Thapsigargin activates univalent- and bivalent-cation entry in human neutrophils by a SK&F 96365- and Gd^{3+} -sensitive pathway and is a partial secretagogue: involvement of pertussis-toxin-sensitive G-proteins and protein phosphatases 1/2A and 2B in the signal-transduction pathway

Katharina WENZEL-SEIFERT*, Dietmar KRAUTWURST†, Ian MUSGRAVE‡ and Roland SEIFERT*§

Institut für Pharmakologie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Thielallee 69/73, D-14195 Berlin, Federal Republic of Germany

The Ca^{2+} -ATPase inhibitor thapsigargin (TG) activates bivalent-cation entry in human neutrophils via depletion of intracellular Ca^{2+} stores, but little is known about the underlying mechanism and the functional role of TG-induced cation entry. We studied the effects of TG on univalent- and bivalent-cation entry, lysozyme release and superoxide-anion (O_2^-) formation in human neutrophils. TG, like the chemotactic peptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), stimulated entry of Ca^{2+} , Mn^{2+} , Ba^{2+} , Sr^{2+} and Na^+ in a 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride (SK&F 96365)- and Gd^{3+} -sensitive manner. The inhibitors of protein phosphatases 1/2A, calyculin A and okadaic acid, diminished TG-induced cation influxes, whereas the inhi-

bitors of protein phosphatase 2B, cyclosporin A and FK-506, were potentiators. Pertussis toxin (PTX) partially inhibited the effects of TG on Ca^{2+} and Mn^{2+} entry. TG and fMLP activated inward currents with a linear current-voltage relationship and a reversal potential at about 0 mV. TG activated lysozyme release and potentiated fMLP-induced O_2^- formation. TG-induced lysozyme release was inhibited by SK&F 96365, PTX and the removal of extracellular Ca^{2+} or Na^+ . Our data show that TG activates a non-selective and SK&F 96365- and Gd^{3+} -sensitive cation entry pathway and is a partial secretagogue. TG-stimulated cation entry involves PTX-sensitive G-proteins and protein phosphatases, with protein phosphatases 1/2A and 2B playing opposite roles.

INTRODUCTION

Thapsigargin (TG) inhibits the Ca^{2+} -ATPase of intracellular Ca^{2+} stores and, consequently, activates Ca^{2+} influx from the extracellular space [1–5]. This store-operated Ca^{2+} -entry mechanism is present in virtually every cell type [1–5]. TG stimulates the entry not only of Ca^{2+} but also of other bivalent cations, specifically of Mn^{2+} [1–5]. TG-stimulated cation entry can be inhibited by imidazoles, e.g. SK&F 96365, and by lanthanides, e.g. Gd^{3+} [6–8]. The molecular mechanism by which TG activates cation entry is still unclear and apparently complex. G-proteins [9,10], steps sensitive to protein phosphatases 1/2A [11–14] and 2B [13] and a putative intracellular signal molecule, operationally referred to as calcium-influx factor ('CIF') [13,14], have been implicated in TG-mediated cation influx. The existence of a calcium-influx factor is, however, not uncontested [15,16].

Human neutrophils and HL-60 cells belong to the most extensively studied model systems concerning TG-mediated cation entry [6–9,12,17,18], but several important questions have remained unanswered. First, it is not known whether the TG-activated cation-entry pathway in neutrophils is permeable only to bivalent cations or to both bivalent and univalent cations. Second, the role of protein phosphatase 2B in the activation process is undefined. Third, the identity of the G-protein postulated to be involved in the cation-entry pathway is obscure.

Fourth, it is not known whether TG-induced cation entry has any consequence for human neutrophil cell functions.

In order to address these questions, we studied the effects of TG on the influx of Ca^{2+} , Mn^{2+} , Ba^{2+} , Sr^{2+} and Na^+ in these cells, using fluorescence techniques, inward currents using electrophysiological techniques and physiological cell functions, i.e. exocytosis and O_2^- formation. We used SK&F 96365, Gd^{3+} , various protein phosphatase inhibitors and pertussis toxin (PTX), which ADP-ribosylates certain G-protein α -subunits, as experimental tools to analyse the mechanisms involved in cation entry. As a reference stimulus, we employed the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). This peptide, via the PTX-sensitive G-proteins G_{12} and G_{13} , leads to activation of phospholipase C with subsequent depletion of intracellular Ca^{2+} stores and cation entry [6,7,19,20].

MATERIALS AND METHODS

Materials

SK&F 96365 (1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride) was kindly provided by Dr. J. E. Merritt (SmithKline Beecham, Welwyn, Herts., U.K.). The B-oligomer of PTX was donated by Dr. M. Yajima (Kyoto, Japan). The cyclosporins CsA, CsD and CsH were gifts from Sandoz (Basel, Switzerland). FK-506 was donated by

Abbreviations used: AM, acetoxymethyl ester; $[Ba^{2+}]_i$, cytosolic free Ba^{2+} concentration; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; Cs, cyclosporin; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; $[Na^+]_i$, cytosolic free Na^+ concentration; O_2^- , superoxide anion; PTX, pertussis toxin; SBF, sodium-binding benzofuran isophthalate; SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride; $[Sr^{2+}]_i$, cytosolic free Sr^{2+} concentration; TG, thapsigargin.

* Present address: Howard Hughes Medical Institute, Beckman Center for Molecular and Genetic Medicine, Room B-159, Stanford University Medical Center, Stanford, CA 94305, U.S.A.

† Present address: Molecular Biology & Genetics, Johns Hopkins University, School of Medicine, Baltimore, MD 21205-2185, U.S.A.

‡ Present address: Pharmacology Group, Prince Henry's Institute of Medical Research, P.O. Box 152, Clayton, Vic. 3168, Australia.

§ To whom correspondence should be addressed, at Stanford.

