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The effects of ionophore A23187 and concanavalin A on the membrane potential of human peripheral blood lymphocytes and rat thymocytes

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Effects of the Ca^{2+}-ionophore A23187 and concanavalin A on the membrane potential of human lymphocytes and rat thymocytes have been studied using the fluorescent potential probe diS-C_3-(5). At concentrations of 10^{-8} to 10^{-6} M A23187 changes the membrane potential, inducing both hyper- and depolarization. Depending on concentrations of A23187 and the external Ca^{2+}, and on the type of lymphocytes, one of these effects predominates. The hyperpolarization induced by A23187 is caused by activation of Ca^{2+}-dependent K^+ channels. It is blocked by quinine and high concentrations of extracellular K^+. The dependence of Ca^{2+}-activated K^+ transport on extracellular Ca^{2+} and its sensitivity to calmodulin antagonists is different for human lymphocytes and for thymocytes. As distinct from lymphocytes, in thymocytes calmodulin is not involved in activation of Ca^{2+}-dependent K^+ transport. The depolarization induced in lymphocytes by A23187 is caused by an increase in Na^+ permeability of the lymphocyte plasma membrane: it is eliminated in a low-Na^+ medium. At mitogenic concentrations concanavalin A does not change the membrane potential of the lymphocytes. The results obtained permit elucidation of the relationship between two early events in lymphocyte activation, namely the increase in intracellular Ca^{2+} concentration and the increase in lymphocyte plasma membrane permeabilities to monovalent cations.

Introduction

Changes in membrane permeability and ion transport are among the earliest events in lymphocyte activation [1,2]. These changes, per se or by changing the membrane potential, may play an important regulatory role in mitogenesis. On the other hand, studying the membrane potential may help to reveal a relationship between changes in the transport of various ions across the lymphocyte plasma membrane. At present, ions are considered the most likely messengers transmitting the signal from the mitogenic factor into the cell [1–4]. A search of possible correlations between changes in transport and intracellular concentrations of K^+, Na^+, Ca^{2+} and other ions is a necessary step in the elucidation of biochemical mechanisms of lymphocyte activation.

In the present work, using the fluorescent potential-sensitive probe diS-C_3-(5), we have studied changes in membrane potential of human lymphocytes and rat thymocytes caused by two mitogens: Ca^{2+}-ionophore A23187 and concanavalin A.

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Abbreviations: diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine; SBS, standard buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Cell preparation

Human lymphocytes were obtained from blood of healthy donors by centrifugation on a Ficoll-Isopaque gradient, according to Ref. 5. Monocytes were removed using their ability to adhere to glass and absorb particles of carbonyl iron [5,6]. The cells (10^7 per ml) were incubated in medium 199 at 37°C. Thymocytes were obtained as in Ref. 7. Thymus glands were removed from decapitated Wistar rats (150–180 g) and the thymocytes were teased out through a nylon mesh into medium 199. Before experiment the cells were spun down, filtered once more through the mesh and resuspended at a density 10^7 cells per ml in SBS containing 5.5 mM (0.5 mM in the experiments with K^+ electrode) KCl, 130 mM NaCl, 1.0 mM (or 0.5 mM, or 0.1 mM) $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM Na_2HPO_4. 10 mM Hepes, 5.5 mM glucose, pH 7.3. In some of the experiments NaCl was partially substituted by choline chloride or Na_2SO_4. Cell viability, assessed by trypan blue exclusion, was greater than 95%. All experiments were performed at 37°C.

Experiments with the fluorescent probe

Fluorescence was measured in a special set-up with a built-in spectrofluorimeter [8,9]. 0.2 ml of cell suspension in SBS was put into the spectrometer cuvette containing 1.8 ml of the same buffer so that cell concentration in the cuvette was $(1–3) \cdot 10^6$ per ml. The wavelengths for excitation and emission were 579 and 672 nm, respectively. The cuvette was thermostated, and the suspension magnetically stirred by glass-covered followers. The stock solution of $3 \cdot 10^{-4}$ M diS-C_3-(5) in ethanol was kept at 4°C in the dark. The final ethanol concentration in the cuvette after addition of the dye was not more than 0.2%. DiS-C_3-(5) concentration in the cuvette varied from $3 \cdot 10^7$ to $6 \cdot 10^{-7}$ M, and this had no effect on the results. Reagents were added into the cuvette after a steady level of diS-C_3-(5) fluorescence in the cell suspension had been reached. The effects of the reagents on diS-C_3-(5) fluorescence in the absence of cells were checked in control experiments. Of all the reagents used only quinine increased slightly the fluorescence intensity of the dye itself, and this was taken into account in the experiments with the cells. Precautions necessary when working with diS-C_3-(5) [10] were undertaken. The value of the membrane potential was estimated by a modified 'null-point' method described in detail elsewhere [6,11].

