Biochimica et Biophysica Acta, 1027 (1990) 130–140
Elsevier

BBAMEM 74942

Membrane depolarization selectively inhibits receptor-operated calcium channels in human T (Jurkat) lymphoblasts

Balázs Sarkadi, Attila Tordai and George Gárdos

National Institute of Haematology and Blood Transfusion, Budapest (Hungary)

(Received 11 January 1990)
(Revised manuscript received 20 April 1990)

Key words: T (Jurkat) lymphoblast; Calcium signal; Calcium release; Calcium ion influx; Receptor-operated calcium channel; Membrane potential

Jurkat lymphoblasts were stimulated by a monoclonal antibody against the CD3 membrane antigen and the evoked calcium signal was followed by the intracellular fluorescent calcium indicator indo-1. The technique applied allowed us to separately investigate the stimulus-induced intracellular calcium release and the calcium-influx pathways, respectively. In the same cells membrane potential was estimated by the fluorescent dye diS-C_3-(5). The resting membrane potential of Jurkat lymphoblasts under normal conditions was between −55 and −60 mV. Membrane depolarization, obtained by increasing external K^+ concentration, removing external Cl^-, or by increasing the Na^+/K^+ leak permeability with gramicidin or PCMBS, did not induce calcium influx in the resting cells and did not influence the CD3 receptor-mediated internal calcium release, while strongly inhibited the receptor-mediated calcium influx pathway. Half-maximum inhibition of this calcium influx was observed at membrane potential values of about −35 to −40 mV and this inhibition did not depend on the external calcium concentration varied between 5 and 2500 μM. Membrane hyperpolarization by valinomycin did not affect either component of the calcium signal. The observed selective inhibition of the receptor-operated calcium influx pathway by membrane depolarization is probably an important modulator of calcium-dependent cell stimulation.

Introduction

Calcium dependent cell stimulation by various hormones or antigens requires an initial increase in the cytoplasmic free calcium concentration and then a sustained phase of this elevated $[Ca^{2+}]_i$. The mechanism of receptor-mediated triggering of calcium liberation is well characterized: upon receptor occupation and a G-protein mediated activation of a specific phospholipase C in the plasma membrane, PIP_2 is cleaved to DAG and IP_3, thereafter this latter compound allows calcium release from intracellular stores (for reviews see Refs. 1–6). There is much debate, however, about the nature and regulation of the plasma membrane calcium influx pathway which opens concomittantly to receptor stimulation. This pathway seems to play a major role in the development of the cellular response to stimuli [2,4], and is variably called in the literature 'receptor-operated calcium channel' (ROCC) or 'second-messenger operated calcium channel' (SMOCC) (see Refs. 7–11), as both the direct opening of such channels through receptor occupation [12,13] and its activation by second messengers, e.g., by the combined action of IP_3 and IP_4 [14,15] or by IP_3 itself [16] have been suggested (in this paper we use the term 'receptor-operated' without the implication of a given transduction mechanism). It seems to be generally accepted, however, that such channels are different from the 'voltage-dependent' calcium channels in the membranes of excitable cells, that is they are not activated by membrane depolarization and not inhibited by the adequately low concentrations of drugs which specifically interfere with the voltage-de-

Abbreviations: aCD3, monoclonal antibody against the CD3 antigen; $[Ca^{2+}]_i$, cytoplasmic free calcium ion concentration; DAG, 1,2-diacylglycerol; DMSO, dimethyl sulfoxide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine; DTT, dithiothreitol; EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-2, interleukin 2; IP_3, inositol 1,4,5-trisphosphate; indo-1/AM, acetoxymethyl ester of indo-1; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzoate sulfonic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate.

Correspondence: G.Gárdos, National Institute of Haematology and Blood Transfusion, H-1113 Budapest, Daróczi u. 24, Hungary.

pendent channels [9–11]. In one report [13], receptor-operated calcium channels were found to be entirely independent of the changes in the membrane potential. However, several studies indicate that stimulation-induced calcium influx, or the cell function depending on the sustained increase of $[Ca^{2+}]_i$, is significantly modulated by membrane hyperpolarization or depolarization [2,17–23].

In the present work, in order to examine the effects of the plasma membrane potential on the receptor-operated calcium channels, we studied the calcium signal response induced by an anti-CD3 monoclonal antibody (aCD3) in Jurkat lymphoblasts. In this model system the cultured T lymphoblast cell line, Jurkat, responds to a stimulation through its antigen receptor pathway (actually evoked by a soluble monoclonal antibody against the CD3 antigen, a component of the T cell antigen receptor complex), by the development of a calcium signal. Cell proliferation in this cell line is not affected by this calcium-dependent stimulation but a functional response is measurable by following stimulation-induced IL-2 or interferon production (see Refs. 24–26). By using this model system and the fluorescent, intracellularly trapped calcium indicator indo-1, we separated the components of the stimulus-induced calcium signal and examined their dependence on the membrane potential. By using various means for producing membrane hyperpolarization and depolarization in the Jurkat lymphoblasts we demonstrate that membrane depolarization, independent of the actual way of achieving such a change, strongly and selectively inhibits the stimulus induced calcium influx.

Materials and Methods

Reagents and cells

EGTA, Hepes, gramicidin, valinomycin, DTT, NEM, and PCMBS were purchased from Sigma, RPMI 1640 cell culture medium and fetal calf serum were from Gibco. Stock solutions of indo-1/AM (Calbiochem), 1 mM, and diS-C_3-(5) (obtained from Dr. A. Waggoner), 0.2 mM, were prepared in DMSO and stored at −20°C. All the basic chemicals used were of reagent grade. Anti CD3 monoclonal antibody (OKT3) was obtained from Ortho.

The standard (NaCl-based) incubation media for the fluorescence measurements contained 120 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 0.04 mM $CaCl_2$, 10 mM Hepes-Na (pH 7.4), 10 mM $NaHCO_3$, 10 mM glucose and 5 mM Na_2HPO_4. These media were supplemented during the fluorescence measurements first with 500 μM EGTA and then with the indicated amount of calcium. For the changes in extracellular K^+ concentrations NaCl was replaced with the indicated amounts of KCl. In choline-Cl based media NaCl was entirely replaced by choline-Cl (120 mM) and the remaining Na^+ concentration was about 25 mM. In Na-glutamate (120 mM) solutions the remaining Cl^- concentration was about 6 mM, all other components were identical to those in the standard medium.

The human leukemic T cell line, Jurkat was maintained under standard conditions in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were harvested in suspension of $(1–2) \cdot 10^6$ cells/ml.

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

For loading with the fluorescent calcium indicator the Jurkat lymphoblasts were resuspended in RPMI + 10% fetal calf serum with a cell number of $(2–3) \cdot 10^6$/ml and incubated at 37°C for 30 min with 1.0 μM final concentration of indo-1/AM. After the loading period the cells were spun down ($600 \times g$, 10 min), and resuspended in indo-1-free RPMI + serum. The cells were kept at room temperature in this medium and the fluorescence measurements were carried out within 2 h.

