shown. The recording time for each potential was 4 min. No channel openings were observed at 0 mV. Similar currents were observed in experiments with five different inside-out patches. (*b*) Current–voltage relationship of single-NSC-channel activity at various holding potentials. Symbols refer to currents recorded in six different inside-out patches. The current–voltage relationship was determined by linear regression analysis ($r = 0.98$).

Table 3. Effects of extracellular Ca^{2+} and Na^+ and SK&F 96365 on β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells

β-Glucuronidase release in HL-60 cells induced by fMLP (1 μM) was assessed under various conditions. SK&F 96365 (30 μM) or solvent (control) were added to cells 5 min before fMLP treatment. The concentration of Ca^{2+} and EGTA was 1 mM, and the concentrations of Na^+ and choline were 138 mM. Results shown are the means ± S.D. of three experiments performed with one preparation of HL-60 cells. Similar results were obtained in three experiments with different preparations of HL-60 cells.

	Stimulated β-glucuronidase release (% of cellular content)	
Additions	Control	SK&F 96365
Ca^{2+} + Na^+	21.0 ± 1.0	3.4 ± 0.4
Ca^{2+} + choline	4.1 ± 0.3	0.6 ± 0.2
EGTA + Na^+	7.3 ± 0.5	2.6 ± 0.2
EGTA + choline	0.2 ± 0.1	0

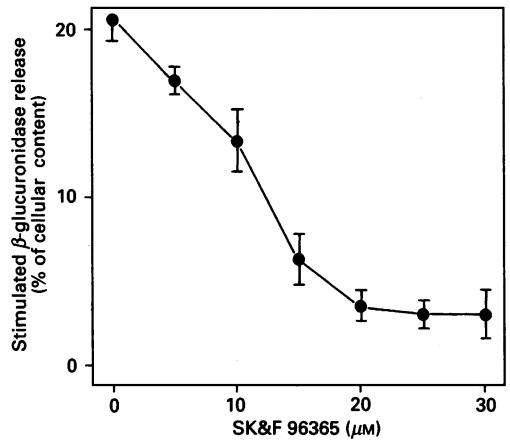

Fig. 9. Concentration–inhibition curve for SK&F 96365 on β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells

β-Glucuronidase release in HL-60 cells was stimulated with fMLP (1 μM) in the presence of SK&F 96365 at various concentrations under the conditions described in detail in the Materials and methods section. Buffer contained 1 mM-Ca^{2+} and 138 mM-Na^+. SK&F 96365 was added to cells 5 min before fMLP. Results shown are the means ± S.D. of three experiments performed with one preparation of HL-60 cells. Similar results were obtained in three experiments with different preparations of HL-60 cells.

absence of both Ca^{2+} and Na^+, stimulation by fMLP of β-glucuronidase release was virtually abolished. Fig. 9 shows a concentration–inhibition curve for SK&F 96365 on fMLP-stimulated β-glucuronidase release in the presence of Ca^{2+} and Na^+. SK&F 96365 inhibited β-glucuronidase release with an IC_{50} of about 12 μM and a maximum at 25 μM.

SK&F 96365 is an imidazole derivative (Merritt *et al.*, 1990), and imidazoles may bind to cytochrome b_{558}, a protein component of the O_2^--producing NADPH oxidase (Iizuka *et al.*, 1985). To exclude the possibility that SK&F 96365 inhibits O_2^- production because of interference with cytochrome b_{558}, the effects of SK&F 96365 on NADPH oxidase-catalysed O_2^- production in a cell-free system with membranes of Me_2SO-differentiated HL-60 cells were studied. In this system, arachidonic acid (0.2 mM) induced O_2^- production at a rate of 43.9 ± 1.0 nmol/min per mg of membrane protein. As reported previously (Seifert & Schultz, 1987), GTPγS (10 μM) enhanced this rate of O_2^- production 2.5-fold. SK&F 96365 (10 μM and 30 μM) did not inhibit O_2^- production under these conditions (results not shown). Additionally, SK&F 96365 (10 μM and 30 μM) did not affect O_2^- production induced by 4β-phorbol 12-myristate 13-acetate (100 ng/ml) in intact Bt_2cAMP-differentiated HL-60 cells (results not shown). These results suggest that SK&F 96365 did not interfere with cytochrome b_{558} or inhibit O_2^- production in an unspecific manner.