Fujisawa (Osaka, Japan). PTX was from either List Biological Laboratories (Campbell, CA, U.S.A.) or Sigma Chemie (Deisenhofen, Germany). TG, fMLP, ferricytochrome *c* (type III from horse heart) and freeze-dried *Micrococcus lysodeicticus* (as substrate for lysozyme) were obtained from Sigma Chemie (Germany). GdCl_3 was purchased from Aldrich (Steinheim, Germany). Calyculin A and okadaic acid were obtained from Boehringer Mannheim (Mannheim, Germany). Fura-2 and sodium-binding benzofuran isophthalate (SBFI) acetoxymethyl esters were from Calbiochem (Frankfurt/Main, Germany) or Molecular Probes (Eugene, OR, U.S.A.). Pluronic F-127 was from Molecular Probes. Other materials were of the highest purity commercially available. Stock solutions of SK&F 96365 (30 mM), SBFI acetoxymethyl ester (AM) and fMLP (10 mM each), fura-2/AM (2 mM), TG, CsA, CsD, CsH, FK-506 (also referred to as tacrolimus), calyculin A and okadaic acid (1 mM each), gramicidin D (1 mg/ml) and Pluronic F-127 (10%, w/v) were prepared in DMSO and stored at -20°C . Preliminary studies showed that DMSO at the concentrations employed, i.e. up to 0.3% (v/v), had no effect on rises in cytosolic free concentrations of Ca^{2+} , Ba^{2+} , Sr^{2+} and Na^+ ($[\text{Ca}^{2+}]_i$, $[\text{Ba}^{2+}]_i$, $[\text{Sr}^{2+}]_i$, $[\text{Na}^+]_i$) and Mn^{2+} entry. For the experiments shown in Figures 1–3 and Tables 1–6, TG and fMLP were employed at maximally effective concentrations. The following buffers (constituents given in mM) were employed. E1: 138 NaCl, 6 KCl, 1 MgSO_4 , 1 Na_2HPO_4 , 5 NaHCO_3 , 5.5 glucose, 20 Hepes/NaOH, pH 7.4, supplemented with 0.1% (w/v) BSA. E2: 138 NaCl, 6 KCl, 1 MgCl_2 , 5.5 glucose, 20 Hepes/NaOH, pH 7.4. E3: 138 *N*-methyl-D-glucamine, 6 KCl, 1 MgCl_2 , 5.5 glucose, 20 Hepes/HCl, pH 7.4. E4: 110 sodium aspartate, 30 NaCl, 1 CaCl_2 , 1 MgCl_2 , 20 Hepes/NaOH, pH 7.4. E5: 110 potassium aspartate, 30 KCl, 1 CaCl_2 , 1 MgCl_2 , 20 Hepes/KOH, pH 7.4. E6: 140 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 10 glucose, 10 Hepes/NaOH, pH 7.4. E7: 140 *N*-methyl-D-glucamine, 5.4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 10 glucose, 10 Hepes/HCl, pH 7.4. I1: 50 KCl, 90 potassium aspartate, 1 MgCl_2 , 3 sodium-ATP, 10 EGTA, 10 Hepes/KOH, pH 7.4. The buffer pairs E2/E3 and E6/E7 were controlled for iso-osmolarity.

Preparation of neutrophils

Heparinized blood was obtained by venipuncture from healthy volunteers of either sex who had taken no drugs for at least 3 weeks. Neutrophils were isolated by dextran sedimentation and centrifugation through Ficoll/Hypaque as described [21]. Cell preparations contained more than 98% viable neutrophils, as assessed by Trypan Blue dye exclusion and Pappenheim-stained smears.

PTX- and B-oligomer treatment

Neutrophils were suspended at 1.0×10^7 cells/ml in E1 supplemented with PTX (5 $\mu\text{g}/\text{ml}$) or B-oligomer (3.8 $\mu\text{g}/\text{ml}$) (control) and were incubated for 2 h at 37°C . When rises in $[\text{Ca}^{2+}]_i$ and Mn^{2+} entry were determined, fura-2/AM (2 μM) was added to cells during the last 60 min of treatment.

Measurement of rises in $[\text{Ca}^{2+}]_i$, Mn^{2+} quenching, $[\text{Ba}^{2+}]_i$ and $[\text{Sr}^{2+}]_i$

Neutrophils (1.0×10^7 cells/ml) were loaded with fura-2/AM (2 μM) for 60 min at 37°C in E1. Loaded cells, which had been washed to remove free dye, were suspended at 1.0×10^6 cells/ml in E2 and were kept at 20°C for up to 3 h. In experiments with calyculin A and okadaic acid, dye and the phosphatase inhibitors

were added to cells which were then incubated as described above. After washing, cells were suspended at 1.0×10^7 cells/ml, and phosphatase inhibitors were re-added at the same final concentrations as during dye loading. Cuvette solutions for fluorescence measurements were not further supplemented with phosphatase inhibitors. Fluorescence of neutrophils (1.0×10^6 cells in 2 ml) was determined at 37°C under constant stirring at 10^3 rev./min in 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 the addition of stimuli. The excitation and emission wavelengths were 340 nm and 510 nm respectively. Fluorescence signals were calibrated as described [18]. $[\text{Ca}^{2+}]_i$ was calculated according to eqn. (6) in Grynkiewicz et al. [22]. With the exception of the original recordings shown in Figure 1, maximum $[\text{Ca}^{2+}]_i$ values stimulated by TG or fMLP, from which basal $[\text{Ca}^{2+}]_i$ values had been subtracted, were calculated. Basal $[\text{Ca}^{2+}]_i$ in freshly loaded neutrophils was 118 ± 26 nM (mean $\pm$ S.D., $n = 28$). Mn^{2+} quenching of fura-2 fluorescence was assessed at the Ca^{2+} -insensitive excitation wavelength of 360 nm [23] and an emission wavelength of 510 nm. E2 was supplemented with 30 μM MnCl_2 . Mn^{2+} influx was calculated by dividing the rate of basal influx over 2 min by the initial rate of stimulated Mn^{2+} influx. For measurements of $[\text{Ba}^{2+}]_i$ and $[\text{Sr}^{2+}]_i$, cells were suspended in E2 and stimulated with TG or fMLP. After 3 min, BaCl_2 or SrCl_2 (1 mM each) was added to cells. The excitation and emission wavelengths were 340 and 510 nm respectively. $[\text{Ba}^{2+}]_i$ and $[\text{Sr}^{2+}]_i$ values obtained 4 min after stimulus addition were calculated according to eqn. (6) in Grynkiewicz et al. [22] by using K_d values of 1.36 μM (Ba^{2+}) and 7.6 μM (Sr^{2+}) [24]. Basal Ba^{2+} and Sr^{2+} influxes were subtracted from stimulated fluxes.

Measurement of $[\text{Na}^+]_i$

$[\text{Na}^+]_i$ was determined by using the Na^+ -sensitive dye SBFI [25]. Portions of SBFI/AM (10 mM) were thawed and mixed with 10% (w/v) Pluronic F-127 (1:1, v/v). This mixture was added to cells (1.0×10^7 neutrophils in E1) to give final concentrations of 20 μM SBFI/AM and 0.02% (w/v) Pluronic F-127. Cells were incubated for 90 min at 30°C in a shaking water bath in the dark. Thereafter, cells were diluted with E1 to a final concentration of 0.5×10^6 cells/ml and were centrifuged at 250 g for 10 min at 20°C . Cells were resuspended in E2 at 4×10^6 cells/ml. Cells were stored in the dark at 20°C for up to 3 h before measurement of $[\text{Na}^+]_i$. This protocol resulted in sufficient dye loading in about 50% of all experiments. Basal $[\text{Na}^+]_i$ in neutrophils was 11.9 ± 3.8 mM (mean $\pm$ S.D., $n = 13$). In preliminary studies we found that decreasing dye and Pluronic F-127 concentrations or incubation temperatures or incubation times resulted in insufficient dye loading. Increases in the concentration of Pluronic F-127 or incubation temperature did not result in more efficient dye loading, but rather induced cell damage. Batches of cells (0.5 ml) were added to fluorescence cuvettes (Sarstedt, Nümbrecht, Germany) and further diluted with E2 to give a final volume of 2 ml. Fluorescence was determined at 37°C under constant stirring at 10^3 rev./min with a LS 50B dual-wavelength fluorimeter (Perkin-Elmer, Beaconsfield, Bucks., U.K.). The excitation wavelengths were 340 nm and 385 nm, and the emission wavelength was 510 nm. Cells were incubated for 3 min in the presence of various substances before addition of stimuli. Fluorescence signals were calibrated by suspending cells in mixtures of E4 and E5, giving final Na^+ concentrations (mM) of 0, 5, 20, 50 and 150, and subsequent stimulation with gramicidin D (10 $\mu\text{g}/\text{ml}$). $[\text{Na}^+]_i$ values were calculated as described by Harootunian et al. [25].

