The Regulation of Store-dependent Ca\(^{2+}\) Influx in HL-60 Granulocytes Involves GTP-sensitive Elements\(^*\)  

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In granulocytes, emptying of intracellular Ca\(^{2+}\) stores activates Ca\(^{2+}\) influx across the plasma membrane. To study the putative role of GTP-binding proteins in this process, we have introduced non-hydrolyzable guanosine phosphate analogues into the cytosol of non-permeabilized HL-60 granulocytes using an endocytosis-hypoosmotic shock procedure. At the cystolic concentrations obtained (100–500 \(\mu\)M), neither guanosine 5'-3-O-(thio)triphosphate (GTPyS) nor guanosine 5'-3-O-(thio)diphosphate (GDPPS) affected basal [Ca\(^{2+}\)]i. Ca\(^{2+}\) release in response to the receptor agonist fMet-Leu-Phe, the Ca\(^{2+}\)-ATPase inhibitor thapsigargin, or the Ca\(^{2+}\) ionophore ionomycin was also unaffected by GTPyS or GDPPS. In contrast, the activation of the Ca\(^{2+}\) influx pathway by fMet-Leu-Phe or thapsigargin was blocked by GTPyS but not by GDPPS. The GTPyS effect was mimicked by NaF. The GTPyS and NaF effects were independent of protein kinase C activation and actin polymerization. Our results demonstrate that a GTP-sensitive element is involved in the signaling between intracellular Ca\(^{2+}\) stores and plasma membrane Ca\(^{2+}\) channels. The identical effects of GTPyS and NaF suggest that the GTP-sensitive element is a heterotrimeric G-protein.

Very little is known about the biochemical basis of the signaling between intracellular Ca\(^{2+}\) stores and plasma membrane Ca\(^{2+}\) channels. The involvement of soluble messengers (6–8), protein phosphatases (6, 9), or cytoskeletal elements (10), or a direct interaction between proteins from Ca\(^{2+}\) stores and the plasma membrane (11) has been suggested. GTP-binding proteins (G-proteins)\(^*\) appear to be ideal candidates to participate in the regulation of store-dependent Ca\(^{2+}\) influx. They have been implicated in the regulation of ion channels and also in the regulation of communication between intracellular organelles (for recent reviews see Refs. 12–16). Previous studies have reported inhibition (17) or stimulation (18) of Ca\(^{2+}\) influx by cholina toxin.

This study was designed to test the involvement of G-proteins in the mediation of store-dependent Ca\(^{2+}\) influx in granulocytes. Our results demonstrate that cytosolic GTPyS but not GDPPS blocks the activation of store-dependent Ca\(^{2+}\) influx. The GTPyS effect is mimicked by fluoride. Our results suggest the involvement of GTP-sensitive elements, most likely a trimeric G-protein, in the regulation of store-dependent Ca\(^{2+}\) influx.

EXPERIMENTAL PROCEDURES  

Materials and Buffers—fMLP, thapsigargin, Me\(_2\)SO, HEPES, and DTPA were purchased from Sigma and fura-2/AM from Molecular Probes (Eugene, OR). RPMI 1640 culture medium and fetal calf serum were purchased from Gibco (Paisley, Scotland, UK). Other chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland) or Sigma. When drugs were added as Me\(_2\)SO solutions, the final concentration of Me\(_2\)SO in the recording medium did not exceed 0.25%. Experiments were performed in a medium containing (in mM): NaCl, 138; KCl, 6; MgCl\(_2\), 1; glucose, 20; HEPES, 20; pH 7.4.

Culture of HL-60 Cells—HL-60 promyelocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and induced to differentiate to granulocytes by addition of 1.3% Me\(_2\)SO (v/v) for 7 days to the culture medium, as described previously (19).

Endocytosis-Osmotic Shock Procedure—The procedure applied in this study is a slight modification of the original procedure described by Rechsteiner (20). 40 × 10\(^4\) HL-60 granulocytes were incubated in 250 \(\mu\)l of a buffer containing 143 mM NaCl, 6 mM KCl, 1 mM Mg\(_2\)SO\(_4\), 20 mM HEPES pH 7.4, 0.1% glucose, 275 mM sucrose, 7.5% polyethylene glycol 1000, 7.5% fetal calf serum. Where indicated, the solutions also contained 50 mM GTPyS or 50 mM GDPPS. The cells were incubated for 15 min at 25°C to allow fluid-phase endocytosis of extracellular material. To induce hypoxosmosis lysis of endosomes, 4 ml of H\(_2\)O was added, and cells were incubated under hypoxic conditions for 60 s. Isoosmolarity was restored by addition of 3.5 ml of 1.8% NaCl. To quantify the efficacy of the endocytosis-hypoosmotic shock procedure, we introduced Lucifer yellow (10 \(\mu\)g/ml) or \([\text{H}]\)mannose (10 x 10\(^3\) cpm/ml) into the cytosol by the same method. After three washes, the cytosolic content of lucifer yellow or \([\text{H}]\)mannose was measured as the amount of the respective compound that could be released by 20 \(\mu\)M digitonin (21). Assuming a cytosolic volume of 0.5 pl/cell (22), the cytosolic concentrations were estimated as 2 ± 1 \(\mu\)g/ml for Lucifer yellow and 0.1 ± 0.02 x 10\(^3\) cpm/ml for \([\text{H}]\)mannose, corresponding to 0.2 ± 0.1 and 1.0 ± 0.2%, respectively, of the extracellular concentration present during the endocytosis-hypoosmotic shock procedure. Inspection of lucifer yellow-loaded granulocytes by fluorescence microscopy showed that approximately 90% of the cells had a homogeneous fluorescence, suggesting a