Before each measurement an aliquot of the cell suspension was rapidly spun down (10 s at $12000 \times g$) in an Eppendorf microfuge, the pellet rinsed without further centrifugations five times with the indicated medium and then the cells resuspended in 2 ml of the same medium (10^6 cell/ml). Indo-1 fluorescence was measured in a Hitachi F-4000 fluorescence spectrophotometer at 37°C with continuous gentle stirring. The excitation wavelength was 331 nm, emission was measured at 410 nm (bandwidth 5 nm). Cytoplasmic free calcium concentration was calculated based on the method in Ref. 27, that is by lysing the cells in digitonin (20 μg/ml) in the presence of 0.5 mM free calcium in order to obtain maximum fluorescence, and then quenching the dye by the removal of Ca^{2+} with 5 mM EGTA at pH 8.5, adjusted with Tris base. We have calibrated the loading procedure with free indo-1 and found the intracellular concentration of free indo-1 to be between 30 and 50 μM in these experiments (the mean cell volume was assumed to be $1 \cdot 10^{-12}$ l).

When we checked the indo-1 response in the intact Jurkat lymphoblasts by the addition of ionomycin (2 μM), we found that the dye was fully responsive to the changes in cytoplasmic calcium concentrations (see later in Fig. 1). The presence of extracellular indo-1 fluorescence was checked by the addition of 0.5 mM EGTA and then 1 mM calcium to the control cells. In each experiment this 0.5 mM EGTA was added to the media containing initially 40 μM Ca^{2+} and any further adjustment of the free calcium concentration was based on calculations by using a computer program taking account of all the constituents of the media (the respective stability constants in this program for the EGTA complexes were as follows: CaEGTA = $5 \cdot 10^{10}$; CaHEGTA = $2.14 \cdot 10^5$; HEGTA = $2.88 \cdot 10^9$; H_2EGTA = $7 \cdot 10^8$; H_3EGTA = 479; H_4EGTA = 100;

MgEGTA = $1.62 \cdot 10^5$; MgHEGTA = 2340; KEGTA = 8).

The leakage of indo-1 proved to be very small during a 10–15-min experimental period, however, this leakage was taken into consideration in the calibration procedure. For cell stimulation aCD3 was used in these experiments at a concentration of 0.1 μg/ml, which gave a maximum response in the calcium signal development.

Membrane potential measurements

Membrane potential in the Jurkat lymphoblasts was estimated by using the fluorescent dye diS-C_3-(5), based on the methods described in Refs. 28–31. In the actual measurements $(2–3) \cdot 10^6$ Jurkat lymphoblasts were suspended in 2 ml of the given media and exactly 2 μl of the dye (0.2 mM, dissolved in DMSO) was added to the cell suspension. The fluorescence measurements were carried out under the same conditions as described for the $[Ca^{2+}]_i$ measurements (in a Hitachi F-4000 instrument, 37°C, gentle stirring) but with the excitation and emission wavelengths of 620 and 670 nm, respectively. The lipid soluble, positively charged diS-C_3-(5) reports the membrane potential because its distribution between the cytoplasm of the cells and the medium is determined by this potential and because its fluorescence inside the cells is largely quenched (see Ref. 29). After the initial equilibration of the dye an increase in fluorescence reports depolarization and a decrease indicates hyperpolarization. The membrane potential reported by this dye has been shown to be largely insensitive to the contribution of intracellular organelles (see Ref. 30). The estimation of the resting membrane potential in Jurkat lymphoblasts was carried out by the valinomycin null-point titration method at varying extracellular K^+ concentrations, as described in Refs. 28–30. The actual fluorescence measurements, the calibration curve obtained in the presence of 1 μM valinomycin and the data for the estimated membrane potential values under various ionic conditions are shown in Figs. 4, 5, and 8 in the Results. In the presence of valinomycin the Nernst equation for K^+ distribution, while in the control cells a Goldman-type equation for K^+ and Cl^- distribution, weighted by their relative permeabilities, was used (according to the choline-Cl experiments (see Fig. 4) Na^+ permeability in these cells has a very low contribution to the membrane potential). The equation used was:

$$V = -58 \cdot \log((P_K \cdot [K^+]_i) + (P_{Cl} \cdot [Cl^-]_o))/((P_K \cdot [K^+]_o) + (P_{Cl} \cdot [Cl^-]_i)).$$

Internal K^+ concentration was measured by flame photometry (the cells were pelleted in K^+-free media, dissolved in 0.1% Triton X-100 containing distilled water and K^+ concentration was measured by a flame photometer with internal lithium standard). The value of cellular K^+ concentration was between 140 and 145 mmol/l of cells in Jurkat lymphoblasts. The determination of intracellular Cl^- concentration was carried out after washing the cells in Na-glutamate by a mercury titration method (the Cl^- concentration of the Triton X-100 dissolved cells was titrated by mercurinitrate solution in the presence of diphenylcarbazone indicator). This titration gave results of 30–40 mM $[Cl^-]_i$, in good agreement with the values found in human lymphocytes [30]. The concentration of valinomycin (1 μM), in agreement with the data in the literature [28–31], proved to be sufficient to produce a maximum response, probably setting the K^+ permeability at least one order of magnitude greater than that of other ions. The low concentration of gramicidin applied (0.1 μM) was sufficient to produce maximum depolarization while higher gramicidin concentrations induced significant cell damage.

It is important to mention, that neither indo-1 fluorescence in the membrane potential measurements, nor diS-C_3-(5) fluorescence in the indo-1 $[Ca^{2+}]_i$ measurements caused any cross-emission and thus most of the membrane potential measurements were carried out with the indo-1-loaded cells, used also for the $[Ca^{2+}]_i$ studies. According to several experiments, indo-1-loaded Jurkat lymphoblasts had the same resting membrane potential as the control cells. The dye, diS-C_3-(5), up to a concentration of 1 μM, reported similar membrane potentials and did not affect cell viability in the framework of the short measurement period. However, diS-C_3-(5) in a concentration greater than 0.1 μM had an inhibitory effect on the aCD3-induced calcium signal development and this inhibition was about 20% at 0.2 μM and 50% at 0.5 μM of the dye. Reports in the literature also indicate that cyanine indicators in greater concentrations and with longer incubation periods may affect cell metabolism and membrane properties [1,32]. In order to obtain a good calibration of the membrane potential and avoid cell damage we used 0.2 μM diS-C_3-(5). Therefore the membrane potential changes seen at the addition of aCD3 (a slight depolarization of about 5–8 mV) are not quantified in the present work.

The data presented in the Figures are representatives of at least six similar experiments carried out with different cell preparations. In this stable cell line the reproducibility of the $[Ca^{2+}]_i$ and membrane potential measurements was extremely good, usually within 5–10% around the mean values.

Results

By using the intracellularly trapped fluorescent calcium indicator indo-1 in low concentrations and by removing or repleting extracellular free calcium, it was possible to separately investigate stimulus-induced

calcium release and calcium influx, respectively. Fig. 1, panels A and B show such experiments, when Jurkat lymphoblasts were incubated in normal, NaCl-based media. In panel A, anti-CD3 monoclonal antibody (aCD3) was applied in the presence of 0.5 mM $[Ca^{2+}]_o$, thus internal calcium release and calcium influx appeared together. In this case, after a rapid rise in $[Ca^{2+}]_i$ up to about 800 nM, the free cytoplasmic calcium level slightly declined and a prolonged plateau-phase was observed. This elevated $[Ca^{2+}]_i$ level, usually stabilizing between 300–350 nM, persisted for more than 30 min (which is the reliable time period of a fluorescent measurement with a given cell population).