Experiments with potassium electrode

Potassium concentration in the medium was continuously monitored with an 'Orion' K^+-selective electrode in a suspension of 10^8 thymocytes per ml. The cuvette was thermostated and the suspension was magnetically stirred.

Materials

Valinomycin, ouabain, EGTA and ionophore A23187 were obtained from Calbiochem, concanavalin A from Pharmacia, Hepes from Fluka, trifluoperazine from Smith, Kline and French, chlorpromazine and quinine were from Sigma Chemical Company. All other reagents were of analytical grade. DiS-C_3-(5) was synthesized in the laboratory of structure and colour of chemical compounds in the Institute of Organic Chemistry, Academy of Sciences of Ukranian SSR (Kiev).

Results

Effects of A23187: dependence on the ionophore concentration

Human lymphocytes. Addition of A23187 to lymphocytes led to a complex pattern of changes in the fluorescence intensity which may be described as a superposition of hyper- and depolarization (Fig. 1). Both effects depended on the concentration of extracellular Ca^{2+} (see next section). Fig. 1 shows the results for 1 mM Ca^{2+} in the medium.

At concentrations of $(3–6) \cdot 10^{-6}$ M the ionophore induced a marked fall in the fluorescence signal, that is, a substantial hyperpolarization. At lower ionophroe concentrations the hyperpolarization became weaker and depolarization appeared. Thus, in the range $(2–7) \cdot 10^{-7}$ M A23187 a considerable increase in fluorescence was observed after a small drop in the signal (Fig. 1, curves 3 and 4).

The described changes in fluorescence under the action of A23187 (Fig. 1) were observed with

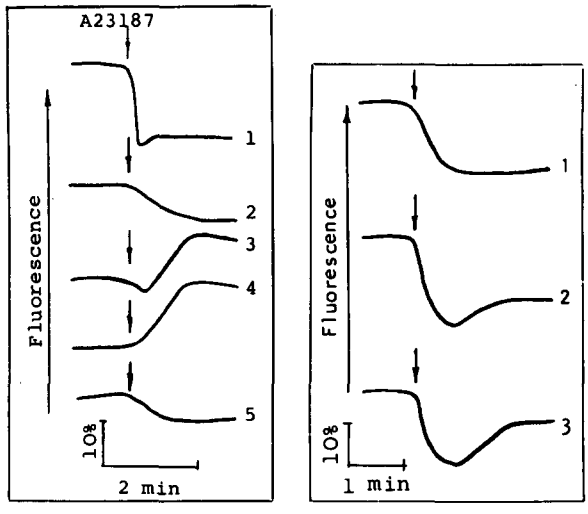

Fig. 1. (left) Changes in the fluorescence of diS-C_3-(5) in lymphocyte suspension upon addition of A23187 at concentrations: (1) $6 \cdot 10^{-6}$ M; (2) $3 \cdot 10^{-6}$ M; (3) $7 \cdot 10^{-7}$ M; (4) $2 \cdot 10^{-7}$ M; (5) $5 \cdot 10^{-8}$ M. Ca^{2+} concentration in the medium is 1 mM. Here and below the 100% level corresponds to the intensity of diS-C_3-(5) fluorescence established in the cell suspension prior to application of reagents.

Fig. 2. Changes in the fluorescence of diS-C_3-(5) in thymocyte suspension upon addition of A23187 at concentrations: (1) $7 \cdot 10^{-7}$ M; (2) $7 \cdot 10^{-8}$ M; (3) $2 \cdot 10^{-8}$ M. Ca^{2+} concentration in the medium is 1 mM.

lymphocytes obtained from six different donors. The general pattern was consistently reproducible; from donor to donor there was variation only in the region of low ($\approx 10^{-8}$ M) A23187 concentrations where hyperpolarization predominated (Fig. 1, curve 5). In some cases the hyperpolarization in this region appeared only in a low-Ca^{2+} medium.