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The abbreviations used are: G-protein, GTP-binding protein; Ca\(^{2+}\), cytosolic free Ca\(^{2+}\) concentration; Me\(_2\)SO, dimethyl sulfoxide; fura-2/AM, fura-2 acetoxyethyl ester; DTPA, N\(_2\),N\(_3\)-dibis(2-iminooethyl)amine; PMA, phorbol 12-myristate 13-acetate.
The cytosolic concentrations of the compound of interest obtained with the method were between 0.2 and 1% of the conservation of store-dependent Ca\(^{2+}\) influx, we have introduced non-procedure. The GTPyS and GDPPS concentrations used during fluorescence decrease within the first 60 s after Mn\(^{2+}\) addition to the membrane (20). It has been recently applied to granulocytes (fura-2 quenching) and subtracted from this value the fluorescence increase after addition of the heavy metal chelator DTPA (= "unquenching" of extracellular fura-2).

**RESULTS**

To study a putative role of GTP-binding proteins in the activation of store-dependent Ca\(^{2+}\) influx, we have introduced non-hydrolyzable guanosine phosphate analogues into the cytosol of HL-60 granulocytes using an endocytosis-osmotic shock procedure. This technique allows the introduction of macromolecules into the cytoplasm of cells without disruption of the plasma membrane (20). It has been recently applied to granulocytes and did not interfere with complex motile functions, indicating that cellular integrity is preserved (23). In our studies, the endocytosis-osmotic shock procedure by itself (i.e. in the absence of added GTP analogues) was not cytotoxic and did not interfere with basal and stimulated cellular Ca\(^{2+}\) homeostasis. The cytosolic concentrations of the compound of interest obtained with the method were between 0.2 and 1% of the concentrations present in the extracellular solution during the procedure. The GTPyS and GDPyS concentrations used during the endocytosis-osmotic shock procedure were 50 nm and therefore yielded intracellular concentrations of approximately 100–500 μm (see "Experimental Procedures" for details).

We first investigated the effect of non-hydrolyzable guanosine phosphate analogues on granulocyte Ca\(^{2+}\) homeostasis in the absence of extracellular Ca\(^{2+}\). At the obtained cytosolic concentrations, neither GTPyS nor GDPyS affected basal Ca\(^{2+}\) levels or Ca\(^{2+}\) release in response to the receptor agonist FMLP, the Ca\(^{2+}\)-ATPase inhibitor thapsigargin, or the Ca\(^{2+}\) ionophore ionomycin (Fig. 1, A–C and G–I, and Table I). The absence of an effect on basal [Ca\(^{2+}\)]\(_i\) levels suggested that, at submillimolar concentrations, GTPyS did not induce a sustained activation of phospholipase C. The normal FMLP-induced Ca\(^{2+}\) release suggested that the achieved cytosolic GDPyS concentrations were not sufficient to block FMLP-induced phospholipase C activation. As the Ca\(^{2+}\) release in response to the Ca\(^{2+}\)-ATPase inhibitor thapsigargin is thought to reflect the basal permeability of Ca\(^{2+}\) stores, the normal thapsigargin-induced Ca\(^{2+}\) release suggested that neither GTPyS nor GDPyS affected the permeability of intracellular Ca\(^{2+}\) stores. The normal ionomycin-induced Ca\(^{2+}\) release indicated that GTPyS and GDPyS had no effect on the total content of intracellular Ca\(^{2+}\) stores.

