Inhibition of calcium signalling in murine splenocytes by polyamines: differential effects on CD4 and CD8 T-cells

Thresia THOMAS,* Uma B. GUNNIA,† Edward J. YURKOW,‡ James R. SEIBOLD† and T. J. THOMAS†§

Departments of *Environmental and Community Medicine and ‡Pharmacology and Toxicology, and the †Program in Clinical Pharmacology, Clinical Research Center, *†University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School, Piscataway, NJ 08854, and ‡Rutgers University, Piscataway, NJ 08854, U.S.A.

Transmembrane Ca^{2+} influx is recognized as a universal second messenger that transduces T-cell activation signals to cytoplasm and nucleus, thereby stimulating transcription and cell division. To examine the role of endogenous factors that regulate mitogenic Ca^{2+} signalling of T-cells, we measured the concanavalin (Con) A-induced increase in cytoplasmic free calcium ($[\text{Ca}^{2+}]_i$) in spleen cells of BALB/c mice, using flow cytometry with an indicator dye, Indo-1 acetoxymethyl ester (Indo-1/AM). Con A is a polyclonal activator of T-cells. Unstimulated splenocytes had a $[\text{Ca}^{2+}]_i$ of 100 nM. $[\text{Ca}^{2+}]_i$ increased with Con A in a dose-dependent manner up to a concentration of 50 $\mu\text{g}/\text{ml}$. In the presence of 50 $\mu\text{g}/\text{ml}$ Con A, $[\text{Ca}^{2+}]_i$ was 350 nM. Natural polyamines (putrescine, spermidine and spermine) inhibited Con A-induced Ca^{2+} influx in a dose-dependent manner. Putrescine

was the most effective polyamine in desensitizing the Ca^{2+} signal, and decreased $[\text{Ca}^{2+}]_i$ from 350 nM in the absence of putrescine to 250 nM in the presence of 100 μM putrescine. This effect was not mimicked by structurally related homologues or inorganic cations, suggesting a specific structural effect of the polyamine. H.p.l.c. analysis showed that polyamines were internalized during incubation of cells *in vitro*. In experiments using monoclonal anti-CD4 and anti-CD8 antibodies, we found a differential effect of putrescine on Ca^{2+} influx in CD4 and CD8 subpopulations of T cells. For CD4⁺ cells, $[\text{Ca}^{2+}]_i$ decreased from 625 nM to 420 nM in the presence of 500 μM putrescine, whereas $[\text{Ca}^{2+}]_i$ was not affected by putrescine in CD8⁺ cells. These data suggest that natural polyamines have cell-specific effects on mitogen-stimulated Ca^{2+} -influx in T-cell subsets.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are organic oligocations present in all living cells (Tabor and Tabor, 1984; Pegg, 1988). In mammalian cells, spermidine and spermine are present in millimolar concentrations, and their levels are tightly regulated by cell cycle, trophic signals and differentiation status of the cell. Putrescine levels are normally lower, but its biosynthesis is induced by cell growth stimuli, including hormones, mitogens and growth factors. Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines (Russell, 1985). Induction of ODC activity and elevation of polyamines are considered to be essential for the progression of cell division and stimulation of transcription. Cellular polyamine concentrations are controlled at different levels, but one of the most important pathways is the delicate regulation of ODC at transcriptional, translational and post-translational levels (Heby and Persson, 1990). Increased levels of polyamines participate in the suppression of their own synthesis by inhibiting the translation of ODC mRNA and by facilitating the degradation of ODC protein. In a number of pathological conditions, however, the tight control mechanism(s) of ODC and polyamines are lost, resulting in the accumulation of high levels of intracellular polyamines. This occurs in malignant cells (Russell and Durie, 1978) and in peripheral mononuclear cells of patients with several of the disorders of the immune system. Recent results show that mononuclear cells of patients with rheumatoid arthritis

(Flescher et al., 1989) and acquired immunodeficiency syndrome ('AIDS') (Colombatto et al., 1989) have intracellular contents of polyamines 2–20-fold higher than that of normal controls. Since these diseases share the feature of a down-regulation of immune function, it is pertinent to ask whether increased level of polyamines contribute to impaired T-cell function by interfering with the mitogenic signal-transduction processes.

Recent research on signal transduction has revealed Ca^{2+} as a universal second messenger for many hormones, neurotransmitters and other external stimuli, transducing cell-surface activation signals to initiate diverse intracellular processes, resulting in the transcription of responsive genes, DNA synthesis and cell division (Altman et al., 1990). Using the fluorescent dye quin 2, Tsien et al. (1982) first demonstrated that mitogens such as concanavalin (Con) A caused a rapid increase in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$). Elevation of $[\text{Ca}^{2+}]_i$ is central to the activation of T-cells mediated by antigenic recognition of T-cell receptors (Gardner, 1989). Important regulatory information is encoded in the intensity, frequency and amplitude of the Ca^{2+} signal. Cytosolic Ca^{2+} signals can be generated from intracellular stores as well as from an extracellular Ca^{2+} pool such as that contained in the growth medium. Transmembrane Ca^{2+} mobilization and the presence of extracellular Ca^{2+} appear to be essential for the production of the lymphotrophic hormone interleukin-2 (IL-2) (Mills et al., 1985).