When aCD3 was applied in a NaCl-based medium in the presence of 0.5 mM EGTA, that is in the virtual absence of external Ca^{2+} (panel B), the only source for the increase in $[Ca^{2+}]_i$ was the release from internal stores. When cellular indo-1 concentration was lower than 30–50 μM and the cells were not significantly depleted of calcium (see Methods), the signal produced by the release usually reached 400–550 nM $[Ca^{2+}]_i$ within 1 min and thereafter rapidly declined to the original free calcium level, or slightly below that. Repletion of external calcium to obtain a $[Ca^{2+}]_o$ of 0.5 mM, produced a second calcium signal, representing the influx of calcium ions from the medium. It is worth noting, that the calcium influx signal was usually slightly greater than that observed during the release and a similar plateau-phase of $[Ca^{2+}]_i$ was maintained as seen in panel A.

As indicated by the results shown in Fig.1, panels C and D, internal calcium stores were strongly depleted after aCD3 stimulation in the absence of external calcium, thus the signal seen after external calcium repletion had to represent a calcium influx. The addition of the calcium ionophore ionomycin (0.25 μM) to the control cells (panel C) produced a rapid calcium release from the internal stores and then the efflux of calcium into the EGTA-containing external medium. After the calcium release signal evoked by aCD3 (panel D) the addition of ionomycin produced only a very small increase in $[Ca^{2+}]_i$. After re-addition of external calcium a rapid influx of calcium was carried by ionomycin. This calcium influx did not depend on the presence of aCD3 but was further increased at increasing ionomycin concentrations.

The peak level of the aCD3-induced calcium signal in the presence of external calcium (Fig. 1, panel A), or after calcium repletion (panel B) was only slightly dependent on the external free calcium concentration: at 5 μM $[Ca^{2+}]_o$ this peak value was about 90% of that seen at 500 μM $[Ca^{2+}]_o$, and there was no measurable change in this value if external calcium concentration was increased up to 2.5 mM. The rate of the restoration of the cytoplasmic free calcium level, however, was somewhat slower at higher external calcium concentrations (data

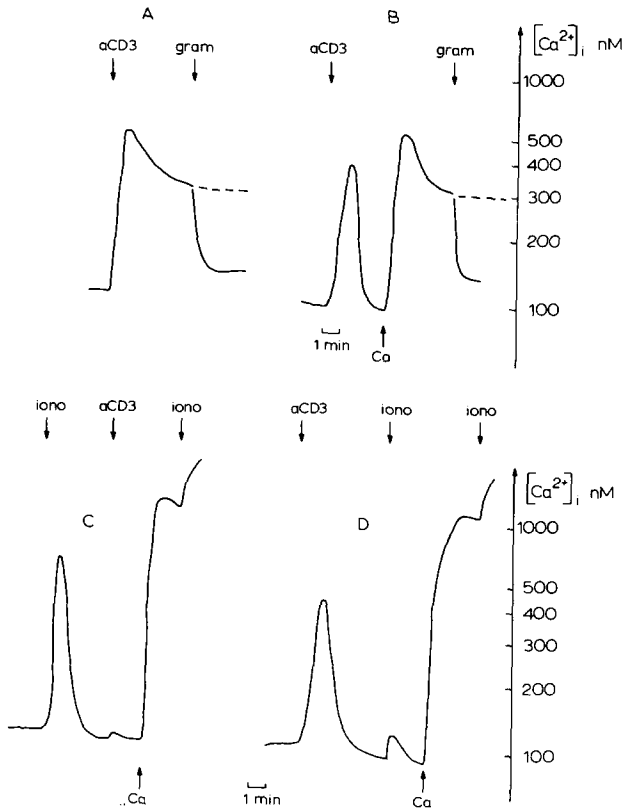

Fig. 1. Development of the aCD3-induced calcium signal in indo-1-loaded Jurkat lymphoblasts. Separation of intracellular calcium release and external calcium influx. Indo-1 fluorescence was measured and $[Ca^{2+}]_i$ changes calibrated as described in Methods. Indo-1-loaded Jurkat lymphoblasts were incubated in the standard NaCl-based media, in panel A in the presence of about 0.5 mM $[Ca^{2+}]_o$ (0.5 mM EGTA + 1.0 mM $CaCl_2$), while in panel B, C and D at an initial $[Ca^{2+}]_o$ lower than 0.1 μM (40 μM $CaCl_2$ and 500 μM EGTA). At the times indicated by the arrows 0.1 μg/ml aCD3, 0.1 μM gramicidin (gram), or 0.25 μM ionomycin (iono) were added to the media. The dashed lines in panels A and B show the time course of the $[Ca^{2+}]_i$ changes without gramicidin. In panels B, C and D, at the time indicated by the lower arrow $[Ca^{2+}]_o$ was restored to about 0.5 mM by the addition of 1 mM $CaCl_2$.

not shown here in detail). In order to compare the various effectors of the calcium signal in the following experiments we used about 0.5 mM $[Ca^{2+}]_o$, that is 1 mM calcium added after 0.5 mM EGTA (see Methods). The $[Ca^{2+}]_i$ in the resting Jurkat lymphoblasts was relatively insensitive to changes in $[Ca^{2+}]_o$, that is if external calcium was repleted in the absence of the stimulus (without the addition of aCD3), the increase in $[Ca^{2+}]_i$ was insignificant as compared to that seen in the stimulated cells and the $[Ca^{2+}]_i$ levels stayed below 120–150 nM within 30 min.

In Fig. 1, panels A and B, gramicidin (0.1 μM) was added to the aCD3 stimulated Jurkat cells during the plateau-phase of increased $[Ca^{2+}]_i$. This ionophore, which allows the permeation of small monovalent cations, such as Na^+ and K^+ (but not, e.g., choline$^+$), depolarized the cell membrane (see below), and strongly

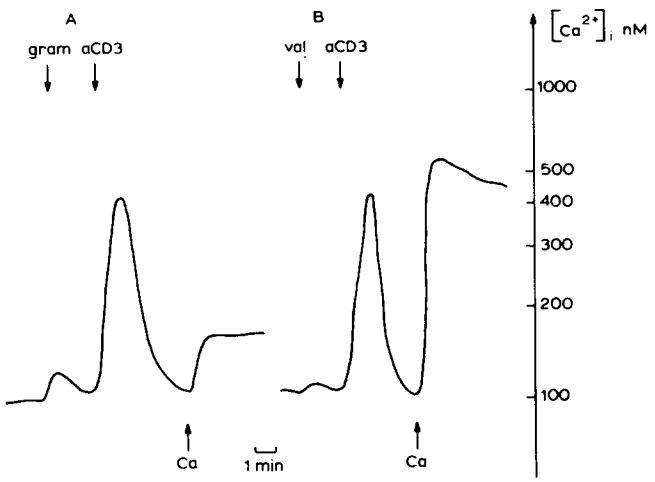

Fig. 2. Effects of gramicidin and valinomycin on the aCD3-induced calcium signal in indo-1-loaded Jurkat lymphoblasts. The cells were incubated in the standard NaCl-based media with the initial $[Ca^{2+}]_o$ of less than 0.1 μM (40 μM $CaCl_2$ and 500 μM EGTA). In panel A 0.1 μM gramicidin (gram), in panel B 1 μM valinomycin (val) was added and then cell stimulation was induced by aCD3. At the times indicated by the lower arrows $[Ca^{2+}]_o$ was increased to about 0.5 mM by the addition of 1 mM $CaCl_2$.

reduced the elevated $[Ca^{2+}]_i$ in the presence of external Ca^{2+}. Since under these conditions this elevated $[Ca^{2+}]_i$ was maintained by an increased calcium influx, these data suggest that gramicidin and/or its membrane depolarizing effect inhibits stimulus-induced calcium influx.