In the presence of extracellular Ca^{2+} and Na^+, fMLP rapidly and effectively activated O_2^- production, although this process ceased after about 8 min (Fig. 10). SK&F 96365 (30 μM) inhibited this O_2^- formation by about 80%. Isradipine (1 μM) did not inhibit fMLP-induced O_2^- production (results not shown). In the absence of Ca^{2+} or Na^+, fMLP-induced O_2^- production was reduced by 70–75%. Similar to the results obtained for β-glucuronidase release, SK&F 96365 substantially inhibited O_2^-

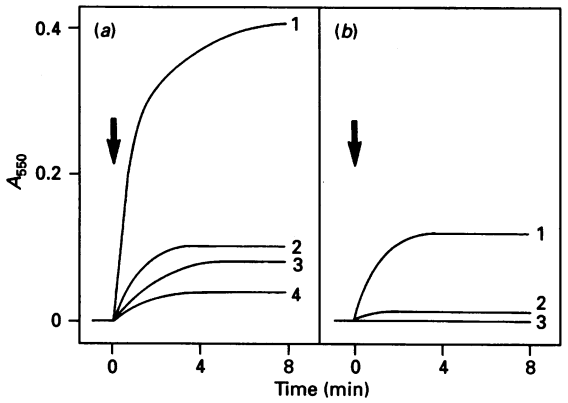

Fig. 10. Effects of extracellular Ca^{2+} and Na^+ and SK&F 96365 on time courses of fMLP-induced O_2^- formation in Bt_2cAMP-differentiated HL-60 cells

O_2^- formation in HL-60 cells induced by fMLP (1 μM) was assessed under various conditions. Arrows indicate the addition of stimulus. Various substances were added to cells 3 min before stimuli. (*a*) Experiments performed in the presence of Na^+ (138 mM). Trace 1, Ca^{2+} (1 mM) plus solvent (control); trace 2, EGTA (1 mM) plus solvent (control); trace 3, Ca^{2+} (1 mM) plus SK&F 96365 (30 μM); trace 4, EGTA (1 mM) plus SK&F 96365 (30 μM). (*b*) Experiments performed in presence of choline (138 mM) instead of Na^+. Trace 1, Ca^{2+} (1 mM) plus solvent (control); trace 2, Ca^{2+} (1 mM) plus SK&F 96365 (30 μM); trace 3, EGTA (1 mM) plus solvent (control) or SK&F 96365 (30 μM). Superimposed original spectrophotometric tracings are shown. Similar results were obtained four times with each preparation of HL-60 cells and in three experiments with different preparations of HL-60 cells.

Table 4. Effects of chloride salts of monovalent cations on basal and fMLP-stimulated GTP hydrolysis in membranes of Bt_2cAMP-differentiated HL-60 cells

High-affinity GTPase activity in membranes from Bt_2cAMP-differentiated HL-60 cells was measured in the presence of chloride salts of monovalent cations at the indicated concentrations without (basal) or with fMLP (10 μM). Values in parentheses in the column 'basal' indicate GTPase activities relative to that measured in the absence of salts (1.00). Values in parentheses in the column 'fMLP' indicate absolute stimulations by fMLP of GTP hydrolysis (pmoles of P_i released in the presence of fMLP minus pmoles of P_i released in the absence of fMLP). Numbers in brackets in the column 'fMLP' indicate relative increases by fMLP of GTP hydrolysis (fMLP-stimulated GTP hydrolysis/basal GTP hydrolysis). Results shown are the means ± S.D. of assay quadruplicates. Similar results were obtained in three separate experiments.