Thymocytes. Application of A23187 produced similar changes in membrane potential of rat thymocytes (Fig. 2). At concentrations of $(3–20) \cdot 10^{-7}$ M the ionophore hyperpolarized the cells, at lower concentrations of A23187 both effects were seen. As compared to lymphocytes, the region of A23187 concentrations where the hyperpolarization predominated was much broader. The effect of depolarization was less pronounced for thymocytes than for lymphocytes: as a rule, we saw only repolarization to the initial value of the membrane potential (Fig. 2, curves 2 and 3).

Effects of A23187: dependence on the external Ca^{2+}

Lymphocytes. The dependence of the fluorescence response to A23187 on the concentration of external Ca^{2+} is shown in Fig. 3. At high concentrations ($\beta \cdot 10^{-6}$ M) the ionophore produced hyperpolarization irrespective of Ca^{2+} concentration in the medium and even in the presence of excess EGTA. At lower A23187 concentrations, the depolarization observed in the presence of 1 mM Ca^{2+} (see above) diminished with lowering of the external Ca^{2+} (Fig. 3C,D). As a result, application of one and the same A23187 concentration may lead to either hyper- or depolarization depending on the concentration of extracellular Ca^{2+} (Fig. 3C,D). On the other hand the effects produced by A23187 were not abolished by subsequent addition of excess EGTA (Fig. 3D).

Addition of Ca^{2+} after A23187 also produced both depolarization and then hyperpolarization (Fig. 4). The relative magnitude of these two effects varied with concentrations of external Ca^{2+} and the ionophore, and from donor to donor. The membrane potential changes in response to application of Ca^{2+} after A23187 indicate that both hyper- and depolarization caused by the ionophore are related to changes in the intracellular Ca^{2+}.

In contrast to lymphocytes, in thymocytes EGTA eliminated the A23187-induced hyperpolarization, which was restored by subsequent addition of excess Ca^{2+} (Fig. 5). Accordingly, in a

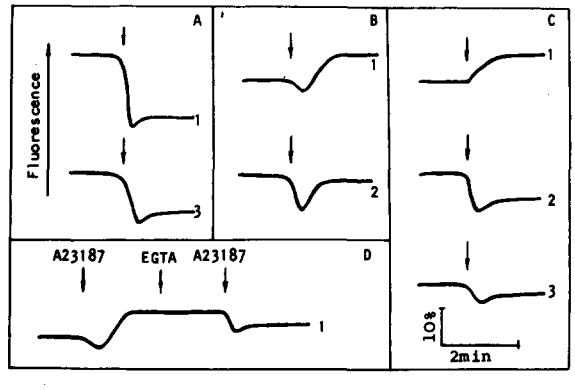

Fig. 3. Effect of extracellular Ca^{2+} on A23187-induced changes in the fluorescence of diS-C_3-(5) in lymphocyte suspension. (1) 1 mM Ca^{2+}; (2) 0.1 mM Ca^{2+}; (3) 0.1 mM Ca^{2+} +1 mM EGTA. The concentrations of A23187 (arrows) are: (A) $6 \cdot 10^{-6}$ M; (B) and (D) $7 \cdot 10^{-7}$ M; (C) $6 \cdot 10^{-8}$ M. EGTA concentration (arrow) is 2 mM.

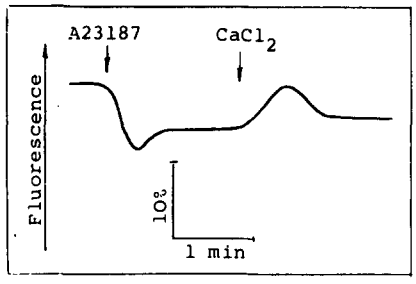

Fig. 4. Changes in the fluorescence of diS-C_3-(5) in lymphocyte suspension upon successive addition of A23187 ($3 \cdot 10^{-6}$ M) and $CaCl_2$ (5 mM). Initially there was 0.1 mM $CaCl_2$ + 1 mM EGTA in the medium.

low-Ca^{2+} medium (1 mM EGTA + 0.1 or even 1 mM Ca^{2+}) application of the ionophore had no effect on the membrane potential.