Fig. 2, panel A, demonstrates an experiment which indicates that the effect of gramicidin was selective for the inhibition of the receptor-operated calcium influx. When gramicidin was added 1 min before aCD3 in the absence of external calcium, the stimulus-induced calcium release from internal stores was practically identical to that seen in the control cells (see Fig. 1B), while calcium influx (after $[Ca^{2+}]_o$ repletion) was strongly reduced. A similar reduction in calcium influx by gramicidin was observed if $[Ca^{2+}]_o$ was varied between 5 μM and 2.5 mM (data not shown).

In Fig. 2, panel B, the effect of another ionophore, the selective K^+-carrier, valinomycin, is demonstrated. Valinomycin which, in contrast to gramicidin, hyperpolarizes the cell membrane (see below), did not significantly modulate the aCD3-induced calcium release or influx, although in this case the plateau-level of $[Ca^{2+}]_i$ after $[Ca^{2+}]_o$-repletion was slightly elevated (the addition of valinomycin or gramicidin to the control cells produced a small, stimulus-independent, transient rise in $[Ca^{2+}]_i$, which may be due to some calcium release from intracellular stores, e.g. mitochondria, directly affected by the ionophores).

In the following experiments we have examined the effects of extracellular cations and anions on the aCD3-induced calcium release and influx, respectively. Jurkat lymphoblasts after the indo-1 loading were resuspended in KCl (Fig. 3, panel A), in choline-Cl (panel B), or in Na-glutamate media (panel C, for the exact composition of these media see Methods). As demonstrated, the addition of aCD3 in all these cases caused a similar rise in $[Ca^{2+}]_i$, produced by the internal calcium release, but the magnitude of the calcium influx after $[Ca^{2+}]_o$ repletion was significantly different. This picture did not change qualitatively if external calcium concentration was varied between 5 μM to 2.5 mM.

In a KCl-based medium (which strongly depolarizes the Jurkat lymphoblast plasma membrane, see below) the influx component was practically abolished and the addition of valinomycin or gramicidin did not produce any significant change in this $[Ca^{2+}]_i$ level. It is important to mention that the addition of K^+ to the media (that is membrane depolarization) either in the absence or presence of external calcium, did not produce a significant change in $[Ca^{2+}]_i$ in non stimulated Jurkat lymphoblasts, thus no voltage-dependent calcium channels seem to be present in these cells (data not shown here). In choline-Cl (panel B), both calcium release and influx were normal, but gramicidin did not reduce the plateau-level of $[Ca^{2+}]_i$. This is most probably due to the fact that gramicidin does not allow the permeation of choline$^+$ [33,34], thus only a small degree of depolarization is expected under these conditions (see Fig. 4). In Na-glutamate (panel C), calcium influx was reduced, but $[Ca^{2+}]_i$ could be increased to a greater level by the addition of valinomycin while decreased to a low value by gramicidin. Any effect of some calcium chelation by glutamate on the calcium influx can be excluded, since changes in the $[Ca^{2+}]_o$ between 5 μM to 2.5 mM in the EGTA-buffered media did not significantly modify the observed calcium influx. Resuspen-

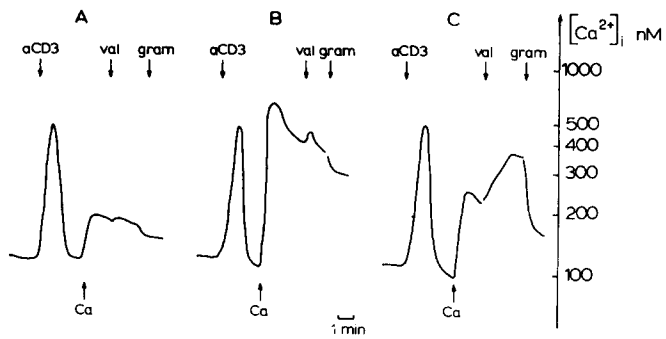

Fig. 3. Effects of the ionic milieu in the incubation media on the aCD3-induced calcium signal in indo-1-loaded Jurkat lymphoblasts. The cells were incubated in 100 mM KCl-based (panel A), in choline-Cl based (panel B) or in Na-glutamate (panel C) media (for the exact compositions see the 'Methods' section). Initial $[Ca^{2+}]_o$ was less than 0.1 μM (40 μM $CaCl_2$ + 500 μM EGTA), which was repleted to 0.5 mM by the addition of 1 mM $CaCl_2$ at the times indicated by the lower arrows. 0.1 μg/ml aCD3, 1 μM valinomycin (val), or 0.1 μM gramicidin (gram) were added at the times indicated by the upper arrows.

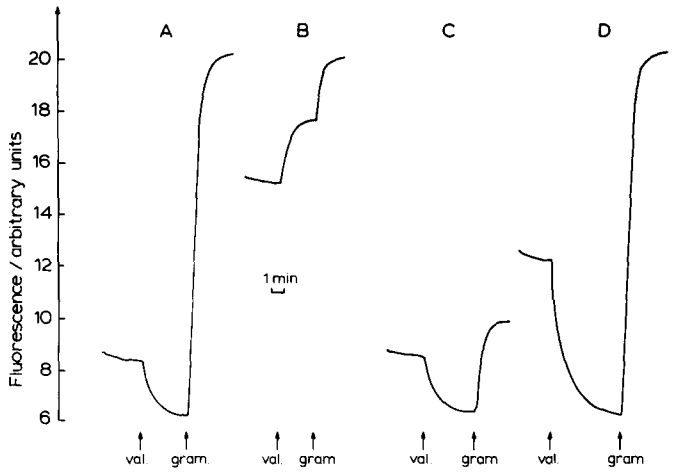

Fig. 4. Fluorescence measurements with the membrane-potential indicator dye, diS-C_3-(5) in Jurkat lymphoblasts. Effects of the ionic milieu and ionophores. The cells were equilibrated with 0.2 μM diS-C_3-(5) as described in Methods and then 1 μM valinomycin (val) or 0.1 μM gramicidin (gram) were added at the times indicated by the arrows. In the four experiments the cuvettes contained exactly the same amount of cells and dye. Panel A, NaCl-based medium; panel B, 100 mM KCl medium; panel C, choline-Cl based medium; panel D, Na-glutamate medium (for the exact compositions of the media see Methods). An increase in fluorescence indicates depolarization, while a decrease reports hyperpolarization of the cell membrane.

sion of the cells in a medium where external Cl^- is replaced by glutamate$^-$, if Cl^--distribution is involved in the determination of the normal membrane potential, is expected to partially depolarize the Jurkat cell membrane. In this medium valinomycin should hyperpolarize, while gramicidin depolarize the cell membrane (see Fig.4).

In order to evaluate the above data we have estimated the membrane potential in Jurkat lymphoblasts by using the fluorescent membrane potential indicator diS-C_3-(5). Fig. 4 shows original fluorescence measurements in which the same number of Jurkat lymphoblasts were resuspended with 0.2 μM of diS-C_3-(5) in NaCl (A), KCl (B), choline-Cl (C) and Na-glutamate (D) media. After the equilibration of the dye (2–3 min), valinomycin, and thereafter gramicidin was added to the media. An increase in the fluorescence indicates depolarization, while decreasing fluorescence shows hyperpolarization of the cell membrane (see Methods). As demonstrated, in KCl and in Na-glutamate media the cells were relatively depolarized. Valinomycin in the NaCl, choline-Cl and Na-glutamate media produced hyperpolarization to about the same extent; the addition of gramicidin in the NaCl, KCl and Na-glutamate media yielded a similar level of depolarization. In KCl medium valinomycin slightly depolarized the cell membrane. In a choline-Cl medium gramicidin (not allowing a rapid choline$^+$ movement) produced only a small degree of depolarization, corresponding to the small amount of sodium (25 mM) in this medium. All these measurements are in good agreement with the expected ion- and ionophore effects (see above).