Salt	GTP hydrolysis (pmol of P_i/min per mg of protein)	
	Basal	fMLP
None	13.2±0.4 (1.00)	18.6±0.2 (5.4) [1.41]
NaCl (25 mM)	15.0±0.5 (1.14)	21.0±0.7 (6.0) [1.40]
NaCl (50 mM)	16.0±0.2 (1.21)	22.6±0.5 (6.6) [1.42]
KCl (50 mM)	16.7±0.7 1.26)	23.4±0.9 (6.7) [1.40]
LiCl (50 mM)	17.0±1.0 (1.29)	23.8±1.1 (6.8) [1.40]
Choline chloride (50 mM)	13.9±0.4 (1.05)	19.9±0.8 (6.0) [1.43]

production under these conditions. In the absence of both Ca^{2+} and Na^+, fMLP-induced O_2^- production was abolished.

Finally, the effects of various chloride salts of monovalent cations on GTPase activity in membranes from Bt_2cAMP-differentiated HL-60 cells were studied. NaCl (25 mM and 50 mM) stimulated basal GTP hydrolysis by 14% and 21% respectively (Table 4). In the absence and presence of NaCl, fMLP (10 μM) stimulated high-affinity GTPase activity by approx. 40%. NaCl significantly enhanced the absolute stimulation of GTP hydrolysis caused by fMLP. KCl and LiCl (50 mM) enhanced basal GTP hydrolysis to a greater extent than did NaCl and choline chloride. KCl, LiCl and choline chloride also enhanced, to different degrees, the absolute extent of GTP hydrolysis caused by fMLP.

DISCUSSION

In Bt_2cAMP-differentiated HL-60 cells, the fMLP-induced increase in $[Ca^{2+}]_i$ depended largely on the presence of extracellular Ca^{2+} and was partially inhibited by SK&F 96365 (see Fig. 1 and Table 1). SK&F 96365 at concentrations as high as 30 μM did not interfere with Ca^{2+} mobilization from intracellular stores (see Figs. 1 and 2 and Table 1). Moreover, fMLP induced Mn^{2+} influx in Bt_2cAMP-differentiated HL-60 cells in an SK&F 96365-sensitive manner (see Fig. 2). Results of recent studies suggested the presence of NSC channels in human neutrophils and Me_2SO-differentiated HL-60 cells (von Tscharner *et al.*, 1986; Andersson *et al.*, 1986; Nasmith & Grinstein, 1987; Merritt *et al.*, 1989, 1990; Pittet *et al.*, 1989; Demaurex *et al.*, 1992; Schumann *et al.*, 1992). These studies showed that fMLP induces transient increases in $[Ca^{2+}]_i$ through Ca^{2+} mobilization from intracellular stores followed by sustained Ca^{2+} influx (Andersson *et al.*, 1986; Nasmith & Grinstein, 1987; Pittet *et al.*, 1989). Additionally, fMLP induces Mn^{2+} influx in neutrophils (Merritt *et al.*, 1989). Ca^{2+} and Mn^{2+} influxes in neutrophils are blocked by SK&F 96365 (Merritt *et al.*, 1990). Taken together, these results indicate that Bt_2cAMP-differentiated HL-60 cells possess NSC channels with properties similar to those of neutrophils and Me_2SO-differentiated HL-60 cells.

fMLP-stimulated inward currents in Bt_2cAMP-differentiated HL-60 cells showed properties which are characteristic of receptor-activated NSC channels (Partridge & Swandulla, 1988). First, currents were activated by receptor agonists but not by voltage (see Fig. 3). Secondly, currents showed no time-dependency of activation or inactivation kinetics (see Figs. 3 and 4). Thirdly, currents had a linear current–voltage relationship and reversed close to 0 mV (see Figs. 3, 7 and 8; Table 2). Fourthly, currents were inhibited by SK&F 96365 but not by blockers of voltage-dependent Ca^{2+} channels (see Figs. 3–5 and 7). The IC_{50} value for SK&F 96365 to inhibit inward currents in HL-60 cells amounted to about 3 μM (see Fig. 5). Similar potencies for SK&F 96365 to inhibit receptor-stimulated Ca^{2+} influx have been reported for neutrophils, platelets, endothelial cells and a neuroglial cell line (Merritt *et al.*, 1990; Montero *et al.*, 1991; Mason *et al.*, 1991).