Recent reports suggest a regulatory role of polyamines in T-cell function, particularly in the production of IL-2 (Flescher et

Abbreviations used: Con A, concanavalin A; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.; ODC, ornithine decarboxylase; IL-2, interleukin-2; DFMO, difluoromethylornithine; HBSS, Hanks balanced salt solution; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Indo-1/AM, Indo-1 acetoxymethyl ester.

§ To whom correspondence should be sent, at: Clinical Research Center, UMDNJ—Robert Wood Johnson Medical School, New Brunswick, NJ 08903-0019, U.S.A.

al., 1989). Increased levels of polyamines down-regulate the production of IL-2, whereas polyamine depletion with an ODC inhibitor difluoromethylornithine (DFMO) increases its production (Bowlin et al., 1987). In the present investigation, we examined whether intracellular polyamines interfere with transmembrane Ca^{2+} mobilization in mouse T-cells stimulated with the mitogenic lectin, Con A. Our experiments show that Con A-induced transmembrane Ca^{2+} mobilization in splenocytes from BALB/c mice is inhibited by exogenous putrescine, spermidine and spermine in a dose-dependent manner. These effects are specific to the polyamine structure, as revealed by a lack of inhibitory effect by closely related polyamine homologues and inorganic cations. Further studies showed that polyamines exert differential effects on Ca^{2+} influx with CD4^+ and CD8^+ subpopulations of T-cells.

EXPERIMENTAL

Preparation of splenocytes and separation of T-cells

BALB/c mice (5 weeks old) were obtained from the Jackson Laboratory, Bar Harbor, ME, U.S.A., and housed in our certified vivarium. At 14 weeks of age, mice were killed by cervical dislocation, spleens were removed, and single-cell suspensions were made in Hanks balanced salt solution (HBSS) containing 1.2 mM Ca^{2+} , by gentle teasing. The cells were filtered through nylon mesh to remove debris and possible tissue contamination.

Purified T-cells were obtained from splenocytes by selectively depleting B-cells with J11D antibody (Accurate Chemical and Scientific Corp., Westbury, NY, U.S.A.). To each 100×10^6 cells, $100 \mu\text{l}$ of J11 D antibody was added and mixed well. After 1–2 min, the cell suspension was treated with 9 vol. of the low-toxic rabbit complement (Accurate Chemical and Scientific Corp.) and diluted in cytotoxicity medium (Cedarlane, Toronto, Ont., Canada). The mixture was incubated for 1 h at 37°C , centrifuged at $400 g$ for 5 min, washed with cytotoxicity media and then with RPMI medium. The cells were counted with a haemocytometer by the Trypan Blue exclusion method. Cells were resuspended in serum-free RPMI supplemented with 0.1% crystalline BSA, at a concentration of 1.5×10^6 cells/ml. These cells were viable for 8 h at 22°C , as determined by Trypan Blue exclusion.

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

Indo-1 acetoxymethyl ester (Indo-1/AM) was obtained from Molecular Probes (Junction City, OR, U.S.A.) in $50 \mu\text{g}$ portions and stored at -70°C . Indo-1/AM was dissolved in anhydrous dimethyl sulphoxide at 0.5 mM concentration and stored at 4°C in the dark. Cells ($1.5 \times 10^6/\text{ml}$) were loaded with $1 \mu\text{M}$ Indo-1/AM by incubating at 37°C in a shaking water bath for 40 min. Loading efficiency was determined by removing samples at 10, 20, 30, 40 and 60 min and measuring the fluorescence ratio, F_{500}/F_{400} , with a flow cytometer. Loading for time periods of up to 30 min produced cells with low fluorescence intensity for accurate determination of the fluorescence ratio. Loading for 60 min produced cells that responded slowly and exhibited $[\text{Ca}^{2+}]_i$ levels lower than that observed with 40 min loading, as a consequence of Indo-1/AM buffering effects (Ng et al., 1988). Loading for 40 min at 37°C produced cells with optimal fluorescence intensity and response to mitogens. The loaded cells were removed from the water bath and maintained at room temperature (22°C) until assay time to minimize dye leakage. We found that keeping cells at room temperature in RPMI supple-

mented with 0.1% BSA was the most efficient way of maintaining cells in terms of viability, minimal dye leakage and optimal response to the Ca^{2+} ionophore ionomycin (Calbiochem, San Diego, CA, U.S.A.).

Cells were analysed with a Coulter Epics 753 Flow Cytometer equipped with a Coherent model Innova 90 argon ion laser. Changes in Indo-1/AM fluorescence were recorded by using 150 mW output of the 356 nm laser light to excite and 400 nm and 500 nm filters on the photomultiplier tube for detection. Cells were gated from debris by using forward-angle light scatter and 90° light scatter (log). When excited near the optimal wavelength of 365 nm , Indo-1/AM showed a shift of the fluorescence emission maximum from 500 nm for free Indo-1/AM to 400 nm for the Ca^{2+} -bound complex. The ratio of the fluorescence intensity at 500 nm to 400 nm could therefore indicate the Ca^{2+} content of the cells, independent of the actual Indo-1/AM concentration within the cell. Ionomycin was used at a concentration of $1\text{--}2 \mu\text{M}$ to check the instrument set-up and cellular dye loading. Since our instrument was set up to compute the fluorescence ratio, F_{500}/F_{400} (R) versus time, increase in Ca^{2+} uptake was detected by a decrease in R . We used a method for calibration *in situ* to convert R into $[\text{Ca}^{2+}]_i$ by using the procedure described by Chused et al. (1987). We computed the mean fluorescence ratio of cells within short time intervals (40 s) by projecting slices of the two parameter histograms on the screen, and calculating the range, mean, S.D. and coefficient of variation within this time interval by using software available from Coulter. The mean fluorescence ratio was converted into $[\text{Ca}^{2+}]_i$ and plotted against time for each experiment.