We next investigated the effect of non-hydrolyzable guanosine phosphate analogues on the activation of the Ca\(^{2+}\) influx pathway (Fig. 1, D–F and J–L, and Fig. 2). We measured the activity of the Ca\(^{2+}\) influx pathway as quenching of cytosolic fura-2 after the addition of Mn\(^{2+}\) to the extracellular medium (19, 24). The rate of fura-2 quenching in unstimulated cells was unaffected by GTPyS and was slightly, but not significantly, increased by GDPyS. The rate of fura-2 quenching increased approximately 3-fold after stimulation with either the receptor agonist or with the Ca\(^{2+}\)-ATPase inhibitor thapsigargin (19, 24). In GTPyS-loaded cells the stimulated increase in Mn\(^{2+}\) influx was inhibited by approximately 70%. The extent of inhibition was comparable for FMLP-induced and for thapsigargin-induced Ca\(^{2+}\) release.
G-proteins and Granulocyte Ca\(^{2+}\) Influx

**FIG. 2.** GTP\(\gamma\)-S blocks the activation of Mn\(^{2+}\) entry by depleted Ca\(^{2+}\) stores. GTP\(\gamma\)-S and GDP\(\beta\)-S were introduced into the cytosol of HL-60 cells using an endocytosis-osmotic shock procedure. Control cells were subjected to the same procedure in the absence of non-hydrolyzable nucleotide analogues. Cells were loaded with fura-2, and fluorimetric Ca\(^{2+}\) measurements were performed in a nominally Ca\(^{2+}\)-free medium. Basal Mn\(^{2+}\) influx (empty columns), and Mn\(^{2+}\) influx in response to 100 nM fMLP (gray columns) and 50 nM thapsigargin (black columns) was assessed. Fura-2 quenching (\(\Delta F \) Mn\(^{2+}\) entry) is expressed as percent of control cells. Basal fura-2 quenching in control cells was 3.7 ± 0.5 fluorescence units/60 s; fMLP- and thapsigargin (TG)-stimulated values were 6.3 ± 0.7 and 8.2 ± 0.8% (after subtraction of basal). The cytosolic GTP\(\gamma\)-S and GDP\(\beta\)-S concentrations were estimated to be between 100 and 500 pmol (see *Experimental Procedures*). The slightly increased Mn\(^{2+}\) influx in GDP\(\beta\)-S-loaded cells was statistically not different from control (p > 0.05).

**FIG. 3.** Increasing concentrations of NaF block the thapsigargin-induced Mn\(^{2+}\) influx independent of an effect on the filling state of intracellular Ca\(^{2+}\) stores. HL-60 granulocytes were loaded with fura-2, and fluorimetric Ca\(^{2+}\) measurements were performed in a nominally Ca\(^{2+}\)-free medium. The indicated NaF concentration was added to cells in Ca\(^{2+}\)-free medium at 0 min, 50 nM thapsigargin at 10 min, and 0.02 mM Mn\(^{2+}\) or 0.5 mM ionomycin at 15 min. The triangles show Mn\(^{2+}\) influx, while the circles show ionomycin-induced Ca\(^{2+}\) release. Addition of equivalent concentrations of NaCl did not have any effects (Mn\(^{2+}\) influx in after addition of 5, 10, or 20 mM NaCl was 109.3 ± 7.7, 102 ± 8.4, and 88.7 ± 4.9% of control). Mn\(^{2+}\) influx is expressed as percent of control, i.e., the thapsigargin-stimulated fura-2 quenching in cells that were incubated without NaF or NaCl addition (8.5 ± 1.2 fluorescence units/60 s). Ionomycin-induced Ca\(^{2+}\) release is expressed as percent of total, with the total Ca\(^{2+}\) release defined as the [Ca\(^{2+}\)] peak after ionomycin in cells that were incubated without thapsigargin and without NaF (584.1 ± 37.5 nm).

Thus, our results demonstrate an exquisite sensitivity of the store-regulated Ca\(^{2+}\) influx to inhibition by GTP\(\gamma\)-S. Two families of G-proteins are thought to mediate GTP\(\gamma\)-S effects: heterotrimeric G-proteins and small G-proteins. To distinguish between heterotrimeric and small G-proteins, we have used fluoride, which can mimic the effect of GTP\(\gamma\)-S on large G-proteins (25, 26) but is not an activator of small G-proteins (27).

2 The quantitatively minor inhibition of thapsigargin-induced Mn\(^{2+}\) influx by PMA might be accounted for simply by the diminished driving force for divalent cation influx secondary to the profound and long lasting PMA depolarization of HL-60 granulocytes (31, 32).
by postulating the involvement of a G-protein in the signaling between Ca\(^{2+}\) stores and Ca\(^{2+}\) channels.

What type of G-protein might be involved in the regulation of store-dependent Ca\(^{2+}\) influx? Although our data do not allow us to exclude a role for small G-proteins, the identical effects of GTP\(\gamma\)S and of fluoride clearly suggest the involvement of a trimeric G-protein. In previous studies, we did not find evidence for an enhancement of basal or stimulated Ca\(^{2+}\) influx by pertussis toxin (31), suggesting that the GTP\(\gamma\)S effect is not mediated by a pertussis toxin substrate; similarly, preliminary studies in our laboratory did not find any marked effect of cholera toxin on basal or stimulated Ca\(^{2+}\) influx. Thus, GTP\(\gamma\)S and F\(-\) most likely act either through the activation of an \(\alpha\) subunit of a pertussis toxin- and cholera toxin-insensitive G-protein or through an increase in free \(\beta/\gamma\) subunits.