Thus, changing the Ca^{2+} concentration in the medium modifies the effects induced by A23187 in both types of lymphocytes. The dependence of the effects on external Ca^{2+} is, however, qualitatively different for human lymphocytes and rat thymocytes.

It is to be noted that changing the Ca^{2+} concentration alone in the medium had no effect on the membrane potential of resting thymocytes or lymphocytes. Using a modified 'null-point' method [6,11], the membrane potential value was shown to be the same for lymphocytes in a low-Ca^{2+} medium (0.1 mM Ca^{2+} + 1 mM EGTA), in the presence of 0.1 mM or 1 mM Ca^{2+}. At 5 mM external Ca^{2+} a weak ($\approx$ 5 mV) hyperpolarization was observed as compared to standard medium with 1 mM Ca^{2+}.

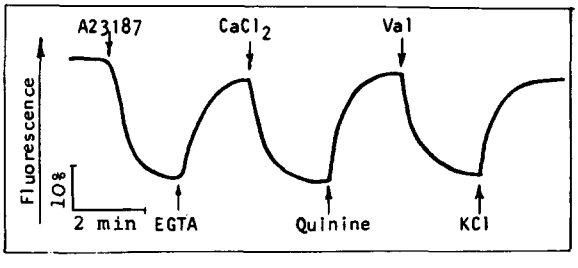

Fig. 5. Changes in diS-C_3-(5) fluorescence in thymocyte suspension upon successive addition of A23187 ($7 \cdot 10^{-7}$ M), EGTA ($5 \cdot 10^{-4}$ M), $CaCl_2$ ($5 \cdot 10^{-4}$ M), quinine ($2.5 \cdot 10^{-4}$ M), valinomycin ($2.5 \cdot 10^{-6}$ M) and KCl ($10 \cdot 10^{-3}$ M). The initial Ca^{2+} concentration in the medium was 0.5 mM

The effects of hyper- and depolarization

Hyperpolarization. The hyperpolarization induced by A23187 is not associated with stimulation of ($Na^+ + K^+$)-ATPase, since the effect did not change in the presence of 1 mM ouabain. The observed hyperpolarization is caused by an increase in K^+ permeability of the lymphocyte plasma membrane. Indeed, the K^+-ionophore valinomycin did not change the membrane potential of lymphocytes in the presence of sufficiently high A23187 concentratoin, though it markedly hyperpolarized control cells (Fig. 6). The membrane potential of lymphocytes became close to E_K upon addition of, for example, $6 \cdot 10^{-6}$ M A23187, that is, the membrane potential value changed from -60 mV for control cells [6] up to approx. -90 mV. On the other hand, A23187 did not hyperpolarize the cells when added after valinomycin (Fig. 6).

Hyperpolarization caused by stimulation of Ca^{2+}-dependent K^+ transport diminished with increase of external K^+: the effect was not observed at 50 mM K^+ in the medium. A23187 did not produce hyperpolarization in the presence of quinine, an inhibitor of Ca^{2+}-activated K^+ channels in red blood cells [12]. And vice versa, the A23187-induced hyperpolarization was abolished by quinine (Figs. 5 and 7). Subsequent application of valinomycin increased K^+ permeability and again induced hyperpolarization (Figs. 5 and 7).

An increase in K^+ efflux from thymocytes in response to A23187 was detected also with a K^+-sensitive electrode (Fig. 8). K^+ efflux from thymocytes caused by A23187 was also essentially eliminated by quinine or EGTA (Fig. 8).

To investigate the possibility of calmodulin involvement in regulation of Ca^{2+}-activated K^+ channels we used the calmodulin antagonists trifluoperazine and chlorpromazine [13]. At concentrations up to 10^{-4} M the phenothiazines did not inhibit but, on the contrary, increased somewhat the A23187-induced K^+ efflux from thymocytes (Fig. 8). This strongly suggests that calmodulin is not involved in stimulation of Ca^{2+}-dependent K^+ transport in rat thymocytes. Similar experiments with lymphocytes are limited by the necessity to use large quantities of volunteers' blood.