Except for the time courses of rises in $[Na^+]_i$, shown in Figure 2, increases in $[Na^+]_i$ observed 5 min after stimulation with TG or fMLP were calculated. For studying the effect of extracellular Na^+ on rises in $[Na^+]_i$, cells were suspended in E3.

Electrophysiology

Neutrophils were transferred into a small chamber (volume 0.2 ml) which was mounted on to an inverted microscope (Zeiss, Oberkochen, Germany). Cells were patched immediately after they had settled to the bottom of the chamber, which was then perfused with E6 at 4 ml/min. Patch pipettes were prepared from glass capillaries (Jencons, Leighton Buzzard, U.K.) as described by Hamill et al. [26] and were filled with I1. The average resistance of electrodes was 5–7 M Ω . Whole-cell membrane currents were measured as described [26], with a List LM/EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). The capacitance of neutrophil membranes was 1.7 ± 0.7 pF (mean $\pm$ S.D., $n = 30$). For studying the effect of Na^+ on currents, E6 was replaced by E7. fMLP and TG were applied via the bath solutions. Currents were continuously recorded on a Sony DTR 1200 digital audio tape recorder (Science Products, Frankfurt/Main, Germany). For off-line analysis, currents were transferred on to a personal computer via an Axon AD/DA converter (Axon, Foster City, CA, U.S.A.).

Measurement of enzyme release

Exocytosis was assessed as described [27]. In brief, neutrophils $[(4.0\text{--}5.0) \times 10^6$ cells in 500 μ l] were incubated in E2 or E3 supplemented with 2 μ M dihydrocytochalasin B and various additions for 10 min at 37 $^\circ$ C before the addition of stimuli. After another 10 min, reaction mixtures were rapidly cooled to 4 $^\circ$ C and were centrifuged for 10 min at 1000 g . The activity of lysozyme in supernatants of reaction mixtures and cell lysates was determined as described [28].

Measurement of O_2^- formation

O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome c reduction inhibitable by superoxide dismutase, by using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) [21]. Neutrophils (1.0×10^6 cells in 500 μ l) were incubated in E2 supplemented with 1 mM $CaCl_2$ for 3 min at 37 $^\circ$ C before addition of stimuli. In the case of co-stimulation with TG and fMLP, TG was added to cells 3 min before fMLP.

Measurement of GTP hydrolysis in HL-60-cell membranes

Membranes from DMSO- or dibutyryl-cAMP-differentiated HL-60 cells were prepared as described by Seifert and Schultz [29]. High-affinity GTP hydrolysis in HL-60 membranes was determined with 500 nM $[\gamma\text{-}^{32}P]\text{GTP}$ (0.1 μ Ci/tube) as substrate under the conditions described by Seifert et al. [30].

RESULTS

In the presence of 1 mM extracellular Ca^{2+} , TG (100 nM) and fMLP (1 μ M) induced large rises in $[Ca^{2+}]_i$ in human neutrophils (Table 1). In the presence of 1 mM extracellular EGTA, the stimulatory effects of TG and fMLP on $[Ca^{2+}]_i$ were decreased by 80 % and 94 % respectively. These findings show that TG and fMLP increased $[Ca^{2+}]_i$ largely via Ca^{2+} influx from the extracellular space. SK&F 96365 (30 μ M) and $GdCl_3$ (10 μ M) virtually abolished the stimulatory effect of TG on Ca^{2+} influx and

Table 1 Effects of removal of extracellular Ca^{2+} , cation-entry blockers and protein phosphatase inhibitors on TG- and fMLP-induced rises in $[Ca^{2+}]_i$ in human neutrophils

Human neutrophils were loaded with fura-2/AM, and the stimulatory effects of TG (100 nM) and fMLP (1 μ M) on $[Ca^{2+}]_i$ were studied as described in the Materials and methods section. Experiments were performed in E2 supplemented with 1 mM $CaCl_2$. At 3 min before stimuli, various substances at the indicated concentrations were added to cells. In the case of addition of EGTA, no $CaCl_2$ was present in E2. Treatments with calyculin A and okadaic acid were performed as described in the Materials and methods section. Data shown are the means $\pm$ S.D. of 5–9 independent experiments performed in duplicate.

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	TG	fMLP
None (control)	309 $\pm$ 37	364 $\pm$ 97
EGTA (1 mM)	63 $\pm$ 7	23 $\pm$ 12
SK&F 96365 (30 μ M)	31 $\pm$ 29	77 $\pm$ 24
$GdCl_3$ (10 μ M)	66 $\pm$ 27	85 $\pm$ 18
Calyculin A (100 nM)	54 $\pm$ 23	30 $\pm$ 16
Okadaic acid (1 μ M)	130 $\pm$ 35	127 $\pm$ 38
CsA (1 μ M)	482 $\pm$ 56	357 $\pm$ 29
FK-506 (100 nM)	469 $\pm$ 44	355 $\pm$ 88

Figure 1 Effect of CsA on TG-induced rises in $[Ca^{2+}]_i$ in human neutrophils

Human neutrophils were loaded with fura-2/AM, and the effects of CsA on TG-induced rises in $[Ca^{2+}]_i$ were assessed as described in the Materials and methods section. (a) Time courses of TG-induced rises in $[Ca^{2+}]_i$. The arrow indicates the addition of TG (30 nM) to cells. CsA (1 μ M) (traces 2 and 4) or solvent (control) (traces 1 and 3) was added to cells 3 min before TG. Experiments were conducted in E2 supplemented with 1 mM $CaCl_2$ (traces 3 and 4) or 1 mM EGTA (traces 1 and 2). Superimposed original fluorimetric recordings from a representative experiment are shown. Similar results were obtained in 8 independent experiments. (b) Concentration–response curve for the potentiating effect of CsA on TG-induced rises in $[Ca^{2+}]_i$. Experiments were conducted in E2 supplemented with 1 mM $CaCl_2$. At 3 min before the addition of TG (30 nM), CsA at the indicated concentrations was added to cells. Data shown are the means of 5 independent experiments. The S.D. values of the data were generally less than 15 % of the means.

decreased that of fMLP by more than 80 %. The protein phosphatase 1/2A inhibitor calyculin A (100 nM) decreased the effects of TG and fMLP on $[Ca^{2+}]_i$ by 83 % and 92 % respectively. Okadaic acid (1 μ M), another inhibitor of protein phosphatases 1/2A, diminished the stimulatory effects of TG and fMLP on $[Ca^{2+}]_i$ by 58 % and 65 % respectively.