BALB/c splenocytes or purified T-cells were prepared and loaded with Indo-1/AM as described above and analysed at typical flow rate of 200 cells/s. For each experiment, base-line unstimulated ratio measurement was followed by addition of the desired stimulus. The cell flow was stopped, and the sample line was back-flushed to remove non-triggered cells before analysis was resumed. This time gap in analysis was kept to a minimum of 15–20 s.

Two-colour flow cytometry

Anti-mouse L3T4-phycoerythrin (PE) (anti-CD4) and Lyt2-fluorescein isothiocyanate (FITC) (anti-CD8) antibodies were purchased from Becton-Dickinson and Co. (San Jose, CA, U.S.A.). Purified splenic T-cells were loaded with Indo-1, treated with L3T4-PE and Lyt2-FITC at a concentration of $4 \mu\text{l}/10^6$ cells and incubated at room temperature (22°C) for 30 min. Samples were then analysed on a Coulter Epics 753 flow cytometer designed to allow simultaneous recordings of u.v., red and green fluorescence. Two lasers with excitation wavelengths at 488 and 355 nm were used at 200 and 100 mW power, respectively. Cells were gated from debris by using forward-angle light scatter. Green fluorescence was measured through 560 nm short-pass dichroic and 525 nm band-pass filters. Red fluorescence was measured through the 560 nm short-pass dichroic and 575 nm band-pass filter. The violet (400 nm) signal of Indo-1/AM for Ca^{2+} was measured with a 450 nm long-pass dichroic and a 400 nm band-pass filter. The blue (500 nm) signal of Indo-1/AM was measured through 450 nm long-pass dichroic and 500 nm band-pass filters. Both 400 and 500 nm signals were processed through a 380 nm laser blocking filter. Four photomultiplier tubes were used for simultaneous detection of the signals. The cells that were surface stained for CD4 and CD8 were gated on each subpopulation. After stimulation with Con A, the intracellular Ca^{2+} determinations were recorded simultaneously for each subset.

Figure 1 Representative histograms showing transmembrane Ca^{2+} mobilization in BALB/c splenocytes after stimulation with $1 \mu\text{M}$ ionomycin (a) and $50 \mu\text{g/ml}$ Con A (b)

The symbols in this Figure represent clusters of cells or events.

Polyamine assay

BALB/c spleen cells were treated with 8% sulphosalicylic acid ($1 \text{ ml}/10^7$ cells) and sonicated in an ice bath for 30 s. The cells were incubated in ice for 1 h to precipitate the proteins completely. The precipitate was removed by centrifugation for 5 min at 12000 g . The supernate was analysed for polyamines by an established h.p.l.c. method (Kabra et al., 1986; Singh et al., 1992) after pre-column conversion into dansyl derivatives.

RESULTS

Effects of Con A and ionomycin on Ca^{2+} mobilization in BALB/c splenocytes

Figure 1 shows two parameter histograms (fluorescence ratio at 500 nm to 400 nm, R , versus time) of ionomycin- and Con A-

Figure 2 Effects of different concentrations of Con A on $[\text{Ca}^{2+}]_i$ (●) and delay times (▲) in BALB/c splenocytes

We computed the mean fluorescence ratio (F_{500}/F_{400}) of cells within short time intervals (40 s) by projecting slices of the two parameter histograms on the screen using software available with the Coulter instrument. The mean fluorescence ratio was converted into $[\text{Ca}^{2+}]_i$ by using a calibration method *in situ*. Peak $[\text{Ca}^{2+}]_i$ and delay times were calculated from plots of $[\text{Ca}^{2+}]_i$ versus time. The delay time is defined as the time interval between Con A addition and attainment of peak mean $[\text{Ca}^{2+}]_i$.

induced Ca^{2+} mobilization in BALB/c splenocytes loaded with the indicator dye Indo-1/AM. The histograms show 9 min recordings in which ionomycin or Con A was added by stopped-flow injection method after recording baseline readings for 40 s. R is generally independent of the intracellular concentration of the dye, and hence monitoring R against time is a convenient method to detect changes in $[\text{Ca}^{2+}]_i$. With $1 \mu\text{M}$ ionomycin, transmembrane Ca^{2+} influx was instantaneous, as indicated by a sharp decrease in R (Figure 1a). At lower concentrations of ionomycin, however, there was a time-dependent Ca^{2+} influx. The response of T-cells to ionomycin-induced Ca^{2+} influx was almost homogeneous, and R attained its minimum value, corresponding to 1000 nM $[\text{Ca}^{2+}]_i$, within 60 s after addition of ionomycin.