Fig. 5 shows a compilation of the diS-C_3-(5) fluorescence measurements and the measured and calculated effects of external K^+ on the membrane potential of Jurkat lymphoblasts. The insert shows the calibration line used for converting the fluorescence changes into

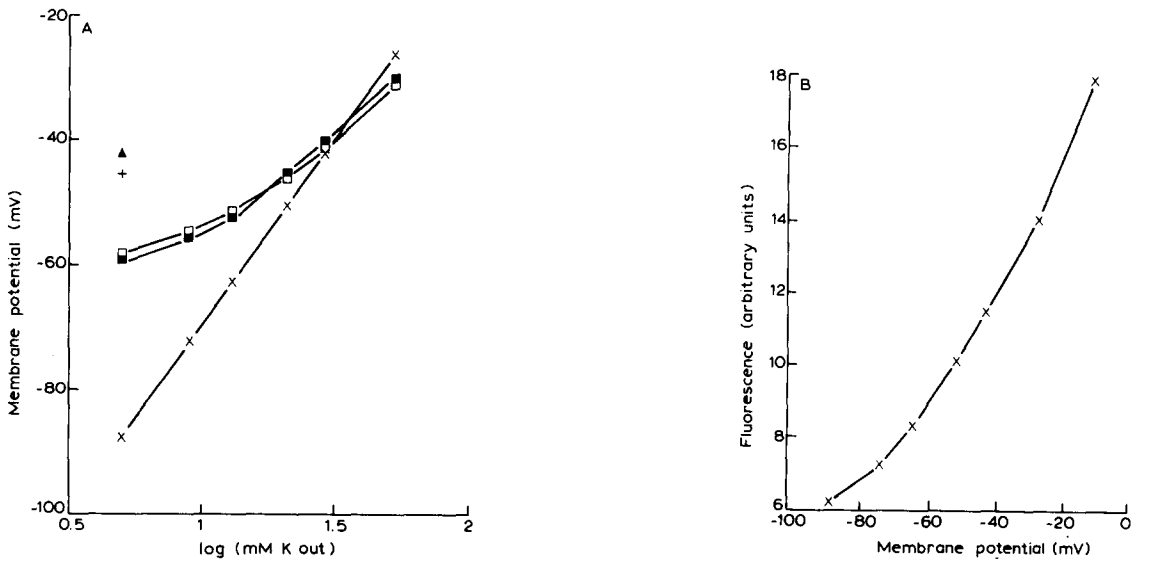

Fig. 5. Membrane potential in Jurkat lymphoblasts as a function of external K^+ concentration (Panel A). The figure presents the measured or calculated membrane potential values as a function of external [K^+] (note the logarithmic scale of the abscissa). ×——×, membrane potential calculated by the Nernst equation in the presence of valinomycin; ■——■, membrane potential measured in the control cells, NaCl replaced with different amounts of KCl; □——□, membrane potential calculated with the Goldman equation described in Methods, by using $[K^+]_i$ of 145 mM, $[Cl^-]_i$ of 40 mM and obtaining the best fit to the measured values by using $P_{Cl}/P_K = 0.7$. ▲, membrane potential measured in Na-glutamate media; +, membrane potential calculated with the same equation as above with external Cl^- concentration of 6 mM.Panel B shows the calibration of the membrane potential by diS-C_3-(5) fluorescence in the presence of 1 μM valinomycin, as described in Methods.

membrane potential values (the calculation is based on the Nernst equation for K^+-distribution in the presence of valinomycin - for the details of the calibration procedure and the possible flaws of using this indicator see Methods).

The membrane potential *measured* in the Jurkat lymphoblasts in a normal, NaCl-based medium is represented in Fig. 5 by the black squares. The first value on the left corresponds to the membrane potential measured at normal external K^+ concentration (5 mM) and its value was found to be between -55 and -60 mV in six independent experiments. Increasing external K^+ concentrations reduced the membrane potential in the demonstrated pattern. Changes in $[Ca^{2+}]_o$ between less than 0.1 μM and 0.5 mM did not affect the resting membrane potential in Jurkat lymphoblasts (data not shown).

The addition of valinomycin (×) increased the membrane potential at 5 mM $[K^+]_o$ to a calculated -88 mV, while gramicidin, which allows the permeation of both K^+ and Na^+, reduced it to practically 0 (see also Fig.4).

When external Cl^- was almost entirely replaced by the non-penetrating glutamate$^-$ anions in the medium (black triangle) the membrane potential of the Jurkat lymphoblasts was reduced to -42 ± 2 mV ($\pm$SD, $n = 5$, see also Fig. 4 panel D). Such a change is expected if the Cl^- distribution is involved in the formation of the membrane potential. By using these data, the known external and intracellular ion concentrations and a Goldman type equation, the calculation of the expected membrane potential (see Methods), is also possible. The calculated membrane potential values (empty squares in a NaCl-based medium, plus sign in a Na-glutamate medium) gave the best fit to the measured values both in NaCl and Na-glutamate media if the Cl^- permeability of the Jurkat lymphoblast cell membrane in the Goldman equation was set to 0.7 times the K^+-permeability value. The proper fit of the measured and calculated values under different conditions reinforce the reliability of the membrane potential measurements in Jurkat lymphoblasts by using the diS-C_3-(5) indicator.

Since the alteration of the external K^+ concentration proved to be a simple and well controlled way to modify the membrane potential of Jurkat lymphoblasts, in the following experiments we examined the aCD3-induced intracellular calcium release and the calcium influx, respectively (the latter was measured after $[Ca^{2+}]_o$ repletion, see Fig. 1) at various external K^+ concentrations. In Fig. 6 the compared peak values of stimulus-induced calcium release (empty squares) and those of calcium influx (triangles) are presented as a function of membrane potential measured by diS-C_3-(5) at the indicated external K^+ concentrations. The figure also demonstrates that, when the membrane was hyperpolarized with valinomycin (× and ■) neither the aCD3-induced calcium release nor the influx was significantly altered