Small NSC-channel currents may result in large rises in $[Ca^{2+}]_i$ (Penner *et al.*, 1988). Bt_2cAMP-differentiated HL-60 cells possess NSC channels with a conductance of 4–5 pS and a reversal potential at about 0 mV as assessed by single-channel recordings in excised inside-out patches (see Fig. 8). von Tscharner *et al.* (1986) identified 4–6-pS and 18–25-pS NSC channels in inside-out patches of human neutrophils with reversal potentials at 0 mV. Possibly, the 18–25-pS channel is not present in Bt_2cAMP-differentiated HL-60 cells (see Fig. 8). Although both human neutrophils and Bt_2cAMP-differentiated HL-60 cells possess NSC channels with conductances of about 5 pS, these channels may be different. This view is supported by the finding that opening of the 4–6-pS NSC channel in neutrophils obligatorily depends on the presence of Ca^{2+} concentrations of above 10 nM in the bath solution of the inside-out patch (von Tscharner *et al.*, 1986), whereas in our hands the 4–5-pS NSC channel in Bt_2cAMP-differentiated HL-60 cells opened in the absence of

Ca^{2+} (see Fig. 8). However, human neutrophils may also possess NSC channels which open independently of rises in $[Ca^{2+}]_i$ (Nasmith & Grinstein, 1987; Schumann *et al.*, 1992). Thus, there are apparently several types of NSC channels in neutrophilic cells. The results of studies with other cell types also support the view that there is considerable variation in conductance, regulation and sensitivity to blockade by SK&F 96365 among NSC channels (Partridge & Swandulla, 1988; Penner *et al.*, 1988; Galietta *et al.*, 1989; Fasolato *et al.*, 1990; Neher, 1992; Schwaner *et al.*, 1992).

Interestingly, removal of extracellular Ca^{2+} had little effect on the conductance and reversal potential of fMLP-stimulated inward whole-cell currents (see Fig. 7 and Table 2). In single-channel recordings, currents also reversed at approx. 0 mV without Ca^{2+} in the patch pipette, i.e. the extracellular medium (see Fig. 8). In contrast, removal of Na^+ strongly diminished fMLP-stimulated currents (see Fig. 7 and Table 2). These results show that fMLP does not only stimulate Ca^{2+} influx via NSC channels but also Na^+ influx; and that Na^+ is the major cation entering the cell. Na^+ influx through NSC channels has also been observed in other cell types including human neutrophils (von Tscharner *et al.*, 1986; Benham, 1990; Avdonin *et al.*, 1991; Borin & Siffert, 1991; Sage *et al.*, 1991). These results support the hypothesis that Na^+, in addition to Ca^{2+}, is an intracellular signal molecule (see below).

Formyl peptide- and ATP receptors interact with pertussis-toxin-sensitive G-proteins, and ATP receptors additionally interact with pertussis-toxin-insensitive G-proteins (Gierschik *et al.*, 1989; Seifert *et al.*, 1989*a*,*b*; Wenzel-Seifert & Seifert, 1990; Cowen *et al.*, 1990; Seifert & Schultz, 1991). Pertussis toxin abolished fMLP-stimulated inward currents and partially inhibited ATP-stimulated currents (see Fig. 6). The pertussis-toxin-insensitive part of ATP-stimulated currents was abolished by GDPβS, suggesting that it was mediated via G-proteins (see Fig. 6). The results obtained for fMLP- and ATP-stimulated inward currents in Bt_2cAMP-differentiated HL-60 cells are in good agreement with the results obtained for phosphoinositide turnover, increases in $[Ca^{2+}]_i$, β-glucuronidase release and O_2^- production in these cells (Dubyak *et al.*, 1988; Seifert *et al.*, 1989*a*; Wenzel-Seifert & Seifert, 1990). Thus, G-proteins are involved in the activation by receptor agonists of NSC channels in Bt_2cAMP-differentiated HL-60 cells.