It appeared that the phenothiazines changed the

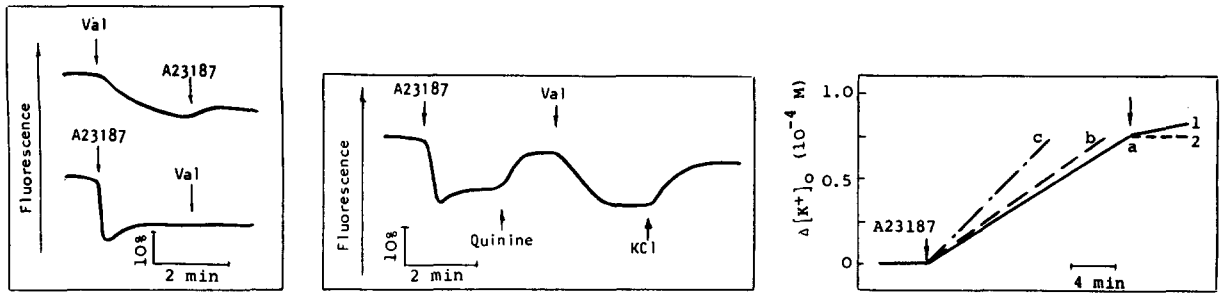

Fig. 6. (left) Effects of A23187 ($6 \cdot 10^{-6}$ M) and valinomycin ($5 \cdot 10^{-6}$ M) on the fluorescence of diS-C_3-(5) in lymphocyte suspension. Ca^{2+} concentration in the medium is 1 mM.

Fig. 7. Changes in diS-C_3-(5) fluorescence in lymphocyte suspension upon successive addition of A23187 ($6 \cdot 10^{-6}$ M), quinine ($2.5 \cdot 10^{-4}$ M), valinomycin ($5 \cdot 10^{-6}$ M) and KCl ($11 \cdot 10^{-3}$ M). Initially there was 0.1 mM $CaCl_2$ + 1 mM EGTA in the medium.

Fig. 8. (right) Effect of A23187 ($1.5 \cdot 10^{-6}$ M) on K^+ efflux from rat thymocytes in the absence of trifluoperazine (a) and in the presence of $3 \cdot 10^{-5}$ M (b) or $1 \cdot 10^{-4}$ M trifluoperazine (c). Ordinate: change of K^+ concentration in the medium; the initial concentration of extracellular K^+ was 0.5 mM. The arrow indicates application of 1 mM quinine (1) or 1 mM EGTA (2).

membrane potential of resting lymphocytes and thymocytes. Application of trifluoperazine or chlorpromazine, even at micromolar concentrations, induced depolarization of the cells as monitored by diS-C_3-(5) (Fig. 9). This effect of depolarization is not caused by an inhibition of the Na^+,K^+-pump (it was preserved in the presence of 1 mM ouabain) or by a decrease in K^+ permeability (Fig. 8); on the other hand, it weakened in a low-Na^+ medium. Our results on the effects of calmodulin antagonists on the membrane potential of lymphocyte and rat thymocytes, for resting cells and under the action of A23187, are presented in detail elsewhere [14].

Depolarization. To investigate the nature of depolarization produced by A23187, we determined whether this effect was preserved in a low-Na^+ medium (SBS with Na^+ substituted by choline). For lymphocytes, the depolarization almost disappeared in such medium, whereas the effect of hyperpolarization remained essentially unchanged (Fig. 10). This indicates that in lymphocytes the depolarization induced by A23187 may be caused by a stimulation of Na^+ transport under the action of Ca^{2+}-ionophore. In contrast with this, for thymocytes lowering the external Na^+ or replacing Cl^- by SO_4^{2-} in the medium did not eliminate the

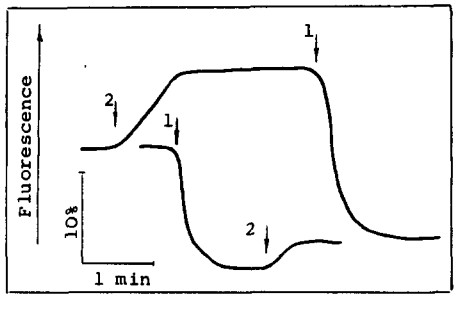

Fig. 9. Changes in diS-C_3-(5) fluorescence in thymocyte suspension upon addition of $5 \cdot 10^{-7}$ M A23187 (1) and $1 \cdot 10^{-5}$ M trifluoperazine (2). Ca^{2+} concentration in medium is 1 mM.