The inhibitor of protein phosphatase 2B (also referred to as calcineurin), CsA, potentiated TG-induced rises in $[Ca^{2+}]_i$ in the absence and presence of extracellular Ca^{2+} (Figure 1). The

Table 2 Effects of cation-entry blockers and protein phosphatase inhibitors on TG- and fMLP-induced Mn^{2+} influx in human neutrophils

Human neutrophils were loaded with fura-2/AM, and the stimulatory effects of TG (100 nM) and fMLP (1 μ M) on Mn^{2+} entry were studied as described in the Materials and methods section. Experiments were performed in E2 supplemented with 30 μ M $MnCl_2$. At 3 min before addition of TG or fMLP (stimulated influx) or water (basal influx), various substances at the indicated concentrations were added to cells. Treatments with calyculin A and okadaic acid were performed as described in the Materials and methods section. Data shown are the means $\pm$ S.D. of 6–8 independent experiments performed in duplicate; N.D., not determined.

Addition	Mn^{2+} influx (stimulated/basal influx)	
	TG	fMLP
None (control)	3.21 $\pm$ 0.37	2.10 $\pm$ 0.37
SK&F 96365 (30 μ M)	1.00 $\pm$ 0.02	1.00 $\pm$ 0.04
GdCl ₃ (10 μ M)	2.42 $\pm$ 0.16	1.00 $\pm$ 0.02
Calyculin A (100 nM)	2.43 $\pm$ 0.27	1.60 $\pm$ 0.47
Okadaic acid (1 μ M)	2.42 $\pm$ 0.36	1.56 $\pm$ 0.19
CsA (1 μ M)	3.63 $\pm$ 0.63	N.D.

Table 3 Effect of PTX on TG- and fMLP-induced rises in $[Ca^{2+}]_i$ and Mn^{2+} influx in human neutrophils

Human neutrophils were treated with the B-oligomer of PTX (control) or PTX and were loaded with fura-2/AM as described in the Materials and methods section. The stimulatory effects of TG (100 nM) and fMLP (1 μ M) on rises in $[Ca^{2+}]_i$ were assessed in E2 supplemented with 1 mM $CaCl_2$. In addition, the effects of TG (30 nM) and fMLP (1 μ M) on Mn^{2+} entry were studied. These experiments were performed in E2 supplemented with 30 μ M $MnCl_2$. Data shown are the means $\pm$ S.D. of 5 independent experiments performed in duplicate.

Treatment	Increase in $[Ca^{2+}]_i$ (nM)		Mn^{2+} influx (stimulated/basal influx)	
	TG	fMLP	TG	fMLP
B-oligomer	298 $\pm$ 47	195 $\pm$ 43	3.00 $\pm$ 0.51	2.45 $\pm$ 0.21
PTX	146 $\pm$ 40	11 $\pm$ 14	2.16 $\pm$ 0.52	1.08 $\pm$ 0.15

potentiating effect of CsA on TG-induced rises in $[Ca^{2+}]_i$ was half-maximal and maximal at 9 nM and 100 nM respectively. CsA (1 μ M) enhanced the stimulatory effect of TG on $[Ca^{2+}]_i$ by 56 % (see Table 1). CsD enhanced TG-induced rises in $[Ca^{2+}]_i$ with an EC_{50} of 65 nM and a maximal effectiveness comparable with that of CsA, whereas CsH up to 1 μ M was ineffective (results not shown). FK-506, like CsA, is a potent and effective inhibitor of protein phosphatase 2B, and potentiated TG-induced rises in $[Ca^{2+}]_i$ with an EC_{50} of 5 nM and a maximal effect at 100 nM. At a maximally stimulatory concentration, FK-506 was similarly effective as CsA (see Table 1). In contrast with TG, CsA and FK-506 were without effect on fMLP-induced rises in $[Ca^{2+}]_i$ (see Table 1) [27,31,32].

TG and fMLP also activated Mn^{2+} entry in human neutrophils (Table 2). SK&F 96365 (30 μ M) abolished the stimulatory effects of TG (100 nM) and fMLP (1 μ M) on Mn^{2+} influx. GdCl₃ (10 μ M) completely inhibited the effect of fMLP, whereas the lanthanide decreased the TG-induced Mn^{2+} entry only by 36 %. Calyculin A (100 nM) and okadaic acid (1 μ M) diminished TG-induced Mn^{2+} entry by 35–36 %. The decrease by the phosphatase inhibitors of fMLP-induced Mn^{2+} influx amounted to 45–49 %. CsA (1 μ M) enhanced TG-induced Mn^{2+} entry by 19 %.

To address the question whether G_i -proteins are involved in TG-induced activation of cation entry, we compared the effects

Figure 2 Time courses of TG- and fMLP-induced rises in $[Na^+]_i$ in human neutrophils

Human neutrophils were loaded with SBFI/AM, and the effects of TG (300 nM) (■) and fMLP (30 nM) (●) on $[Na^+]_i$ were assessed as described in the Materials and methods section. Experiments were performed in the presence of E2. The arrow indicates the addition of stimuli to cells. Data shown are the means $\pm$ S.D. of 8–10 independent experiments.

of PTX and its B-oligomer, i.e. PTX devoid of the enzymically active A-promoter, on TG- and fMLP-induced rises in $[Ca^{2+}]_i$ and Mn^{2+} influx. Compared with the B-oligomer, PTX decreased the stimulatory effects of fMLP (1 μ M) on $[Ca^{2+}]_i$ and Mn^{2+} entry by 94 % (Table 3). These findings support the notion that G_i -proteins are involved in the signal-transduction pathway activated by fMLP [30,33]. PTX decreased the stimulatory effect of TG (100 nM) on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} by 51 %. The TG-induced Ca^{2+} mobilization was also diminished by 50 % (results not shown). PTX decreased the stimulatory effect of TG on Mn^{2+} entry by 42 % (see Table 3).

In HL-60 membranes, fMLP induces a large PTX-sensitive stimulation of high-affinity GTP hydrolysis, reflecting the enzymic activity of G_i -protein α -subunits [30]. In contrast, we could not detect any stimulatory effect of TG (0.01–10 μ M) on GTPase in membranes of dibutyryl-cAMP- and DMSO-differentiated HL-60 cells (results not shown).