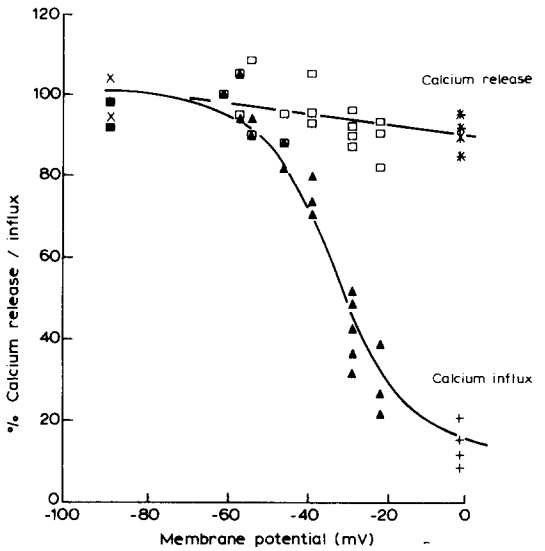

Fig. 6. Dependence of the aCD3-induced intracellular calcium release and external calcium influx, respectively, on the membrane potential in Jurkat lymphoblasts. The calcium release induced by aCD3 was measured in the absence of extracellular calcium, while calcium influx was estimated after $[Ca^{2+}]_o$ repletion, as shown in Fig. 1. The peak values of the $[Ca^{2+}]_i$ were considered and the value measured in the standard, NaCl-based media were taken as 100%. The data points show mean values obtained in 2–5 sets of experiments with different cell batches. Membrane potential in the control cells was altered by changing the external K^+ concentration. Maximum hyperpolarization was induced by valinomycin, maximum depolarization by gramicidin. When applied, the concentration of valinomycin was 1 μM, that of gramicidin 0.1 μM. □, intracellular calcium release in control cells; ▲, external calcium influx in control cells; ■, internal calcium release in valinomycin-treated cells; ×, external calcium influx in valinomycin-treated cells; *, internal calcium release in gramicidin-treated cells; +, external calcium influx in gramicidin-treated cells.

(see also Fig. 2). The complete depolarization of the cell membrane, achieved with gramicidin, hardly affected aCD3-induced calcium release (*), while practically abolished receptor-operated calcium influx (+). Half-maximum inhibition of the stimulus-induced calcium influx was seen at about -35 to -40 mV. The approx. 50% reduction of the aCD3-induced calcium influx in Na-glutamate media (see Fig. 3, panel C) when the membrane potential is about -40 mV, closely corresponds to this value.

Earlier data indicated a selective inhibition of the calcium influx pathway by non-penetrating SH-reagents in platelets [35]. Therefore, in the following experiments we have examined the effects of such an agent, PCMBS, on the stimulus-induced calcium movements and on the membrane potential in Jurkat lymphoblasts. As shown in Fig. 7, Panel A, the addition of 50 μM PCMBS did not influence the development of the aCD3-induced calcium signal in the absence of external calcium, while practically abolished the calcium influx after $[Ca^{2+}]_o$ repletion. The addition of DTT to the medium significantly reversed this inhibition even after several minutes

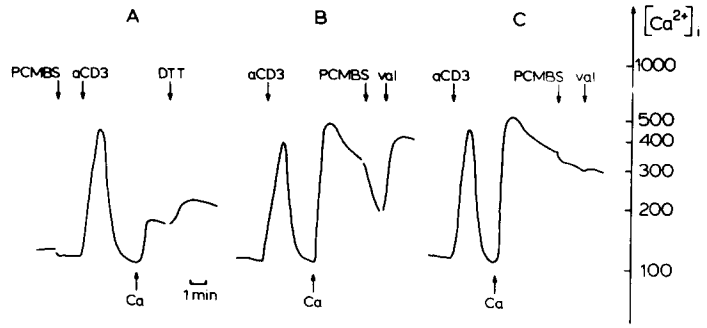

Fig. 7. Effects of PCMBS on the aCD3-induced calcium signal in indo-1-loaded Jurkat lymphoblasts. The cells were incubated in standard, NaCl-based media (panels A and B) or in choline-Cl based medium (panel C), with an initial external $[Ca^{2+}]_o$ of less than 0.1 μM (40 μM $CaCl_2$ + 500 μM EGTA). At the times indicated by the lower arrows $[Ca^{2+}]_o$ was repleted to 0.5 mM by the addition of 1 mM $CaCl_2$. The upper arrows indicate the addition of 50 μM PCMBS, 0.1 μg/ml aCD3, 1 mM DTT, and 1 μM valinomycin (val), respectively.

of the exposure to PCMBS (longer incubations than 8–10 min with PCMBS eliminated the DTT reversal). If PCMBS was added to the medium in the presence of 0.5 mM $[Ca^{2+}]_o$, the aCD3-induced calcium signal in Jurkat lymphoblasts developed but the level of the plateau of $[Ca^{2+}]_i$ was strongly reduced (data not shown here).

In Fig. 7, panels B and C, the effect of PCMBS on the calcium influx pathway was further examined. In these experiments PCMBS was added during the plateau phase of the aCD3-induced calcium signal after calcium repletion either in a NaCl (panel B), or a choline-Cl based (panel C) medium. As shown, in a NaCl medium PCMBS induced a rapid decline in $[Ca^{2+}]_i$ in the stimulated cells, which probably reflects the inhibition of the calcium influx pathway. The addition of valinomycin, which hyperpolarizes the cells (see Fig. 8)

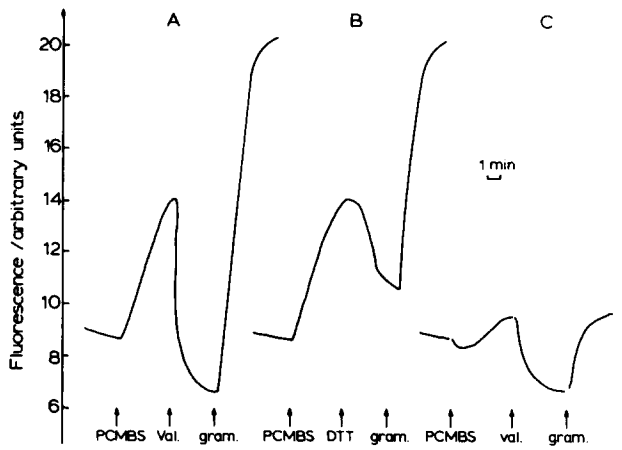

Fig. 8. Fluorescence measurements with the membrane-potential indicator dye, diS-C_3-(5) in Jurkat lymphoblasts. Effects of PCMBS, DTT and ionophores. The cells were equilibrated with 0.2 μM diS-C_3-(5) as described in Methods and then 50 μM PCMBS, 1 μM valinomycin (val), 1 mM DTT, or 0.1 μM gramicidin (gram) were added at the times indicated by the arrows. The cuvettes contained exactly the same amount of cells and dye. Panels A and B, standard, NaCl-based media; panel C, choline-Cl based medium (for the exact compositions of the media see Methods). An increase in fluorescence indicates depolarization, while a decrease reports hyperpolarization of the cell membrane.

but would not interfere with the SH-groups in the cell membrane, rapidly counteracted the effect of PCMBS. As shown in panel C, in a choline-Cl medium, that is at low external Na^+, PCMBS was ineffective on the stimulus-induced increase in $[Ca^{2+}]_i$, and under these conditions valinomycin was also without effect. These data indicate that the inhibition of the calcium influx by PCMBS is not a direct action on the calcium channels but related to the changes in membrane potential through modulation of the Na^+ permeability of the membrane.

The membrane potential measurements shown in Fig. 8 entirely support the above suggestion: in a NaCl medium PCMBS significantly depolarized the Jurkat lymphoblast plasma membrane (panels A and B), while in a choline-Cl medium PCMBS had little effect on the membrane potential (panel C). Valinomycin, by greatly increasing the selective K^+-permeability, reverted the PCMBS-induced change in the membrane potential. Addition of DTT, by reducing the SH-groups affected by PCMBS, also had a reverting effect on membrane depolarization (DTT in itself had no effect on the calcium signal in the Jurkat lymphoblasts). This reversal by DTT was slower and incomplete, as compared to that caused by valinomycin.