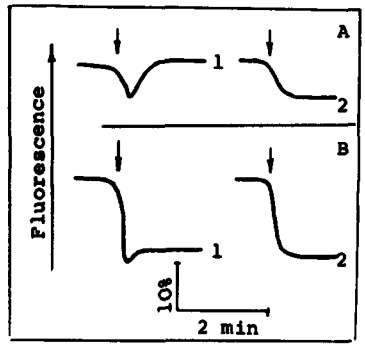

Fig. 10. Changes in the fluorescence of diS-C_3-(5) upon addition of A23187 to lymphocytes suspended in the medium with normal (1) and low (2) Na^+ concentration: (1) 142 mM Na^+; (2) 22 mM Na^+, 120 mM choline$^+$. Ca^{2+} concentration in the medium is: (A) 0.1 mM; (B) 1 mM. A23187 concentrations are: (A) $7 \cdot 10^{-7}$ M, (B) $6 \cdot 10^{-6}$ M.

contribution of repolarization in the effects induced by A23187.

Effect of concanavalin A

At concentrations up to 50 μg/ml concanavalin A had no effect on the fluorescence of diS-C_3-(5) in suspension of lymphocytes or rat thymocytes. An estimation by the modified 'null-point' method [6,11] showed that possible variations of the membrane potential value under the action of concanavalin A in this concentration range were less than the error limits of the method (±4 mV). Only at much higher concentrations (150–200 μg/ml) concanavalin A induced a weak (≈7 mV) depolarization of lymphocytes.

Discussion

The data obtained show that the ionophore A23187 can induce both hyper- and depolarization of lymphocytes. Which of these effects predominates depends on the concentrations of A23187 and the external Ca^{2+}, and on the type of lymphocytes (Figs. 1–3).

The hyperpolarization of human lymphocytes and rat thymocytes is caused by activation of Ca^{2+}-dependent K^+ transport under the action of A23187 (Figs. 5–8). In the present work changes in the membrane potential of these cells were monitored using the fluorescent probe diS-C_3-(5). Previously, Ca^{2+}-activated K^+ channels in lymphocytes have not been detected by means of cyanine fluorescent dyes. Using diS-C_3-(5) only A23187-induced depolarization was observed in mouse splenocytes [11]. Hyperpolarization caused by A23187 was observed in another type of lymphocytes with the use of an oxonol dye [15]. It is known that cyanine fluorescent probes can interfere with Ca^{2+}-activated K^+ channels [16]. However, the kinetics of the dye-channel interaction is rather slow. The inhibition of Ca^{2+}-dependent K^+ transport by diS-C_3-(5) depends essentially on the concentration of external K^+ and is observed only in a low-K^+ (< 2 mM) medium [11,16–18]. The results of the present work demonstrate that, taking all this into account, one can successfully use diS-C_3-(5) to study Ca^{2+}-dependent K^+ channels in lymphocytes. The fact that A23187-induced hyperpolarization was not detected in mouse splenocytes [11] may be explained not by diS-C_3-(5) interaction with Ca^{2+}-dependent K^+ channels but probably by predominance of the effect of depolarization at the A23187 concentration used in that work. Note that data obtained with oxonol dyes should also be considered with caution, since these dyes can stimulate Ca^{2+}-dependent K^+ transport [19].

Previously, Ca^{2+}-activated K^+ channels have been found in mouse thymocytes [15,20], human lymphocytes [21–23] and (probably) pig mesenteric lymphocytes [20]. In the present work this type of K^+ transport was studied by monitoring changes in both the membrane potential and the rate of K^+ efflux. For rat thymocytes, the picture of membrane potential changes caused by alteration of Ca^{2+}-dependent K^+ transport (Fig. 5) is identical with that observed for the red cells [18]. In lymphocytes, as distinct from thymocytes and red blood cells, application of EGTA does not eliminate the hyperpolarization induced by A23187 (Fig. 3). The increase in cytosolic Ca^{2+} in lymphocytes is probably due to Ca^{2+} release from intracellular stores [24]. Other properties of Ca^{2+}-activated K^+ channels in human lymphocytes and rat thymocytes, namely their inhibition by quinine (Figs. 5, 7, 8) or high concentrations of external K^+, are similar to those in the red cells [25].