In addition to Mn^{2+} , Ba^{2+} and Sr^{2+} were employed as substitutes for Ca^{2+} in previous studies on cation entry mechanisms [24,34,35]. TG (100 nM) and fMLP (1 μ M) increased $[Ba^{2+}]_i$ by 130 $\pm$ 74 nM and 616 $\pm$ 212 nM (means $\pm$ S.D., $n = 13$) respectively. The corresponding values for rises in $[Sr^{2+}]_i$ were 3391 $\pm$ 889 and 2092 $\pm$ 978 nM (means $\pm$ S.D., $n = 8$) respectively. SK&F 96365 (30 μ M) and GdCl₃ almost completely or completely inhibited the stimulatory effects of TG and fMLP on Ba^{2+} and Sr^{2+} entry in human neutrophils (results not shown).

To clarify whether TG and fMLP also stimulate the entry of univalent cations into neutrophils, we studied their effects on $[Na^+]_i$, using the Na^+ -sensitive dye SBFI. After exposure to TG (300 nM) or fMLP (30 nM), $[Na^+]_i$ steadily increased to a plateau which was reached after approx. 4.5 min (Figure 2). Removal of extracellular Na^+ (E3) resulted in complete inhibition of TG- and fMLP-induced rises in $[Na^+]_i$ (results not shown), indicating that Na^+ entered the cytosol by influx from the extracellular space. SK&F 96365 (30 μ M) decreased the stimulatory effects of TG (300 nM) and fMLP (30 nM) on $[Na^+]_i$ by 93 % and 82 % respectively (Table 4). The corresponding values for GdCl₃ (10 μ M) were 55 % and 69 % respectively. Chloride salts of various bivalent cations (1 mM each) differentially inhibited TG- and fMLP-induced rises in $[Na^+]_i$. The most prominent dif-

Table 4 Effects of SK&F 96365, GdCl₃, chloride salts of bivalent cations and CsA on TG- and fMLP-induced rises in [Na⁺]_i in human neutrophils

Human neutrophils were loaded with SBFI/AM, and the stimulatory effects of TG (300 nM) and fMLP (1 μ M) on [Na⁺]_i were studied as described in the Materials and methods section. Experiments were performed in E2. At 3 min before stimuli, various substances at the indicated concentrations were added to cells. Data shown are the means $\pm$ S.D. of 3–5 independent experiments performed in triplicate: ND, not determined.

Addition	Increase in [Na ⁺] _i (mM)	
	TG	fMLP
None (control)	5.8 $\pm$ 2.5	8.5 $\pm$ 3.2
SK&F 96365 (30 μ M)	0.4 $\pm$ 0.2	1.5 $\pm$ 1.1
GdCl ₃ (10 μ M)	2.7 $\pm$ 0.4	2.6 $\pm$ 1.6
CaCl ₂ (1 mM)	1.3 $\pm$ 1.7	5.9 $\pm$ 1.7
MnCl ₂ (1 mM)	0.3 $\pm$ 0.1	7.1 $\pm$ 0.9
BaCl ₂ (1 mM)	3.1 $\pm$ 1.6	5.2 $\pm$ 2.5
SrCl ₂ (1 mM)	3.3 $\pm$ 2.6	3.3 $\pm$ 2.1
CsA (1 μ M)	7.4 $\pm$ 1.5	ND

ferences are apparent when CaCl₂ and MnCl₂ are considered. Specifically, CaCl₂ decreased the stimulatory effect of TG by 78 %, whereas that of fMLP was diminished by only 31 %. The inhibition of TG-stimulated Na⁺ entry by MnCl₂ amounted to 95 %, but only 16 % for fMLP. CsA (1 μ M) enhanced TG-induced Na⁺ entry by 28 %.

We also employed an electrophysiological approach to study cation entry in neutrophils. In the whole-cell configuration, fMLP (100 nM) activated inward currents with a lag time of 13 $\pm$ 7 s (mean $\pm$ S.D.; 5 out of 6 cells were responders). In the presence of extracellular Na⁺ (E6), fMLP activated an inward current, which was largely decreased by removal of extracellular Na⁺ (E7) and was re-activated by re-addition of Na⁺ (E6) (Figure 3a). These findings indicate that Na⁺ is an important charge carrier for the fMLP-activated current and confirm the data obtained with SBFI (see Figure 3 and Table 4). The current–voltage relationship under fMLP (100 nM) measured during ramp pulses from -100 mV to $+50$ mV was linear and showed apparent reversal potentials at about 0 mV (Figure 3b and Table 5). Removal of extracellular Na⁺ (E7) decreased the fMLP-induced current by 70 % and shifted the reversal potential by -21 mV. TG (1 μ M) stimulated inward currents with a lag time of 48 $\pm$ 33 s (mean $\pm$ S.D.; 8 out of 11 cells were responders). Removal of extracellular Na⁺ (E7) decreased the TG-induced inward current by 21 % and shifted the linear current–voltage relationship by -12 % (Figure 3c, Table 5).

In order to assess the functional role of TG- and fMLP-stimulated cation entry, we studied their effects on exocytosis and O₂⁻ formation. Both TG and fMLP (1 μ M each) stimulated lysozyme release, but the chemotactic peptide was considerably more effective than the Ca²⁺-ATPase inhibitor (Table 6). PTX treatment abolished fMLP-induced enzyme release and diminished TG-stimulated exocytosis by 72 % (results not shown). SK&F 96365 (30 μ M) inhibited the stimulatory effects of TG and fMLP on lysozyme release by 50 % and 41 % respectively (see Table 6). Removal of extracellular Ca²⁺ decreased TG- and fMLP-stimulated lysozyme release by 95 % and 61 % respectively. Removal of extracellular Na⁺ (E3) decreased the stimulatory effect of TG by 45 %, and that of fMLP by 62 %. TG (1 nM–1 μ M) itself did not activate O₂⁻ formation, whereas fMLP was stimulatory (Figure 4). fMLP activated O₂⁻ formation with an EC₅₀ of 30 nM and a plateau at 0.3–1 μ M. TG (1 μ M)

Figure 3 TG- and fMLP-induced whole-cell inward currents in human neutrophils

Inward currents in human neutrophils were determined as described in the Materials and methods section. (a) Time course of fMLP-induced inward current. The time course of the fMLP-induced inward current was continuously recorded at a holding potential of -60 mV in Na⁺-containing buffer (E6). Application times of fMLP (100 nM) and Na⁺-free bath solution (0 Na⁺) (E7) are indicated by horizontal bars. The zero-current level is indicated by the dotted line. At the times indicated by numbers, current–voltage relationships were measured. (b) Current–voltage relationships were measured during 200 ms lasting voltage-ramp pulses from a holding potential of -60 mV at the times indicated by numbers in (a). Currents in the absence of fMLP (1, 2) were subtracted from fMLP-stimulated currents (3, 4). Currents were determined in the presence of E6 (con) (current 3—current 2) or E7 (0 Na⁺) (current 4—current 1). (c) Current–voltage relationships were measured during 100 ms lasting voltage-ramp pulses from a holding potential of -60 mV. TG (1 μ M) was applied via the bath solution. Currents measured under E6 or E7 before activation by TG were subtracted from stimulated currents. In (a–c), original tracings from a single experiment are shown. Similar results were obtained in 5–8 independent experiments.

potentiated the effect of fMLP at sub-maximally and maximally stimulatory concentrations and increased the potency of the chemotactic peptide (EC₅₀ = 10 nM) (see Figure 4). Moreover, TG rendered cells responsive to fMLP at sub-threshold concentrations (3–10 nM). In the presence of fMLP at a sub-maximally effective concentration (50 nM), TG enhanced O₂⁻ formation up to 2-fold with an EC₅₀ of 30 nM (see Figure 4).