Figure 1(b) shows the effect of $50 \mu\text{g/ml}$ Con A on transmembrane Ca^{2+} influx in BALB/c splenocytes. Con A-induced Ca^{2+} mobilization occurred at a slower rate than that of ionomycin, and the peak value of $[\text{Ca}^{2+}]_i$ was approx. 50% of that with ionomycin. The Con A-induced increase in $[\text{Ca}^{2+}]_i$ was dose-dependent up to a concentration of $50 \mu\text{g/ml}$ Con A (Figure 2). Therefore we conducted all subsequent measurements at $50 \mu\text{g/ml}$ Con A.

The increased level of $[\text{Ca}^{2+}]_i$ observed in our experiments could be due to influx of extracellular Ca^{2+} or a result of Ca^{2+} release from intracellular pools, or a combination of the two processes. A role for Ca^{2+} influx from extracellular sources in T-lymphocyte activation was suggested by its dependence on extracellular Ca^{2+} (Alcovier et al., 1986). In contrast, in human tumour T-cell lines such as REX, substantial increase in $[\text{Ca}^{2+}]_i$ was effected even in the absence of extracellular Ca^{2+} , suggesting Ca^{2+} mobilization from intracellular stores (Alcovier et al., 1986). The requirement for extracellular Ca^{2+} for the rise in $[\text{Ca}^{2+}]_i$ has also been reported to vary with experimental systems (Imboden and Stobo, 1985; Oettgen et al., 1985). To confirm the source of increased $[\text{Ca}^{2+}]_i$ in our system, we conducted fluorescence measurements in the presence of a Ca^{2+} chelator, EGTA. For these experiments, samples of Indo-1-loaded splenic T cells (1.5×10^6 cells/ml) were suspended in HBSS containing 1 mM Ca^{2+} or that containing 1 mM $\text{Ca}^{2+} + 2 \text{ mM}$ EGTA. The cells

were then analysed for their ability to mobilize Ca^{2+} by stimulation with ionomycin or with Con A. Cells suspended in Ca^{2+} -containing HBSS showed results similar to those presented in Figure 1. In contrast, there was no significant change in the fluorescence ratio R when cells were stimulated with Con A in the presence of EGTA. Similarly, there was no change in the fluorescence ratio of Con A-stimulated cells when cells were suspended in Ca^{2+} -free HBSS and repeatedly washed with the same medium. These results may indicate an essential requirement for extracellular Ca^{2+} in order to cause an increase in $[\text{Ca}^{2+}]_i$. However, the possibility of buffering of low-intensity Ca^{2+} transients by Indo-1/AM should also be considered. In experiments using ionomycin, there was a transient rise in Ca^{2+} even in the presence of EGTA (results not shown), suggesting intracellular Ca^{2+} mobilization. The amount of $[\text{Ca}^{2+}]_i$ in this case was, however, less than 20% of the total Ca^{2+} rise when the cells were stimulated with ionomycin in the absence of EGTA. It therefore appears that ionomycin could mobilize intra- and extra-cellular Ca^{2+} in mouse splenocytes. In contrast, the Con A-stimulated Ca^{2+} rise appears to be from the extracellular Ca^{2+} pool under our experimental conditions.

Effects of polyamines on Ca^{2+} influx in Con A-stimulated splenocytes

In the next set of experiments, we examined the effect of the exogenous polyamines putrescine, spermidine and spermine on Con A-stimulated Ca^{2+} influx. In these experiments, different concentrations of polyamines (10–1000 μM) were added to Indo-1/AM-loaded cells, incubated for 30 min at room temperature, and $[\text{Ca}^{2+}]_i$ was determined in the presence of 50 $\mu\text{g}/\text{ml}$ Con A. Figure 3 shows the effects of different concentrations of polyamines on $[\text{Ca}^{2+}]_i$. All three polyamines inhibited Ca^{2+} influx in a dose-dependent manner. Putrescine was the most effective, and decreased $[\text{Ca}^{2+}]_i$ from a peak of 350 ± 25 nM in untreated cells to 250 ± 20 nM in the presence of 100 μM putrescine ($n = 4$). Above this concentration, there was no major alteration in $[\text{Ca}^{2+}]_i$. With spermidine and spermine, the maximum inhibition of Ca^{2+} influx occurred at 500 μM concentrations, and the $[\text{Ca}^{2+}]_i$ values reached a plateau at approx. 270 nM. The delay time, or the time required for cells to attain the maximal mean value of $[\text{Ca}^{2+}]_i$ after addition of Con A, is also shown in Figure 3.

We also examined whether polyamines exerted an inhibitory effect on ionomycin-induced Ca^{2+} mobilization. Ionomycin is known to by-pass the membrane receptor pathway (Truneh et al., 1985; Chatila et al., 1989), and hence its ability to mobilize transmembrane Ca^{2+} influx is not altered by agents that specifically affect the structure and transport properties of membrane receptors. Our experiments with putrescine, spermidine and spermine showed that polyamines had no inhibitory effect on transmembrane Ca^{2+} influx in cells stimulated with ionomycin (results not shown).