Calmodulin antagonists do not inhibit K^+ efflux induced by A23187 in thymocytes (Figs. 8 and 9). This indicates that calmodulin is not involved in activation of Ca^{2+}-dependent K^+ channels in these cells. The A23187-induced K^+ efflux is even potentiated slightly in the presence of the phenothiazines (Fig. 8), which can be explained by their inhibition of Ca^{2+}-ATPase leading to an elevation of cytosolic Ca^{2+} [26].

In lymphocytes, in contrast, trifluoperazine and chlorpromazine cause a reduction in A23187-induced K^+ efflux, as follows from the data of Refs. 21 and 22. It should be noted that the data on calmodulin involvement in regulation of Ca^{2+}-dependent K^+ transport are, in general, controversial: both inhibition [27,28] and stimulation [26,29] of A23187-induced K^+ efflux by phenothiazines has been observed. Thus, the conclusion can be made that calmodulin is not necessary for the work of Ca^{2+}-dependent K^+ channels.

The depolarization induced in lymphocytes by

A23187 is eliminated in a low-Na^+ medium (Fig. 10), hence the effect is caused by an increase in Na^+ permeability. On the other hand, the depolarization depends essentially on the external Ca^{2+} (Fig. 3). These data suggest the possibility for Ca^{2+} regulation of not only potassium but also sodium permeability of lymphocyte plasma membrane. This may be of importance because the increase in Na^+ permeability probably plays a role in mitogenic lymphocyte stimulation [30,31]. A possible mechanism for the increase in Na^+ permeability may be via stimulation of Na^+/H^+ exchange [32,33]. Na^+ influx is increased on activation of a number of stimulus-secretion coupling processes and cell proliferation. For instance, increasing the concentration of cytosolic Ca^{2+} in acinar pancreatic cells by a stimulus or A23187 results in an increase in Na^+ permeability [34]; the serum or growth factors induce an increase in Na^+ influx in fibroblasts which depends on both intra- and extracellular Ca^{2+} [35,36].

Thus, an increase in the intracellular Ca^{2+} results in an increase in both K^+ and Na^+ permeabilities of lymphocyte plasma membrane. This can explain the interplay of the effects of hyper- and depolarization leading to variability of the patterns of fluorescence response to application of A23187.

As shown by flow cytometry [37] and penetrating ion [38] techniques, concanavalin A and its derivative succinyl-Con A do not change the membrane potential of lymphocytes at mitogenic concentrations; a weak depolarization has been observed only in a small subpopulation of lymphocytes [37]. Our results for lymphocytes obtained with the fluorescent potential probe are consistent with these data. Concanavalin A is known to increase both K^+ and Na^+ permeabilities of lymphocyte plasma membrane [2], and to change its properties [1]. These simultaneous and oppositely directed effects possibly compensate for each other and therefore the membrane potential of lymphocytes is nearly unchanged under the action of concanavalin A.

Mitogenic concentrations of concanavalin A induce hyperpolarization in mouse thymocytes [15,20]; in rat thymocytes we observed no changes in membrane potential upon addition of concanavalin A. Our data and the results of others show that depending on the type of lymphocytes and the mitogen used one can observe both hyper- [15,20] and depolarization [31,39] or no change in the membrane potential [37,38] on mitogenic stimulation of lymphocytes. Hence, it seems unlikely that membrane potential changes per se play a role in lymphocyte activation.

Calcium ions are the most likely secondary messengers in mitogenic stimulation of lymphocytes [3,4]. However, the interdependence between changes in the concentration of cytosolic Ca^{2+} and other events of lymphocyte activation, in particular stimulation of K^+ and Na^+ transport, remains unclear. The results obtained allow elucidation of the relationship between the increase in intracellular Ca^{2+} concentration and the increase in lymphocyte plasma membrane permeabilities to monovalent cations.

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