Table 5 Effects of various extracellular ionic conditions on TG- and fMLP-stimulated inward-current conductances and reversal potentials in human neutrophils

Conductances and reversal potentials of inward currents in human neutrophils activated by TG (1 μ M) or fMLP (100 nM) under various ionic conditions were determined. Experiments were performed in Na⁺-containing buffer (E6) or Na⁺-free buffer (E7). Currents were recorded in the whole-cell patch-clamp configuration at a holding potential of -60 mV before and during activation, and the difference current amplitude (I_{-60}) was then referred to the respective cell's membrane capacitance. The reversal potentials (E_{rev}) of the currents were measured during 100 ms-lasting (TG) or 200 ms-lasting (fMLP) linear ramp pulses from -100 mV to +50 mV. Data shown are the means $\pm$ S.D. of 5–8 independent experiments.

Extracellular solution	TG		fMLP	
	I_{-60} (pA/pF)	E_{rev} (mV)	I_{-60} (pA/pF)	E_{rev} (mV)
E6 (+Na ⁺)	112 $\pm$ 85	+3 $\pm$ 8	73 $\pm$ 66	-2 $\pm$ 3
E7 (-Na ⁺)	77 $\pm$ 63	-9 $\pm$ 9	22 $\pm$ 16	-23 $\pm$ 14

Table 6 Activation of lysozyme release by TG and fMLP in human neutrophils: effects of various ionic conditions and SK&F 96365

The stimulatory effects of TG and fMLP (1 μ M each) on lysozyme release were determined as described in the Materials and methods section. Experiments were performed in the presence (E2) or absence (E3) of extracellular Na⁺. The concentration of EGTA and CaCl₂ was 1 mM each and the concentration of SK&F 96365 was 30 μ M. Data shown are the means $\pm$ S.D. of assay triplicates. Similar results were obtained in 4 independent experiments.

Extracellular solution	Stimulated lysozyme release (% of cellular content)	
	TG	fMLP
E2 (+Na ⁺) + CaCl ₂	21.1 $\pm$ 1.0	52.9 $\pm$ 4.8
E2 (+Na ⁺) + CaCl ₂ + SK&F 96365	10.6 $\pm$ 0.8	30.7 $\pm$ 1.9
E2 (+Na ⁺) + EGTA	1.1 $\pm$ 0.3	20.6 $\pm$ 2.5
E3 (-Na ⁺) + CaCl ₂	11.6 $\pm$ 0.9	20.1 $\pm$ 1.7

DISCUSSION

In accordance with results obtained by other groups [6–8,10,12,17], we found that TG activates Ca²⁺ and Mn²⁺ entry in human neutrophils (see Tables 1 and 2). The TG-activated cation-entry pathway is also permeable to other bivalent cations, in particular Sr²⁺, and to the univalent cation, Na⁺ (see Figures 3 and 4 and Tables 4 and 5). In addition, the current-voltage relationship of the TG-stimulated inward current is linear and has a reversal potential of approx. 0 mV (see Figure 3 and Table 5). Thus, in terms of ion permeability and reversal potential, the TG-activated cation-entry pathway of neutrophils behaves like a non-selective cation channel. Another property of the TG-stimulated cation-entry mechanism is its sensitivity to blockade by imidazoles and lanthanides (see Tables 1, 2 and 4) [6–8].

The mechanism by which TG activates cation entry is still obscure. One current hypothesis states that protein phosphatases 1/2A are involved in the signal-transduction pathway. Support in favour of this hypothesis comes from the finding that the inhibitors of protein phosphatases 1/2A, calyculin A and okadaic acid, inhibit TG-stimulated Ca²⁺ and Mn²⁺ entry (see Tables 1 and 2) [12]. Similarly to its effect in neutrophils, okadaic acid inhibits TG-induced Ca²⁺ influx in HeLa S3 cells [11]. In contrast, okadaic acid potentiates TG-induced Ca²⁺ influx in 132N1 astrocytoma cells [13]. These data show that the regulation of TG-activated cation entry by protein phosphatases 1/2A is cell-

Figure 4 Effects of TG and fMLP on O₂⁻ formation in human neutrophils

O₂⁻ formation was assessed in human neutrophils as described in the Materials and methods section. (a) Concentration-response curves for fMLP in the absence of TG (control) (●) or in the presence of TG (100 nM) (■). The black triangle designates that, under these experimental conditions, no O₂⁻ was generated. TG was added to cells 3 min before fMLP. (b) Concentration-response curves for TG in the absence of fMLP (control) (●) or in the presence of fMLP (50 nM) (■). In co-stimulation experiments, TG was added to cells 3 min before fMLP. Data shown are the means of assay triplicates. The S.D. values of the data were generally less than 15% of the means. Similar results were obtained in 5 independent experiments.

type-specific. The molecular target of action of protein phosphatases 1/2A may be a putative low-molecular-mass calcium-influx factor, a regulatory protein involved in cation entry, or an ion channel itself.

Protein phosphatase 2B has been implicated in the regulation of TG-stimulated cation entry as well. This assumption is based on the finding that CsA, which inhibits this phosphatase via cyclophilin [36], potentiates TG-stimulated cation entry in 132N1 astrocytoma cells [13]. Similarly, we found CsA to enhance TG-stimulated Ca²⁺, Mn²⁺ and Na⁺ entry in human neutrophils (see Figure 1 and Tables 2 and 4). The potency of CsA to potentiate TG-induced rises in [Ca²⁺]_i in neutrophils correlates well with its affinity to cyclophilin [36]. The effects of CsD and CsH on TG-induced increases in [Ca²⁺]_i are also in agreement with their affinities to cyclophilin [37]. Moreover, FK-506, which inhibits protein phosphatase 2B via FK-506-binding protein [36], is a potent enhancer of TG-induced rises in [Ca²⁺]_i in human neutrophils. Collectively, these data point to a role of protein phosphatase 2B in the regulation of TG-activated cation entry in human neutrophils. In this system, protein phosphatases 1/2A and 2B play opposite roles in the regulation of TG-stimulated cation entry. Intriguingly, CsA potentiated not only TG-induced cation entry but also Ca²⁺ mobilization (see Figure 1). These findings may indicate that protein phosphatase 2B is involved in Ca²⁺-ATPase regulation. In fact, there is evidence for regulation by phosphorylation of Ca²⁺-ATPases [38]. Thus, as Ca²⁺-store depletion is a prerequisite for activation of Ca²⁺ entry by TG [1–5], CsA may act at the level of the intracellular Ca²⁺ stores.