Internalization of polyamines in splenocytes

In order to confirm whether polyamines were internalized during our incubation of cells before Con A stimulation, we determined intracellular polyamine pools in parallel sets of splenocytes after incubation with putrescine, spermidine and spermine for different time points. The cells were then washed in PBS to remove extracellular polyamines, and were analysed for polyamine pools by h.p.l.c. after pre-column derivative formation to their dansyl derivatives (Kabra et al., 1986); the results are presented in Table

Figure 3 Effects of putrescine (●, ○), spermidine (▲, △) and spermine (■, □) on Con A-stimulated Ca^{2+} influx and delay times in BALB/c splenocytes

Cells were incubated with indicated concentrations of polyamines for 30 min and Con A (50 $\mu\text{g}/\text{ml}$) was added. $[\text{Ca}^{2+}]_i$ and delay times were calculated from the histograms. Similar results were obtained in three separate experiments.

Table 1 Internalization of polyamines on incubation with BALB/c splenocytes

Results shown are means $\pm$ S.D. of triplicate measurements

Polyamine added	Intracellular polyamine concn. (pmol/ 10^6 cells)		
	Putrescine	Spermidine	Spermine
None	46 $\pm$ 8	249 $\pm$ 41	316 $\pm$ 50
100 μM putrescine, 30 min	180 $\pm$ 26	274 $\pm$ 35	352 $\pm$ 42
1 mM putrescine, 30 min	232 $\pm$ 15	226 $\pm$ 20	360 $\pm$ 40
1 mM spermidine, 30 min	55 $\pm$ 10	450 $\pm$ 24	440 $\pm$ 56
1 mM spermine, 30 min	56 $\pm$ 10	302 $\pm$ 30	625 $\pm$ 60
1% putrescine <i>in vivo</i>	250 $\pm$ 25	315 $\pm$ 21	352 $\pm$ 18

1. Incubation of splenocytes with 1 mM putrescine, spermidine or spermine increased their intracellular concentrations by 5-, 2- and 2-fold respectively within 30 min. These results are comparable with those in recent reports showing rapid uptake of diamines by rat lung epithelial cells, mouse splenocytes and malignant-cell lines (Oreffo et al., 1991; Singh et al., 1992).

Effect of administration of putrescine *in vivo* on Ca^{2+} influx

We further examined whether administration of putrescine *in vivo* could produce a similar increase in intracellular putrescine levels and the inhibition of mitogenic Ca^{2+} signalling. For this purpose, we treated a group of BALB/c mice with 1% putrescine in drinking water for 1 week and determined Ca^{2+} influx of splenic T cells after Con A stimulation. Peak $[\text{Ca}^{2+}]_i$ was only 250 ± 25 nM ($n = 3$), compared with 350 ± 25 nM in splenocytes from untreated animals (results not shown). These results confirm that administration of polyamines *in vivo* also inhibits the ability of splenic T-cells to transduce the mitogenic Ca^{2+} signal. Polyamine analysis of cells from mice treated with putrescine *in vivo* showed a 5-fold increase in intracellular putrescine concentration compared with splenocytes from untreated mice (Table 1). Thus supplementation of splenic T-cells with exogenous polyamines, either by addition *in vitro* or by administration *in vivo*, results in

Table 2 Effect of bivalent cations on Con A-induced Ca^{2+} mobilization in BALB/c splenocytes

Cells were stimulated with 50 $\mu\text{g}/\text{ml}$ Con A after 30 min incubation with bivalent cations, of which 100 μM concentrations were used. Results are means $\pm$ S.D. ($n = 3$).

Cation added	$[\text{Ca}^{2+}]_i$ (nM)	Delay time (s)
None	350 ± 25	180 ± 20
Putrescine	250 ± 20	320 ± 25
1,3-Diaminopropane	380 ± 35	185 ± 25
1,5-Diaminopentane	360 ± 35	215 ± 20
1,6-Diaminohexane	390 ± 30	210 ± 25
Mg^{2+}	375 ± 25	200 ± 15
Zn^{2+}	365 ± 25	215 ± 25

an increase of intracellular polyamine pools and inhibition of mitogenic signalling through the Ca^{2+} second messenger.

Structural effects of polyamines on Ca^{2+} influx

The observed inhibitory effect of a polyamine such as putrescine on transmembrane Ca^{2+} mobilization may be a result of ionic effects due to the competition between putrescine $^{2+}$ and Ca^{2+} for anionic sites on the cell membrane. Another possibility is that putrescine may be exerting a specific effect linked to its structural geometry and the number of hydrophobic methylene groups. To distinguish between these two possibilities, we examined the effects of three structurally related homologues of putrescine and two bivalent cations (Mg^{2+} and Zn^{2+}) on Con A-stimulated Ca^{2+} influx at the optimum putrescine concentration (100 μM). Results of this set of experiments are presented in Table 2. Our results show that lower and higher putrescine homologues differing by even one methylene group had no effect on the ability of Con A to mobilize extracellular Ca^{2+} . Furthermore, the inorganic cations Mg^{2+} and Zn^{2+} had no effect on Ca^{2+} influx. These results clearly show that the effect of putrescine on the mitogenic Ca^{2+} -signalling pathway is highly specific to its chemical structure. The structure specificity of putrescine observed in this desensitization of Ca^{2+} mobilization is similar to the effect of polyamines in certain macromolecular interactions, such as polyamine-induced B-DNA to Z-DNA transition (Thomas and Messner, 1988).

