Early plasma-membrane-potential changes during stimulation of lymphocytes by concanavalin A

Suzanne M. FELBER and Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. We have monitored the plasma-membrane potential of lymphocytes by measuring the accumulation of the lipophilic cation methyltriphenylphosphonium (TPMP⁺) in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP). 2. The mitogen concanavalin A causes a decrease in TPMP⁺ accumulation by pig lymphocytes corresponding to a 3 mV depolarization within 2½ min. Concanavalin A does not alter ⁸⁶Rb⁺ uptake in the first 30 min. 3. In contrast concanavalin A increased TPMP⁺ accumulation and the rate of Rb⁺ uptake in mouse thymocytes. This is consistent with a previous proposal that the mitogen induces a hyperpolarization of mouse thymocytes as a result of stimulation of a Ca²⁺-dependent K⁺ channel. 4. Studies with the calcium ionophore A23187 and quinine (an inhibitor of the Ca²⁺-dependent K⁺ channel) suggest that the channel is partially closed in mouse resting thymocytes but is almost fully active in pig resting cells. Thus concanavalin A hyperpolarizes mouse thymocytes by activating the Ca²⁺-dependent K⁺ channel but cannot do so in pig lymphocytes because the channel is already maximally activated. 5. The 3 mV depolarization of pig cells cannot be explained by a decrease in electrogenic K⁺ permeability.

Changes in $\Delta\psi_p$ may be important in the control of cell proliferation (Cone, 1971; Sachs *et al.*, 1974; Cone & Cone, 1976).

A depolarization of $\Delta\psi_p$ is among many events reported to occur early in the course of stimulation of human peripheral lymphocytes (Taki, 1970; Shapiro *et al.*, 1979) and mouse spleen cells (Kiefer *et al.*, 1980). However, as yet, none of the techniques applied to determination of $\Delta\psi_p$ in these lymphocytes has provided a reliable quantitative measure of changes that occur on treatment with mitogen.

In contrast, an early hyperpolarization of $\Delta\psi_p$ has been observed in mouse thymocytes (Tsien *et al.*, 1982) by using the fluorescent dye bis-oxonol. These workers have carried out a crude calibration of the dye signal and report that the hyperpolarization is of the order of 20 mV. They have shown that this early

increase in $\Delta\psi_p$ accompanies a rise in free cytoplasmic [Ca²⁺] and have attributed it to stimulation of a Ca²⁺-activated K⁺ conductance in the plasma membrane.

The question of whether a change in $\Delta\psi_p$ could constitute a signal for lymphocyte mitogenesis remains to be answered. The variation in the response of the lymphocyte $\Delta\psi_p$ to mitogens reported above may result from the use of different techniques to monitor $\Delta\psi_p$. If, however, lymphocytes from different species or tissues do not have a common $\Delta\psi_p$ response, then an alteration in $\Delta\psi_p$ in itself is unlikely to be an important general mechanism for triggering activation of these cells.

We have measured previously the pig lymphocyte $\Delta\psi_p$ and described the factors contributing to this potential in the resting cell (Felber & Brand, 1982a). We have now extended this study to mitogen-treated cells. In the present paper we investigate and quantify the effects of the mitogen concanavalin A on the pig lymphocyte $\Delta\psi_p$. We also study the effect of concanavalin A on TPMP⁺ accumulation by mouse thymocytes. In addition we try to relate the observed changes in $\Delta\psi_p$ to alteration in K⁺ fluxes that may be occurring as a result of mitogen action. Our observation that

Abbreviations used: $\Delta\psi_p$, difference in electrical potential across the plasma membrane (positive outside), given a positive sign throughout the present paper; $\Delta\psi_m$, difference in electrical potential across the mitochondrial membrane (positive outside); TPMP⁺, methyltriphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

concanavalin A causes a very early depolarization of pig lymphocytes has already been published as an abstract (Felber & Brand, 1982b).

Experimental

Materials

$^{86}\text{RbCl}$, $[^3\text{H}]\text{inulin}$ and $^3\text{H}_2\text{O}$ were from Amersham International, Amersham, Bucks., U.K. $[^3\text{H}]\text{TPMP}^+$ bromide was from New England Nuclear Chemicals, Dreieich, Germany. RPMI 1640 culture medium was from Flow Laboratories, Irvine, Scotland, U.K. Ouabain, FCCP and quinine hydrochloride were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. A23187 was from Eli Lilly and Co., Indianapolis, IN, U.S.A. Concanavalin A was from Miles Laboratories, Research Products Division, Stoke Poges, Slough, Bucks., U.K. Bis-oxonol [bis-(1,2-diethylthiobarbiturate)trimethine oxonol] was a gift from Dr. T. J. Rink, Department of Physiology, University of Cambridge. All other reagents were of analytical grade.