G-proteins have also been suggested to play a part in TG-mediated cation entry in HL-60 cells and hepatocytes [9,10]. Since G_i-proteins are the most abundant G-proteins in neutrophils and HL-60 cells, and because they can be functionally impaired by PTX [39,40], we studied the effect of this toxin on cation entry. PTX partially decreased the stimulatory effect of TG (see Table 3). Thus our findings imply that G_i-proteins are involved in TG-induced Ca²⁺ entry in human neutrophils. The

question of whether the PTX-insensitive portion of TG-stimulated cation entry is attributable to a PTX-insensitive G-protein or a G-protein-independent mechanism remains unanswered. In agreement with our data, inhibitory effects of PTX on TG-stimulated Ca^{2+} entry have been reported for rat hepatocytes [9]. Jaconi et al. [10] did not study the effect of PTX on TG-stimulated Ca^{2+} in HL-60 cells. Interestingly, PTX not only inhibited Ca^{2+} entry, but also suppressed Ca^{2+} mobilization from intracellular stores in human neutrophils, indicating a role of G_i -proteins in Ca^{2+} -ATPase regulation. In fact, G_i -protein α -subunits are localized not only in the neutrophil plasma membrane, but also in neutrophil cytosol and in intracellular compartments [41,42].

In this context, the question arises of whether TG is a direct activator of G_i -proteins. TG is a lipophilic substance, may affect the function of membrane proteins and must cross the plasma membrane before reaching the Ca^{2+} -ATPase of intracellular Ca^{2+} stores [1,2,43,44]. G-proteins are also plasma-membrane-attached, and various lipophilic substances directly activate G_i -proteins in HL-60 membranes [30,45–47]. However, TG did not activate high-affinity GTP hydrolysis in HL-60 membranes. These findings argue against TG being a direct G_i -protein activator. Accordingly, the involvement of G_i -proteins in TG-mediated activation of Ca^{2+} entry may be indirect. One could envisage that, in order to be in a TG-activatable state, the store-operated cation entry pathway requires basal G_i -protein activity, which is impaired by PTX. In agreement with such an hypothesis is the fact that the basal GTPase activity of G_i -proteins in membranes of neutrophils and HL-60 cells is decreased by PTX [45,46,48].

Like TG, fMLP activates a SK&F 96365- and lanthanide-sensitive non-selective cation-entry pathway in human neutrophils and HL-60 cells (see Tables 1, 2 and 4) [6,7,18,33,49,50]. There are, however, a number of differences between TG and fMLP. For example, protein phosphatase 2B does not play a crucial role in the fMLP-activated signal-transduction pathway, as CsA and FK-506 are without effect on formyl-peptide-stimulated rises in $[\text{Ca}^{2+}]_i$ (see Table 1) [27,31,32]. Additionally, Gd^{3+} (10 μM) abolished the stimulatory effect of fMLP on Mn^{2+} entry, but only partially inhibited the effect of TG (see Table 2). In addition, fMLP and TG were differentially effective in stimulating the influx of various cations, in particular Ba^{2+} . Moreover, various bivalent cations differently inhibited TG- and fMLP-induced Na^+ entry (see Table 4). These findings show that the TG- and fMLP-activated cation-entry pathways in neutrophils underly differential regulation. Evidence in favour of heterogeneity of cation-entry pathways was also obtained for HL-60 cells [18,19,33]. Currently, we cannot answer the question of whether the differential regulation of cation-entry pathways by TG and fMLP is attributable to differential regulation of a single type of ion channel or to differential activation of various types of ion channels.

In order to assess the functional role of TG-induced cation entry in neutrophils, we studied the effects of TG and fMLP on lysozyme release and O_2^- formation. fMLP is a full secretagogue, i.e. it activates both exocytosis and O_2^- formation (see Figure 4 and Table 6) [33,39,40]. TG was a less effective activator of exocytosis than fMLP, and failed to activate O_2^- formation. Thus TG is a partial secretagogue. Similar to other partial secretagogues, i.e. platelet-activating factor and leukotriene B_4 [40], TG was an effective potentiator of fMLP-stimulated O_2^- formation (see Figure 4). Our findings imply that cation entry is not a sufficient signal for activation of O_2^- formation and maximal activation of exocytosis, but rather an optional feed-forward signal.

The fact that TG activated lysozyme release allowed us to study the role of cation entry in this process by adding SK&F 96365 and by the removal of extracellular Ca^{2+} or Na^+ . In a previous study we showed that fMLP-activated exocytosis in dibutylryl-cAMP-differentiated HL-60 cells depends on Ca^{2+} and Na^+ entry [33]. In agreement with the results of the latter study, SK&F 96365 and the removal of extracellular Ca^{2+} and Na^+ inhibited TG- and fMLP-induced lysozyme release in human neutrophils (see Table 6). Moreover, as is true for cation entry (see Table 2), TG- and fMLP-stimulated exocytosis was PTX-sensitive. Ca^{2+} entry plays a key role in the activation of exocytosis by TG, as its removal from the extracellular medium almost completely abolished lysozyme release. Compared with TG, fMLP-stimulated lysozyme release was less dependent on Ca^{2+} entry, and the chemotactic peptide was a more effective stimulus than the Ca^{2+} -ATPase inhibitor (see Table 6) [51]. These findings corroborate the assumption that, in addition to Ca^{2+} entry, additional signals are needed for maximal activation for lysozyme release [52]. Na^+ entry is also required for exocytosis, as omission of this cation partially decreased the stimulatory effects of TG and fMLP. The mechanisms by which Na^+ entry could lead to activation of exocytosis were discussed previously [33]. In view of the fact that SK&F 96365 completely inhibited Ca^{2+} entry and that removal of extracellular Ca^{2+} almost completely inhibited TG-stimulated exocytosis, we expected SK&F 96365 to exhibit a very large inhibitory effect on lysozyme release. Most intriguingly, this was not the case (see Table 6). This dissociation of the effects of SK&F 96365 on Ca^{2+} entry and exocytosis may thus indicate that TG activates an additional, as yet unknown, signal-transduction pathway which is important for activation of exocytosis.

We express gratitude to Mrs. E. Glass for expert technical assistance and to Dr. D. Daunt, Dr. A. Lückhoff and Dr. G. Schultz for critical reading of the manuscript. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie and the Fonds der Chemischen Industrie.