The question arises of whether NSC-channel opening in Bt_2cAMP-differentiated HL-60 cells is indirectly or directly regulated by G-proteins, i.e. of whether or not intracellular signal molecules are involved. To address this problem, experiments with inside-out patches from Bt_2-cAMP-differentiated HL-60 cells were performed. The bath solution, i.e. the solution on the cytosolic side of the patch, contained GTPγS but no Ca^{2+} or ATP (see the Materials and methods section). Under these conditions, NSC-channel openings were observed (see Fig. 8). These results suggest that opening of 4–5-pS NSC channels in Bt_2cAMP-differentiated HL-60 cells occurs independently of intracellular Ca^{2+} and/or activation of protein kinases such as cyclic AMP-dependent protein kinase or protein kinase C (see Fig. 8). Rather, cyclic AMP-dependent protein kinase and protein kinase C play inhibitory roles in the regulation of NSC channels in neutrophilic cells (McCarthy *et al.*, 1989; Seifert *et al.*, 1992*a*; Schumann *et al.*, 1992). It cannot be completely excluded, however, that other intracellular signal molecules which may stimulate cation channels, such as arachidonic acid, were formed in fMLP-stimulated inside-out patches (for review see Schultz *et al.*, 1990). In addition, our results do not exclude the possibility that NSC channels in Bt_2cAMP-differentiated HL-60 cells are regulated by Ca^{2+}.

To study the role of NSC channels in the activation of β-glucuronidase release and O_2^- production in Bt_2cAMP-differentiated HL-60 cells, the effects of SK&F 96365 and of omissions of extracellular Ca^{2+} and Na^+ were studied. SK&F 96365, although not being very potent on a molar basis (see Figs. 1, 5 and 9 and Table 1), is a suitable drug for this purpose as it is not cytotoxic and does not interfere with NADPH oxidase or enzyme release in an unspecific manner. Additionally, unlike in human neutrophils, SK&F 96365 concentrations as high as 30 μM did not interfere with Ca^{2+} mobilization from intracellular stores in Bt_2cAMP-differentiated HL-60 cells (see Figs. 1 and 2 and Table 1) (Merritt *et al.*, 1990). SK&F 96365 inhibited fMLP-induced β-glucuronidase release and O_2^- production in the presence of Ca^{2+} and Na^+ by 80–85% (Fig. 10). In agreement with these findings, SK&F 96365 (30 μM) reduced fMLP-stimulated inward currents by approx. 90% (see Fig. 5). In addition, SK&F 96365 inhibited fMLP-stimulated β-glucuronidase release and reduced inward currents with a similar potency (see Figs. 5 and 9). NSC channels are permeable to Ca^{2+} and Na^+, and SK&F 96365 inhibited fMLP-stimulated inward currents regardless of the ionic conditions employed (see Fig. 7). In accordance with these findings both extracellular Ca^{2+} and Na^+ were required for maximal activation of β-glucuronidase release and O_2^- production, and in the absence of Ca^{2+} and Na^+ fMLP was ineffective. Intriguingly, SK&F 96365 inhibited the small but significant β-glucuronidase release and O_2^- production stimulated by fMLP in the absence of Ca^{2+} or Na^+. These results imply that influx of Na^+ was required for cell activation in the absence of Ca^{2+} and vice versa and that Ca^{2+} and Na^+ play different roles in cellular activation. Taken together, these results suggest that NSC channels permeable for Ca^{2+} and Na^+ are involved in the activation of β-glucuronidase release and O_2^- production in Bt_2cAMP-differentiated HL-60 cells. The fact that extracellular Ca^{2+} is also required for maximal activation of O_2^- production by fMLP in neutrophils supports the notion that NSC channels are involved in the activation of NADPH oxidase (see Fig. 10) (for review see Seifert & Schultz, 1991). However, the role of NSC channels in the regulation by fMLP of β-glucuronidase release in human neutrophils is less clear as EGTA, unlike in Bt_2cAMP-differentiated HL-60 cells, is without inhibitory effect (Smolen *et al.*, 1981).