Taken together, our results would be best compatible with a tonic inhibition of the store-dependent Ca\(^{2+}\) channel by a heterotrimeric G-protein. The activity of this G-protein might determine the sensitivity of the Ca\(^{2+}\) channel to the depletion of Ca\(^{2+}\) stores; alternatively, however, inhibition of the activity of this G-protein might be involved in the mechanism of activation of store-dependent Ca\(^{2+}\) influx.

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REFERENCES

1. Krause, K.-H., Demaurex, N., Jaconi, M. E. E., and Lew, D. P. (1993) Blood Cells (NY) 19, 165-175
2. Putney, J. W., Jr. (1990) Cell Calcium 11, 611-624
3. Penner, B., Fasolato, C., and Hoth, M. (1990) Ann. Rev. Neurosci. 3, 368-374
4. Krause, K.-H., Angel, T., and Lewis, D. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6295-6299
5. Paredes, A. B., Terhau, H., and Stühlmann, W. (1993) Nature 364, 814-818 (abstr.)
6. Randriamampita, C., and Tisch, R. Y. (1993) Nature 364, 598-614 (abstr.)
7. Clapham, D. E. (1993) Nature 364, 763-764 (abstr.)
8. Vostal, J. G., Jackson, W. L., and Shulman, N. R. (1991) J. Biol. Chem. 266, 16911-16916
9. Roser, M. F., Bird, G. J., and Putney, J. W., Jr. (1991) Biochem. J. 274, 643-650
10. Irvine, R. P. (1990) FERS Lett. 28, 5-9
11. Birnbaumer, L. (1992) Cell 71, 1069-1072
12. Iniguez-Lluhi, J., Kleuss, C., and Gilman, A. G. (1993) Trends Cell Biol. 3, 223-236
13. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Science 253, 802-808
14. Hepler, J. R., and Gilman, A. G. (1992) Trends Biochem. Sci. 17, 383-387
15. Hall, A. (1990) Science 249, 635-640
16. Gouy, H., Cefau, D., Christensen, S. B., Debepe, P., and Bismuth, G. (1991) J. Immunol. 147, 757-766
17. Nilsson, V., Holewka, D., Frewterle, C., and Baird, B. (1988) J. Biol. Chem. 263, 18926-18932
18. Demaurex, N., Lew, D. P., and Krause, K. H. (1992) J. Biol. Chem. 267, 2318-2324
19. Rechatiner, M. (1992) Methods Enzymol. 149, 42-48
20. Prentki, M., Wollheim, C. B., and Lew, D. P. (1984) J. Biol. Chem. 259, 13777-13783
21. Demaurex, N., Griesinger, S., Jaconi, M. E. E., Schlegel, W., Lew, D. P., and Krause, K. H. (1993) J. Physiol. (Lond.) 466, 329-344
22. Hendey, B., Klein, C. B., and Maxfield, F. R. (1992) Science 258, 286-289
23. Pittet, D., Lew, D. P., Mayr, G. W., Monod, A., and Schlegel, W. (1989) J. Biol. Chem. 264, 7251-7261
24. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615-649
25. Rodbell, M. (1992) Curr. Top. Cell Regul. 32, 1-47
26. Kahn, R. A. (1991) J. Biol. Chem. 266, 15595-15597
27. Monterro, M., Garcia-Sanchez, J., and Alvarez, J. (1993) J. Biol. Chem. 268, 13053-13061
28. Kikuchi, A., Ikeda, K., Kosawa, O., and Takai, Y. (1987) J. Biol. Chem. 262, 6766-6770
29. Bengtsson, T., Sarradahl, E., Stendahl, O., and Andersson, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2921-2925
30. Krause, K. H., Schlegel, W., Wallheim, C. B., Andersson, T., Waldegr, F. A., and Lew, D. P. (1990) J. Clin. Invest. 86, 1488-1504
31. Demaurex, N., Schlegel, W., Varnai, P., Mayr, G. W., Lew, D. P., and Krause, K. H. (1992) J. Clin. Invest. 90, 830-839
32. Bird, G. J. S., and Putney, J. W., Jr. (1991) J. Biol. Chem. 266, 21486-21488
33. Fasolato, C., Hoth, M., and Penner, R. (1993) J. Biol. Chem. 280, 20737-20740

M. E. E. Jaconi, D. P. Lew, A. Monod, and K-H. Krause, unpublished observation.