REFERENCES

- 1 Thastrup, O. (1990) *Agents Actions* **29**, 8–15
- 2 Putney, J. W., Jr. and Bird, G. S. J. (1993) *Cell* **75**, 199–201
- 3 Penner, R., Fasolato, C. and Hoth, M. (1993) *Curr. Opin. Neurobiol.* **3**, 368–374
- 4 Felder, C. C., Singer-Lahat, D. and Mathes, C. (1994) *Biochem. Pharmacol.* **48**, 1997–2004
- 5 Fasolato, C., Innocenti, B. and Pozzan, T. (1994) *Trends Pharmacol. Sci.* **15**, 77–83
- 6 Demaurex, N., Lew, D. P. and Krause, K.-H. (1992) *J. Biol. Chem.* **267**, 2318–2324
- 7 Demaurex, N., Monod, A., Lew, D. P. and Krause, K.-H. (1994) *Biochem. J.* **297**, 595–601
- 8 Koch, M., Faurot, G. F., Kopanitsa, M. V. and Swinney, D. C. (1994) *Biochem. J.* **302**, 187–190
- 9 Fernando, K. C. and Barritt, G. J. (1994) *Biochem. J.* **303**, 351–356
- 10 Jaconi, M. E. E., Lew, D. P., Monod, A. and Krause, K.-H. (1993) *J. Biol. Chem.* **268**, 26075–26078
- 11 Berlin, R. D. and Preston, S. F. (1993) *Cell Calcium* **14**, 379–386
- 12 Montero, M., Garcia-Sancho, J. and Alvarez, J. (1994) *J. Biol. Chem.* **269**, 3963–3967
- 13 Randriamampita, C. and Tsien, R. Y. (1995) *J. Biol. Chem.* **270**, 29–32
- 14 Thomas, D. and Hanley, M. R. (1995) *J. Biol. Chem.* **270**, 6429–6432
- 15 Gilon, P., Bird, G. S. J., Bian, X., Yakel, J. L. and Putney, J. W., Jr. (1995) *J. Biol. Chem.* **270**, 8050–8055
- 16 Clapham, D. E. (1995) *Nature (London)* **375**, 634–635
- 17 Geiszt, M., Kaldi, K., Szeberenyi, J. B. and Ligeti, E. (1995) *Biochem. J.* **305**, 525–528
- 18 Musgrave, I., Seifert, R. and Schultz, G. (1994) *Biochem. J.* **301**, 437–441
- 19 Montero, M., Garcia-Sancho, J. and Alvarez, J. (1994) *J. Biol. Chem.* **269**, 29451–29456

- 20 Montero, M., Garcia-Sancho, J. and Alvarez, J. (1994) *Biochem. J.* **303**, 559–566
- 21 Seifert, R., Burde, R. and Schultz, G. (1989) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **306**, 101–106
- 22 Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- 23 Merritt, J. E., Jacob, R. and Hallam, T. J. (1989) *J. Biol. Chem.* **264**, 1522–1527
- 24 Kwan, C.-Y. and Putney, J. W., Jr. (1990) *J. Biol. Chem.* **265**, 678–684
- 25 Harootunian, A. T., Kao, J. P. Y., Eckert, B. K. and Tsien, R. Y. (1989) *J. Biol. Chem.* **264**, 19458–19467
- 26 Hamill, O. P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. J. (1981) *Pflüger's Arch.* **391**, 85–100
- 27 Wenzel-Seifert, K., Grünbaum, L. and Seifert, R. (1991) *J. Immunol.* **147**, 1940–1946
- 28 Absalom, D. R. (1986) *Methods Enzymol.* **132**, 95–180
- 29 Seifert, R. and Schultz, G. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1296–1302
- 30 Seifert, R., Hagelüken, A., Höer, A., Höer, D., Grünbaum, L., Offermanns, S., Schwaner, I., Zingel, V., Schunack, W. and Schultz, G. (1994) *Mol. Pharmacol.* **45**, 578–586
- 31 Wenzel-Seifert, K. and Seifert, R. (1993) *J. Immunol.* **150**, 4591–4599
- 32 Wenzel-Seifert, K. and Seifert, R. (1993) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **348**, 7–13
- 33 Krautwurst, D., Seifert, R., Hescheler, J. and Schultz, G. (1992) *Biochem. J.* **288**, 1025–1035
- 34 Schilling, W. P., Rajan, L. and Strobl-Jäger, E. (1989) *J. Biol. Chem.* **264**, 12838–12848
- 35 Hu, Y., Vaca, L., Zhu, X., Birnbaumer, L., Kunze, D. L. and Schilling, W. P. (1994) *Biochem. Biophys. Res. Commun.* **201**, 1050–1056
- 36 Fruman, D. A., Burakoff, S. J. and Bierer, B. E. (1994) *FASEB J.* **8**, 391–400
- 37 Handschumacher, R. E., Harding, M. W., Rice, J. and Drugge, R. J. (1984) *Science* **226**, 544–547
- 38 Carafoli, E. (1992) *J. Biol. Chem.* **267**, 2115–2118
- 39 Dillon, S. B., Verghese, M. W. and Snyderman, R. (1988) *Virchows Arch. B* **55**, 65–80
- 40 Seifert, R. and Schultz, G. (1991) *Rev. Physiol. Biochem. Pharmacol.* **117**, 1–338
- 41 Rotrosen, D., Gallin, J. I., Spiegel, A. M. and Malech, H. L. (1988) *J. Biol. Chem.* **263**, 10958–10959
- 42 Rudolph, U., Koesling, D., Hinsch, K.-D., Seifert, R., Bigalke, M., Schultz, G. and Rosenthal, W. (1989) *Mol. Cell. Endocrinol.* **63**, 143–153
- 43 Nelson, E. J., Li, C. C. R., Bangalore, R., Benson, T., Kass, R. S. and Hinkle, P. M. (1994) *Biochem. J.* **302**, 147–154
- 44 Buryi, V., Morel, N., Salomone, S., Kerger, S. and Godfraind, T. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **351**, 40–45
- 45 Hagelüken, A., Grünbaum, L., Nürnberg, B., Harhammer, R., Schunack, W. and Seifert, R. (1994) *Biochem. Pharmacol.* **47**, 1789–1795
- 46 Hagelüken, A., Grünbaum, L., Klinker, J. F., Nürnberg, B., Harhammer, R., Schultz, G., Leschke, C., Schunack, W. and Seifert, R. (1995) *Biochem. Pharmacol.* **49**, 901–914
- 47 Hagelüken, A., Nürnberg, B., Harhammer, B., Grünbaum, L., Schunack, W. and Seifert, R. (1995) *Mol. Pharmacol.* **47**, 234–240
- 48 Feltner, D. E., Smith, R. H. and Marasco, W. A. (1986) *J. Immunol.* **137**, 1961–1970
- 49 Krautwurst, D., Hescheler, J., Arndts, D., Lösel, W., Hammer, R. and Schultz, G. (1993) *Mol. Pharmacol.* **43**, 655–659
- 50 Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. and Rink, T. J. (1990) *Biochem. J.* **271**, 515–522
- 51 Smolen, J. E., Korchak, H. M. and Weissmann, G. (1981) *Biochim. Biophys. Acta* **677**, 512–520
- 52 Packham, D. E., Jiang, L. and Conigrave, A. D. (1995) *Cell Calcium* **17**, 399–408