Preparation of pig mesenteric lymphocytes and mouse thymocytes

Pig lymphocytes were prepared from lymph nodes dissected from the mesentery of a freshly slaughtered pig as described previously (Felber & Brand, 1982a). The cells responded normally to concanavalin A ($2.5\mu\text{g}/\text{ml}$) as assayed by turnover of phosphatidylinositol by using an adaptation of the method of Moore *et al.* (1982). Mouse thymocytes were prepared from 6–8-week-old mice [Balb/c or CFLP (Anglia Lab.)] as in Felber & Brand (1982a).

Determination of $[^3\text{H}]\text{TPMP}^+$ and $^{86}\text{Rb}^+$ accumulation by pig and mouse lymphocytes

Cells were pre-incubated in standard medium (RPMI 1640/10mM-Hepes, pH 7.4) for 30 min at 37°C at a concentration of $(2\text{--}6) \times 10^7$ cells $\cdot \text{ml}^{-1}$. Each experiment was started by mixing cells with an equal volume of medium with appropriate additions. Final concentrations of these were: $3\mu\text{M}$ -tetraphenylboron, $0.1\mu\text{Ci}$ of $[^3\text{H}]\text{TPMP}^+/\text{ml}$ (50 nM) and $0.02\mu\text{Ci}$ of $^{86}\text{RbCl}/\text{ml}$. Other additions are described in the Figure legends. Samples (1 ml) were taken in duplicate at relevant times and centrifuged for 2 min in an Eppendorf bench centrifuge. Samples of supernatant and whole pellets were assayed for radioactivity as described previously (Felber & Brand, 1982a). Parallel determinations of intra- and extra-cellular pellet spaces were performed with each experiment. The procedure was as described above except that cells were incubated with $0.02\mu\text{Ci}$ of $^{86}\text{RbCl}/\text{ml}$ and either $0.8\mu\text{Ci}$ of $[^3\text{H}]\text{inulin}/\text{ml}$ ($56\mu\text{M}$) or $1.5\mu\text{Ci}$ of $^3\text{H}_2\text{O}/\text{ml}$. $\Delta\psi_p$ and accumulation ratios of TPMP^+ and Rb^+ were calculated

with corrections for extracellular space and for TPMP^+ binding as in Felber & Brand (1982a).

Fluorescence experiments using bis-oxonol

These experiments were performed in a Perkin-Elmer MPF 44A spectrophotometer by the method of Rink *et al.* (1980) as described in Felber & Brand (1982a). Details of dye and other additions are given in Figure legends.

Results

In the present paper $\Delta\psi_p$ was monitored by following distribution of the radiolabelled lipophilic cation TPMP^+ in the presence of the mitochondrial uncoupler FCCP. We have discussed this technique fully in a recent paper (Felber & Brand, 1982a) and have shown it to give a reliable measure of the resting potential of pig mesenteric lymphocytes.

Effect of concanavalin A on pig lymphocyte $\Delta\psi_p$

Pig mesenteric lymphocytes were found to depolarize by 3 mV when incubated with the mitogen concanavalin A (Fig. 1). The depolarization was apparent within 2½ min and was maintained for at least 3 h (results not shown). The concentration of concanavalin A optimal for pig lymphocyte mitogenesis, measured by $[^3\text{H}]\text{thymidine}$ uptake at

Fig. 1. Effect of concanavalin A on pig lymphocyte $\Delta\psi_p$. The $[^3\text{H}]\text{TPMP}^+$ accumulation ratio was measured as described in the Experimental section in the presence of $5\mu\text{M}$ -FCCP. Concanavalin A ($2.5\mu\text{g}/\text{ml}$) was present where indicated. The results are means of seven experiments. The difference in TPMP^+ accumulation ratio between control and concanavalin A-treated cells is significant at $P < 0.01$ (Wilcoxon's signed rank test).

Fig. 2. Effect of concanavalin A concentration on pig lymphocyte $\Delta\psi_p$

The [³H]TPMP⁺ concentration ratio was measured at 10 min at different concentrations of concanavalin A in the presence of 5 μM-FCCP as described in the Experimental section. The results are from two experiments.

Fig. 3. Effect of concanavalin A on TPMP⁺ accumulation by pig lymphocytes

The [³H]TPMP⁺ concentration ratio was measured as described in the Experimental section. Concanavalin A (2.5 μg/ml) was present where indicated. The results are means of four experiments. The difference in TPMP⁺ accumulation ratio between control and concanavalin A-treated cells is significant at $P < 0.01$ (Wilcoxon's signed rank test).

42–48 h, is variable but normally around 2.5 μg/ml (Hesketh *et al.*, 1977; Metcalfe *et al.*, 1980). We found that concentrations of concanavalin A ranging from 2.5 μg/ml up to 25 μg/ml caused only a slight additional depolarization (Fig. 2). However, higher concentrations did improve the reproducibility of the effect from day to day.