Cell-specific effects of putrescine on CD4^+ and CD8^+ subpopulations of T-cells

We next examined whether the effects of putrescine could be segregated within the T-cell subpopulations identified by the cell-surface markers CD4 and CD8. For these experiments, splenic T-cells were purified, loaded with Indo-1/AM and treated with monoclonal anti-CD4 (PE) and anti-CD8 (FITC) antibodies. The fluorescence ratio was not change by addition of antibodies to CD4 or CD8, thereby ruling out a stimulatory effect by these antibodies alone under our experimental conditions. Using a dual-laser set-up, these cells were gated individually on each subset (CD4, red; CD8, green). The fluorescence ratios were then recorded simultaneously in the presence and absence of putrescine on Con A-stimulated cells. Considerable heterogeneity was evident in response to Con A in all cell populations, even after segregating them to the CD4^+ and CD8^+ subpopulations. The response of CD4^+ subset to Con A-induced Ca^{2+} mobilization

Figure 4 Effect of putrescine on mean $[\text{Ca}^{2+}]_i$ in Con A-stimulated CD4^+ (a) and CD8^+ (b) T-cell subsets

$[\text{Ca}^{2+}]_i$ at 0 ($\circ$), 0.5 mM ($\blacktriangle$) and 1 mM ($\bullet$) putrescine are shown. The mean fluorescence ratios and $[\text{Ca}^{2+}]_i$ were computed as described in the legend to Figure 2 and in the text.

was higher than that of the CD8^+ subset. Calculation of the mean Ca^{2+} influx in CD4^+ and CD8^+ subpopulations showed a differential effect of putrescine on these populations: putrescine inhibited Ca^{2+} mobilization in CD4^+ cells, but had no effect on CD8^+ cells (Figure 4a and 4b). Thus $[\text{Ca}^{2+}]_i$ decreased from 625 ± 40 to 420 ± 25 nM ($n = 4$) in CD4^+ cells after treatment with 500 or 1000 μM putrescine. In contrast, $[\text{Ca}^{2+}]_i$ in CD8^+ cells

remained virtually unchanged at 400 ± 35 nM in the absence and presence of putrescine.

Con A-induced Ca^{2+} influx in BALB/c T-cells is heterogeneous even within CD4 and CD8 subsets of T-cells, probably due to differential uptake of Ca^{2+} by different subpopulations within these subsets. In order to evaluate this heterogeneity in CD4 and CD8 subsets, we constructed overlay plots of cell population versus $[\text{Ca}^{2+}]_i$. Our analysis showed that the percentage of cells with poor or no response to Con A-induced Ca^{2+} influx was increased by 2-fold in putrescine-treated CD4⁺ cells. In contrast, there was no change in the cell population at different $[\text{Ca}^{2+}]_i$ levels with CD8⁺ cells as a result of putrescine treatment.

DISCUSSION

Our results show a concentration-dependent inhibition of mouse splenocytes to Con A-induced transmembrane Ca^{2+} influx in the presence of exogenous putrescine, spermidine and spermine. Putrescine is the most effective polyamine to inhibit Ca^{2+} influx in splenic cells and decreases $[\text{Ca}^{2+}]_i$ to 70% of that of untreated cells at 100 μM concentration. This effect is highly specific, since homologues differing by one methylene group or inorganic cations such as Mg^{2+} and Zn^{2+} could not mimic the inhibitory effect of putrescine on Ca^{2+} influx. Spermidine and spermine were less efficient, and decreased $[\text{Ca}^{2+}]_i$ to 270 nM at 500 μM . The inhibitory effect of polyamines on $[\text{Ca}^{2+}]_i$ influx was observed whether the cells were incubated with putrescine *in vitro* or putrescine was administered *in vivo*. Within the T-cell subpopulations, CD4⁺ cells are sensitive to putrescine, whereas CD8⁺ cells are refractory to the effects of polyamines. Overall, our results suggest that cellular polyamine concentrations could regulate the extent of the $[\text{Ca}^{2+}]_i$ response in a subpopulation of T-cells and consequently modulate the function of these cells. In all cases, considerable heterogeneity of cell population with respect to $[\text{Ca}^{2+}]_i$ is observed. Heterogeneity of Ca^{2+} response with T-cell subsets has been reported by other investigations also (Rabinovitch et al., 1986). Information on this heterogeneity is an advantage of flow cytometry compared with conventional fluorimetric methods.

Ca^{2+} influx in CD4⁺ cells could be affected by the binding of monoclonal anti-CD4 antibody. For example, using CD4⁺ hybridoma cell lines, Rosoff et al. (1987) showed a 50% decrease in Ca^{2+} influx after incubating these cells with monoclonal anti-CD4 antibody. In contrast with the observations on hybridoma cells (Rosoff et al., 1987), Budd et al. (1990) reported the absence of any inhibitory action of anti-CD4⁺ antibody on Ca^{2+} influx in CD4 cells isolated from MRL-*lpr/lpr* mice. In our experiments, $[\text{Ca}^{2+}]_i$ in CD4 cells is nearly twice the mean $[\text{Ca}^{2+}]_i$ value in T-cells, and hence it is unlikely that anti-CD4-antibody inhibition of Ca^{2+} influx played a major role in our system.