Discussion

The data presented above demonstrate that in a human T lymphoblast (Jurkat) cell line, by using the fluorescent calcium indicator, indo-1, it is possible to separate the aCD3-induced intracellular calcium release and the external calcium influx, respectively. A similar simple technique has been used by our group to investigate these two types of calcium movements and their functional role in platelet aggregation [35]. As shown in Figs. 1 and 2, in the aCD3-stimulated Jurkat lymphoblasts the level of the plateau-phase of the calcium signal in the presence of external calcium correlates with the magnitude of the calcium influx, if exter-

nal calcium is repleted after the cells had been stimulated in the absence of external calcium. In order to obtain a maximum calcium signal in the absence of $[Ca^{2+}]_o$, it is necessary to use low concentrations of indicators with a high fluorescence intensity, such as indo-1, so as to avoid significant intracellular calcium buffering and metabolic effects, experienced, e.g., with quin-2 (see Refs. 27 and 36). In experiments not presented here we have examined the relative magnitude of the calcium liberation and calcium influx signals (measured as shown in Fig. 1, panel B) in Jurkat lymphoblasts containing various intracellular concentrations of indo-1 and found that their ratio was about the same between 5 and 50 μM of indo-1, while a significant decrease in the calcium release signal could be observed at higher indo-1 levels. In order to avoid calcium depletion of the Jurkat lymphoblasts, and a consequent decrease in the calcium release signal, it was necessary to carry out all the loading and preincubation procedures in RPMI + fetal calf serum media, containing normal levels of $[Ca^{2+}]_o$ and metabolic substrates. The 5–15 min incubation of the Jurkat lymphoblasts in a low-calcium (EGTA-buffered) medium during the fluorescence measurements did not seem to produce any significant effect on the calcium homeostasis of the unstimulated cells, as calcium repletion did not induce a significant calcium influx and, after this period, upon the addition of aCD3, a calcium release signal similar to that seen in the control cells could be evoked (data not shown in detail). Alternative methods for measuring stimulus-induced calcium influx, e.g., by internal calcium depletion [37], or following extracellular manganese influx through the calcium transport pathway and the consequent quenching of the intracellular fluorescence of indo-1 or fura-2 [12,38] have also been suggested in the literature. All these techniqes have inherent flaws and difficulties (e.g., modulation of the signal-development by $[Ca^{2+}]_i$ or $[Mn^{2+}]_i$, relatively high background permeability for manganese, need of rapid wavelength-alterations in the fluorescence measurements, etc.), which probably do not make them superior to the simple method presented herein.

Another methodological question is the reliability of the fluorescent membrane potential measurements applied in these studies. Carbocyanine dyes are relatively easy to use, stable compounds and their intracellular quenching gives the distribution measurements a good resolution at relatively low dye concentrations (50–500 nM). The dye used in this study, diS-C_3-(5), has been reported to give reliable membrane potential measurements in lymphocytes and other cell types [28–31]. In contrast, some reports indicate that 0.1–1 μM of diS-C_3-(5) or similar carbocyanines significantly inhibit cell metabolism and certain specific transport processes [32]. In accordance with the data in the literature [1,32] we also found that diS-C_3-(5) concentrations greater than 100 nM reduced the aCD3-induced calcium signal in Jurkat lymphoblasts; however, during the measurement periods, the presence of the dye did not affect the resting calcium level of the cells (see Methods). Therefore, by using low diS-C_3-(5) concentrations and a careful calibration procedure we consider the diS-C_3-(5) membrane potential measurements in the non-stimulated cells to be reliable, while we do not interpret the potential changes (a slight depolarization) seen during aCD3-stimulation. When using oxanol dyes, which may have no toxic effects on lymphocytes (see Ref. 1), the calibration of the membrane potential, due to an interference with valinomycin and gramicidin, is much less reliable, and isotope distribution experiments are not suitable for following relatively rapid changes in the membrane potential, such as induced by PCMBS.

The series of experiments by using various ionic conditions, ionophores and membrane-affecting compounds, such as PCMBS, indicate that aCD3-induced calcium release from internal stores in Jurkat lymphoblasts is hardly affected by all these treatments. That is, the receptor induced formation of second messengers and their calcium mobilizing action is practically unchanged if external Na^+ is replaced by K^+ or choline, if external Cl^- is replaced by the non-penetrating glutamate$^-$, or even if internal K^+ is largely exchanged by Na^+, as expected in the presence of gramicidin (see also Refs. 19–22). In contrast, any of the above treatments, when inducing membrane depolarization, practically abolishes the aCD3-induced calcium influx component. By comparing the changes in membrane potential and the magnitude of stimulus-induced calcium influx, we found a half-maximum inhibition of this calcium transport pathway between -35 and -40 mV. The absolute level of this threshold potential may be affected by the methods of the potential measurements and may be different for various cell types, but it is certainly in the range of potential values which may occur under certain physiological or pharmacological/therapeutical conditions (see below). In our experiments we found no evidence for the presence of voltage-dependent (that is, depolarization-induced) calcium channels in Jurkat lymphoblasts, as the resting level of $[Ca^{2+}]_i$ was unchanged upon rapid depolarizing effects on the cell membrane and 10–20 μM verapamil hardly affected the stimulus-induced calcium signal (data not shown — see also Ref. 23).

In Results we demonstrated that gramicidin or PCMBS did not directly inhibit stimulus-induced calcium movements but, by increasing the leak permeability for small monovalent ions, in NaCl-based media they decreased the cell membrane potential and selectively abolished the receptor-operated calcium influx. PCMBS and some other SH-group reagents have long been known to introduce small monovalent cation leakage in the red cell membrane (see Ref. 39), which is

reversible upon SH-reduction, e.g., by DTT. Thus the inhibitory effect of non-penetrating SH-group reagents on the thrombin-induced calcium influx component, seen in platelets [35], is probably also secondary to membrane depolarization. When the permeating SH-reagent NEM (50–100 μM) was added to the Jurkat lymphoblasts, both aCD3-induced calcium release and calcium influx were strongly reduced (data not shown), thus in this case the combined effect of this drug on the internal and external membrane functions appeared (see also Ref. 35).

Replacement of external Cl^- by glutamate$^-$ or other slowly penetrating anions significantly decreased the resting membrane potential in Jurkat lymphoblasts (see Fig. 4) and this may be the basis of the recently reported findings [40] that in these cells stimulus-induced calcium influx was significantly inhibited by external Cl^- removal. According to the same report, DIDS, an inhibitor of Cl^- transport, had a similar blocking effect on this particular calcium movement. Since DIDS in the concentrations applied is known to affect SH-groups [41], membrane depolarization may be a common source of the observed changes in receptor-stimulated calcium influx.

The possible regulatory role of membrane potential on the calcium-dependent stimulation of lymphoid cells has been examined in a series of reports by Gelfand et al. [2,19,37,42]. They found an inhibitory effect of membrane depolarization, evoked, e.g., by an increase in external K^+ concentration, both on stimulus-induced calcium influx and lymphocyte proliferation. In chemotactic peptide stimulated human neutrophils [21], carbachol-treated parotid acinar cells [20], or most recently in vasopressin-stimulated hepatocytes [22] similar findings were reported. The present paper, reinforcing and extending these observations by a careful separation of the components of the stimulus-induced calcium signal and concomittantly measuring the membrane potential in human lymphoblasts, demonstrates and quantitates the selective and powerful inhibition of the calcium influx pathway by membrane depolarization. It is important to mention that the reduction of the inward calcium gradient caused by this membrane depolarization may not be the basis of the observed effects as changes in $[Ca^{2+}]_o$ of more than two orders of magnitude (between 5 and 2500 μM) did not significantly alter the rate of stimulus-induced calcium influx.