Cytosolic Ca^{2+} may regulate the activity of several proteins such as phospholipases A_2 and C, protein kinase C, proteases, calmodulin and, thereby, other proteins in neutrophils (for reviews see Rossi, 1986; Sandborg & Smolen, 1988; Seifert & Schultz, 1991). Our results suggest a stimulatory role for Na^+ influx in the activation of β-glucuronidase release and O_2^- formation (see Figs. 9 and 10 and Table 3). With regard to O_2^- formation, our results are in agreement with those obtained by Simchowitz & Spilberg (1979). By contrast, Della Bianca *et al.* (1983) suggested that Na^+ may play an inhibitory role in the regulation of β-glucuronidase release and O_2^- production. The discrepancies in the effects of extracellular Na^+ in the latter study and in this study may be due to the fact that Della Bianca *et al.* (1983) replaced Na^+ by K^+, i.e. a cation which permeates NSC channels, whereas choline does not (see Fig. 7) (Schumann *et al.*, 1992).

The mechanism by which Na^+, once it reaches the cytosol, enhances activation of β-glucuronidase release and O_2^- production is as yet unknown but several possibilities have to be considered. Na^+ is thought to stimulate Ca^{2+} mobilization from intracellular stores and Na^+/Ca^{2+} exchange and, thereby, to potentiate the effects of Ca^{2+} (Nordmann & Steunkel, 1991). Additionally, Na^+ may directly affect exocytosis, e.g. via fusion of exocytotic vesicles with the plasma membrane (Rahamimoff *et al.*, 1978; Nordmann & Stuenkel, 1991). As Na^+ modulates the function of G-proteins in Me_2SO-differentiated HL-60 cells (Gierschik *et al.*, 1989), we asked the question of whether Na^+

could enhance G-protein activation in Bt_2cAMP-differentiated HL-60 cells. Of interest in this context is the finding that $[Na^+]_i$ in stimulated human blood cells may be as high as 60 mM (Simchowitz, 1985; Borin & Siffert, 1991; Sage *et al.*, 1991). We found that NaCl (25–50 mM) significantly enhanced basal GTP hydrolysis and the absolute extent of fMLP-stimulated GTP hydrolysis of G-proteins in membranes from Bt_2cAMP-differentiated HL-60 cells (see Table 4). The effects of NaCl were apparently due to Na^+ rather than to Cl^- or an increase in ionic strength. This notion is supported by the findings that chloride salts of other monovalent cations differentially enhanced GTP hydrolysis (see Table 4). In this context, it should be emphasized that fMLP very effectively and rapidly activates inward currents, β-glucuronidase release and O_2^- production, i.e. signal amplification is apparently very effective (Seifert *et al.*, 1989*a*; Wenzel-Seifert & Seifert, 1990; Baggiolini & Wymann, 1990) (see Figs. 3 and 10). Thus, stimulation by fMLP of Na^+ influx may provide a feed-forward signal for enhanced activation of G-proteins, resulting in increased β-glucuronidase release and O_2^- production.

Using membranes from Me_2SO-differentiated HL-60 cells Gierschik *et al.* (1989) reported on reduction by Na^+ of basal GTP hydrolysis. The order of effectiveness of cations in inhibiting GTP hydrolysis was $Na^+ > Li^+ > K^+ >$ choline (Gierschik *et al.*, 1989). In contrast, we found that monovalent cations enhanced basal GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells in the order of effectiveness $Li^+ > K^+ > Na^+ >$ choline (see Table 4). However, in both types of HL-60 membranes Na^+ increased the absolute extent of GTP hydrolysis caused by fMLP (see Table 4) (Gierschik *et al.*, 1989). The differences in regulation by monovalent cations of GTP hydrolysis between Me_2SO- and Bt_2cAMP-differentiated HL-60 cells may be caused by the functional non-equivalence of G-proteins in these cells (Tohkin *et al.*, 1991).

In conclusion, we have shown that receptor agonists stimulate inward currents through NSC channels in Bt_2cAMP-differentiated HL-60 cells. These channels are activated via G-protein-dependent mechanisms and are blocked by SK&F 96365. Ca^{2+} and Na^+ inward currents are involved in the activation of β-glucuronidase release and of O_2^- production. An increase in $[Na^+]_i$ may enhance activation of G-proteins, providing a mechanism for signal amplification.

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