A preliminary report by Flescher et al. (1991) showed that anti-CD-3-induced intracellular Ca^{2+} mobilization in normal human peripheral lymphocytes was suppressed by spermidine. They also found significantly decreased Ca^{2+} influx in T-cells of rheumatoid-arthritis patients that contained constitutively higher levels of polyamines than that of normal lymphocytes. Cell-specific inhibition of Ca^{2+} mobilization in splenic T-cells was also reported recently with regard to the opiate-induced immunosuppression (Sei et al., 1991). They also observed a maximum effect of morphine on CD4⁺ cells, and concluded that the morphine effect was probably effected through increased glucocorticoids. Our data, as well as those of Sei et al. (1991), show that CD8⁺ cells are not affected by polyamines and opiate, respectively, indicating a relative insensitivity of this cell population.

There are several reports that link polyamines to the Ca^{2+} signalling, particularly to Ca^{2+} transport in mitochondria and sarcoplasmic reticulum (Schuber, 1989; De Meis, 1991). Micromolar concentrations of spermidine and spermine were found to block $\text{Ins}(1,4,5)\text{P}_3$ -independent unidirectional Ca^{2+} efflux induced by Ca^{2+} -releasing drugs in sarcoplasmic reticulum (Palade, 1987). It was also found that polyanions and polycations had opposing (stimulatory versus inhibitory) effects on the uptake of Ca^{2+} by cardiac microsomes (Xu and Kirchberger, 1989). Szöllösi et al., (1989) also observed delayed Ca^{2+} response in the presence of excess polyamines in A172 human glioblastoma cells. Koenig et al. (1983a,b), on the other hand, found that a transient increase in ODC activity and polyamine concentrations is necessary for hormonal stimulation of $[\text{Ca}^{2+}]_i$ fluxes and the mediation of several Ca^{2+} -dependent membrane transport processes, suggesting the role of polyamines as second messengers.

A possible mechanism for the action of polyamines in Ca^{2+} signalling might involve the binding of polyamines with membrane phospholipids, probably through electrostatic interactions between the phosphate groups and positively charged amino groups. For example, spermine interacts with negatively charged phospholipids, particularly polyphosphoinositides, and phosphatidylinositol, but not InsP_3 (Tadolini and Varani, 1986). Veraga et al. (1986) showed that spermine blocked Ca^{2+} signalling in electrically stimulated muscle fibres by interfering with the production of InsP_3 . Other enzymes that might be affected by polyamines include InsP_3 5-phosphatase as well as kinases involved in the regulation of phosphatidylinositol (Schacht, 1976; Seyfred et al., 1984). These reports suggest that polyamines might interact with phosphoinositide, inhibit its metabolism and thereby block a pathway mediating Ca^{2+} entry into the cell. However, since the role of InsP_3 in transmembrane Ca^{2+} mobilization is still controversial (Ng et al., 1988; Lewis and Cahalan, 1989; Guoy et al., 1990), other mechanisms may also be operative in the action of polyamines in Ca^{2+} signalling in T-cells.

Polyamines have been recognized as cell growth-regulatory molecules with multiple functions which are not as yet well defined (Pegg, 1986; Tabor and Tabor, 1984). Activity of a variety of enzymes involved in DNA replication, transcription and translation is modulated by polyamines (Tabor and Tabor, 1984). They selectively modulate the transcription of growth-associated genes (Celano et al., 1989) and the function of T-cell subpopulations (Ehrke et al., 1986). Polyamines are essential for cell growth; depletion of polyamines by inhibiting the activity of their biosynthetic enzymes such as ODC by DFMO results in cell growth inhibition (Metcalf et al., 1978). Paradoxically, polyamines act as immunosuppressants in T-cell subpopulations. For example, Flescher et al. (1989) found that exogenous polyamines suppressed IL-2 production in Con A-stimulated T-cells. Polyamine-induced immunosuppression and inhibition of IL-2 production are comparable with that by well-characterized immunosuppressants such as cyclosporin (Nordmann et al., 1989) and FK108 (Lin et al., 1991). Bowlin et al. (1987) further showed that DFMO-mediated polyamine depletion *in vivo* and *in vitro* enhanced Con A-induced IL-2 production. The finding that Ca^{2+} mobilization is a prerequisite for the induction of IL-2 (Mills et al., 1985; Tamura et al., 1990) provides a link between Ca^{2+} influx, IL-2 production and the immunosuppressive effects of polyamines.

In summary, our results show that increased levels of polyamines down-regulate mitogen-stimulated transmembrane Ca^{2+} mobilization in murine T-cells and suggest a major role for polyamines in Ca^{2+} signal-transduction pathways leading to T-cell proliferation and differentiation. The effects of polyamines

are specific within T-cells subpopulations, the CD4⁺ subset being the most sensitive. The remarkable structural specificity of putrescine in blocking transmembrane Ca²⁺ influx further suggests that natural polyamines are capable of specific interactions with components of the cellular machinery that drives the Ca²⁺ influx.

This work was supported, in part, by grants CA42439, ES05022 (NIEHS Center) and AR 39020 from the National Institutes of Health and by the W. H. Conzen Endowment of the Schering-Plough Foundation.