According to the data in the literature (see Ref. 43), the modulation of the properties of the membrane receptors and/or the components of the calcium signal transduction pathway through protein kinase action may have a basic physiological importance. Recently we have demonstrated the elimination of the aCD3-induced calcium signal in Jurkat lymphoblasts when protein kinase C was activated by phorbol ester or by a soluble DAG [44]. However, in these cases the development of the signal is completely blocked and the activation of protein kinase C in the Jurkat lymphoblasts by the above agents did not yield a significant membrane depolarization (Sarkadi and Tordai, unpublished data).

A recent report in Nature [13] indicates, that receptor-operated (thrombin-induced) calcium channels in excised platelet membrane fragments do not show a voltage sensitivity. In contrast, the above cited studies and the data presented here suggest that *in situ*, in the living cell membrane, such channels are not voltage-dependent, but they are certainly voltage sensitive — that is not opened but inhibited by membrane depolarization. Thus the induction of an increased conductive Na^+ and/or Cl^- permeability in the cell membrane (the latter is known to occur e.g. during volume regulation of various cell types — see Refs. 30, 31 and 45) should affect calcium-dependent cell stimulation. Several endogenous modulators or pharmacological agents may specifically depolarize certain cell types (see, for example, reports on cyclosporin action [46,47]), thus the further exploration of such effects is a challenging project in studying cellular regulation.

Acknowledgements

The authors are grateful for the technical assistance of Mrs. M. Sarkadi and for the help in cell culturing by E. Bitai. We also wish to thank Drs. Gy. Görög, M. Benczúr and Tünde Kovács for the consultations and help during this work. The learning of the fine details of the membrane potential measurements by the first author in the collaborative work with Drs. A. Rothstein, S. Grinstein and E. Gelfand is gratefully acknowledged. This work has been supported by a grant of OKKFT-Tt 1.5.1.3. of the Hungarian Academy of Sciences.

References

1 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 69–71.
2 Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159–193.
3 Gelfand, E.W., Mills, G.B., Cheung, R.K., Lee, J.W.W., and Grinstein, S. (1987) Immunol. Rev. 95, 59–87.
4 Putney, J. (1986) Cell Calcium 7, 1–12.
5 Penner, R., Matthews, G. and Neher, E. (1988) Nature 334, 499–504.
6 Catt, K.J. and Balla, T. (1989) Annu. Rev. Med. 40, 487–509.
7 Hosey, M.M. and Lazdunsky, M. (1988) J. Membr. Biol. 104, 81–105.
8 Neher, E. (1987) Nature 326, 242.
9 Meldolesi, J. and Pozzan, T. (1987) Exp. Cell Res. 171, 271–283.
10 Rink, T.J. (1988) Nature 334, 649–650.
11 Petersen, O.H. (1988) Nature 336, 528.
12 Benham, C.D. and Tsien, R.W (1987) Nature 328, 275–278.
13 Zschauer, A., van Breemen, C., Buhler, F.R. and Nelson, M.T. (1988) Nature 334, 703–705.
14 Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Nature 330, 653–655.
15 Irvine, R.F. and Moor, R.M. (1987) Biochem. Biophys. Res. Commun. 146, 284–290.

16 Kuno, M. and Gardner, P. (1987) Nature 326, 301–304.
17 Oettgen, H.C., Terhorst, C., Cantley, L.C. and Rosoff, P.M. (1985) Cell 40, 583–590.
18 Sage, S.O. and Rink, T.J. (1986) Eur. J. Pharmacol. 128, 99–107.
19 Gelfand, E.W., Cheung, R.K., Mills, G.B. and Grinstein, S. (1987) J. Immunol. 138, 527–531.
20 Merritt, J.E. and Rink, T.J. (1987) J. Biol. Chem. 262, 17362–17369.
21 Di Virgilio, F., Lew, D.P., Andersson, T. and Pozzan, T. (1987) J. Biol. Chem. 262, 4574–4579.
22 Savage, A.L., Biffen, M. and Martin, B.R. (1989) Biochem. J. 260, 821–827.
23 Gray, L.S., Gnarra, J.R., Russell, J.H. and Engelhart, V.H. (1987) Cell 50, 119–127.
24 Imboden, J.B., Weiss, A. and Stobo, J.D. (1985) J. Immunol. 134, 663–665.
25 Imboden, J.B. and Weiss, A. (1987) Biochem. J. 247, 695–700.
26 Ng, J., Fredholm, B.B., Jondal, M. and Andersson, T. (1988) Biochim. Biophys. Acta 971, 207–214.
27 Grynkievicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
28 Hladky, S.B. and Rink, T.J. (1976) J. Physiol. 263, 287–319.
29 Waggoner, A.S. (1979) Methods Enzymol. 55, 689–695.
30 Grinstein, S., Clarke, C.A., DuPre, A. and Rothstein, A. (1982) J. Gen. Physiol. 80, 801–823.
31 Sarkadi, B., Attisano, L., Grinstein, S., Buchwald, M., and Rothstein, A. (1984) Biochim. Biophys. Acta 774, 159–168.
32 Montecucco, C., Pozzan, T. and Rink, T.J. (1979) Biochim. Biophys. Acta 552, 552–557.
33 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530.
34 Sarkadi, B., Mack, E. and Rothstein, A. (1984) J. Gen. Physiol. 83, 513–527.
35 Magócsi, M., Sarkadi, B., Kovács, T. and Gárdos, G. (1989) Biochim. Biophys. Acta 984, 88–96.
36 Jy, W. and Haynes, D.H. (1987) Biochim. Biophys. Acta 929, 88–102.
37 Gelfand, E.W., Cheung, R.K., Mills, G.B. and Grinstein, S. (1988) Eur. J. Immunol. 18, 917–922.
38 Sage, O.S., Merritt, J.E., Hallam, T.J. and Rink, T.J. (1989) Biochem. J. 258, 923–926.
39 Rothstein, A. (1981) in The Function of Red Blood Cells: Erythrocyte Pathobiology, (Wallach, D.F.H., ed.), pp. 105–131, Alan R. Liss, New York.
40 Rosoff, P.M., Hall, C., Gramates, L.S. and Terlecky, S.R. (1988) J. Biol. Chem. 263, 19535–19540.
41 Knauf, P.A. (1979) in Current Topics in Membranes and Transport, Vol. 12 (Bronner, F. and Kleinzeller, A., eds.), pp.251–365, Academic Press, New York.
42 Gelfand E.W., Macdougals, S.L., Cheung, R.K. and Grinstein, S. (1989) J. Exp. Med. 170, 315–320.
43 Nishizuka, Y. (1988) Nature 334, 661–665.
44 Tordai, A., Sarkadi, B., Görög, Gy. and Gárdos, G. (1989) Immunol. Lett. 20, 47–52.
45 Grinstein, S., Rothstein, A., Sarkadi, B. and Gelfand, E.W. (1984) Am. J. Physiol. 246, C204–215.
46 Gelfand, E.W., Cheung, R.K. and Mills, G.B. (1987) J. Immunol. 138, 115–120.
47 Damjanovich, S., Aszalos, A., Mulhern, S.A., Szölösi, J., Balázs, M., Trón, L. and Fulweyler, M.J. (1987) Eur. J. Immunol. 17, 763–768.