REFERENCES

- Alcovie, A., Weiss, M. J., Delay, J. F. and Reiner, E. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2614–2618
- Altman, A., Coggeshall, K. M. and Mustelin, T. (1990) *Adv. Immunol.* **48**, 227–359
- Bowlin, T. L., McKown, B. J. and Sunkara, P. S. (1987) *Immunopharmacology* **13**, 143–147
- Budd, R. C., Winslow, G., Inokuchi, S. and Imboden, J. B. (1990) *J. Immunol.* **145**, 2862–2872
- Celano, P., Baylin, S. B. and Casero, R. A., Jr. (1989) *J. Biol. Chem.* **264**, 8922–8927
- Chatila, T., Silverman, L., Miller, R. and Geha, R. (1989) *J. Immunol.* **143**, 1283–1289
- Chused, T. M., Wilson, H. A., Greenblatt, D., Ishida, Y., Edison, L. J., Tsien, R. Y. and Finkelman, F. D. (1987) *Cytometry* **8**, 396–404
- Colombatto, S., De Agostini, M., Corsi, O. and Sinicco, A. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 745–748
- De Meis, L. (1991) *J. Biol. Chem.* **266**, 5736–5742
- Ehrke, M. J., Porter, C. W., Eppolito, C. and Mihich, E. (1986) *Cancer Res.* **46**, 2798–2803
- Flescher, E., Bowlin, T. L., Ballester, A., Houk, R. and Talal, N. (1989) *J. Clin. Invest.* **83**, 1356–1362
- Flescher, E., Ledbetter, J. A., Shieven, G. L., Fossum, D., Alboukrek, D. and Talal, N. (1991) *Arthritis Rheum.* **34**, S80 (abstr.)
- Gardner, P. (1989) *Cell* **59**, 15–20
- Gouy, H., Cefai, D., Christensen, S. B., Debre, P. and Bismuth, G. (1990) *Eur. J. Immunol.* **20**, 2269–2275
- Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158
- Imboden, J. B. and Stobo, J. D. (1985) *J. Exp. Med.* **161**, 446–455
- Kabra, P. M., Lee, H. K., Lubich, W. P. and Marton, L. J. (1986) *J. Chromatogr. Biomed. Appl.* **380**, 19–32
- Koenig, H., Goldstone, A. D. and Lu, C. Y. (1983a) *Nature (London)* **305**, 530–534
- Koenig, H., Goldstone, A. D. and Lu, C. Y. (1983b) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7210–7214
- Lewis, R. S. and Cahalan, M. D. (1989) *Cell Regul.* **1**, 99–112
- Lin, C. S., Boltz, R. C. and Siekierka, J. J. (1991) *Cell. Immunol.* **133**, 269–284
- Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. and Vever, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2557
- Mills, G. B., Cheung, R. Y., Grinstein, S. and Gelfand, E. W. (1985) *J. Immunol.* **134**, 1640–1643
- Ng, J., Fredholm, B. B., Jondal, M. and Andersson, T. (1988) *Biochim. Biophys. Acta* **971**, 207–214
- Nordmann, R., Anderson, E., Trussard, R. and Mazer, N. A. (1989) *Biochemistry* **28**, 1791–1797
- Oettgen, H. C., Terhorst, C., Cantley, L. C. and Rosoff, P. M. (1985) *Cell* **40**, 583–590
- Oreffo, V. I. C., John, R. A. and Richards, R. J. (1991) *Biochem. Pharmacol.* **41**, 1209–1215
- Palade, P. (1987) *J. Biol. Chem.* **262**, 6149–6154
- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- Rabinovitch, P. S., June, C. H., Grossman, A. and Ledbetter, J. A. (1986) *J. Immunol.* **137**, 952–961
- Rosoff, P. M., Burakoff, S. J. and Greenstein, J. L. (1987) *Cell* **49**, 845–853
- Russell, D. H. (1985) *Drug Metab. Rev.* **16**, 1–88
- Russell, D. H. and Durie, B. G. M. (1978) *Prog. Cancer Res. Ther.* **8**, 15–41
- Schacht, J. (1976) *J. Neurochem.* **27**, 1119–1124
- Schuber, F. (1989) *Biochem. J.* **260**, 1–10
- Sei, Y., McIntyre, E., Frider, E., Yoshimoto, K., Skolnick, P. and Arora, P. K. (1991) *FASEB J.* **5**, 2194–2199
- Seyfred, M. A., Farell, I. E. and Wells, W. W. (1984) *J. Biol. Chem.* **259**, 13204–13208
- Singh, A. B., Thomas, T. J., Thomas, T., Singh, M. and Mann, R. A. (1992) *Cancer Res.* **52**, 1840–1847
- Szöllösi, J., Feuerstein, B. G., Pershadsingh, H. A., Basu, H. S. and Marton, L. J. (1989) *Proc. Am. Assoc. Cancer Res.* **31**, 77
- Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- Tadolini, B. and Varani, E. (1986) *Biochem. Biophys. Res. Commun.* **135**, 58–64
- Tamura, T., Mizuguchi, J. and Nariuchi, H. (1990) *J. Immunol.* **145**, 78–84
- Thomas, T. J. and Messner, R. P. (1988) *J. Mol. Biol.* **201**, 463–467
- Truneh, A., Aibert, F., Golstein, P. and Schmitt-Verhulst, A. M. (1985) *Nature (London)* **313**, 318–322
- Tsien, R. Y., Pozzan, T. and Rink, T. J. (1982) *Nature (London)* **295**, 68–71
- Veraga, J., Tsien, R. and Delay, M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6352–6356
- Xu, Z.-C. and Kirchberger, M. A. (1989) *J. Biol. Chem.* **264**, 16644–16651