To eliminate the possibility that the $\Delta\psi_p$ response was influenced by the FCCP needed for the assay we looked at the effect of concanavalin A on steady-state TPMP⁺ accumulation in the absence of the uncoupler. Under these conditions TPMP⁺ accumulates into the mitochondria and thus the TPMP⁺ accumulation ratio at equilibrium is a function of both $\Delta\psi_p$ and $\Delta\psi_m$ (Deutsch *et al.*, 1979; Hoek *et al.*, 1980; Scott & Nicholls, 1980; Davis *et al.*, 1981; Felber & Brand, 1982a). Concanavalin A induced a small decrease in accumulation of the cation (Fig. 3). Assuming that $\Delta\psi_m$ remains constant over 30 min and that $\Delta\psi_p$ of control cells is 56–60 mV (Felber & Brand, 1982a; the present work) then the drop in TPMP⁺ accumulation corresponds to a plasma-membrane depolarization of about 3 mV. Thus the mitogen-induced depolarization of pig lymphocytes (Fig. 1) is neither dependent on, nor reduced in magnitude by, FCCP.

Effect of concanavalin A on TPMP⁺ accumulation by mouse thymocytes

Tsien *et al.* (1982) observed a 20 mV hyperpolarization of mouse thymocytes a few minutes after treatment with concanavalin A. We have confirmed this effect by using bis-oxonol to monitor $\Delta\psi_p$, although our observations suggest a rather smaller hyperpolarization of less than 10 mV (results not shown). We investigated the apparent discrepancy between these results and our results with pig cells. We found that addition of mitogen to mouse cells caused an increase in the accumulation ratio of TPMP⁺ at 5 min from 127 ± 10 ($n = 5$) to 150 ± 11 ($n = 5$) (means $\pm$ S.E.M.), quite the opposite of the effect on pig cells in Fig. 3. This increase could be due to hyperpolarization of $\Delta\psi_p$. We could not see a direct effect of concanavalin A on $\Delta\psi_p$ measured by TPMP⁺ distribution in the presence of uncoupler. Using bis-oxonol we found that FCCP itself hyperpolarizes $\Delta\psi_p$ of mouse thymocytes (results not shown), perhaps owing to the severe ATP depletion observed on treatment of mouse cells with mitochondrial poisons (Pozzan *et al.*, 1980). This is not the case in pig cells (Felber & Brand, 1982a).

Thus concanavalin A hyperpolarizes mouse thymocytes whichever measuring technique is used.

Why do pig lymphocyte and mouse thymocyte $\Delta\psi_p$ respond differently to concanavalin A?

Tsien *et al.* (1982) proposed that concanavalin A alters $\Delta\psi_p$ of mouse cells by causing a rise in cytoplasmic free $[Ca^{2+}]$, which activates a plasma-membrane K^+ conductance of the sort described in red cells by Lew & Ferreira (1978). Our measurements of the Rb^+ influx rates of concanavalin A-treated cells are compatible with their proposal. We

see an increase in the rate of Rb^+ accumulation into mouse cells after addition of mitogen (Fig. 4). Furthermore stimulation of Rb^+ influx does not occur in the presence of quinine, an inhibitor of the Ca^{2+} -activated K^+ channel in red cells (Armando-Hardy *et al.*, 1975), in barnacle photoreceptors (Hanani & Shaw, 1977) and in pancreatic islet cells (Atwater *et al.*, 1979). Thus it is reasonable to

Fig. 4. Effect of concanavalin A on Rb^+ uptake into mouse thymocytes

The $^{86}\text{Rb}^+$ accumulation ratio was measured as described in the Experimental section. ●, Control; ○ and △, $+2\mu\text{g}$ of concanavalin A/ml; ▲ and △, $+0.5\text{mM}$ -quinine. The results are from one representative experiment.

Fig. 5. Effect of quinine and A23187 on Rb^+ uptake into pig resting lymphocytes

The $^{86}\text{Rb}^+$ accumulation ratio was measured as described in the Experimental section. Quinine (0.5mM) and 500nM -A23187 were present, where indicated. The results are from one representative experiment.

Table 1. Effect of quinine and A23187 on $\Delta\psi_p$ and Rb^+ accumulation by resting and concanavalin A-treated pig lymphocytes

$[^3\text{H}]\text{TPMP}^+$ and $^{86}\text{Rb}^+$ accumulation ratios were measured at 30 min as described in the Experimental section in the presence of $5\mu\text{M}$ -FCCP. Concanavalin A was added at $5\mu\text{g}/\text{ml}$, quinine and A23187 were added at 0.5mM and 500nM respectively. The results are means of two experiments.

Addition	$\Delta\psi_p$ (mV)		$^{86}\text{Rb}^+$ accumulation ratio	
	Concanavalin		Concanavalin	
	Control	A	Control	A
—	64.3	61.3	2.23	2.23
Quinine	40.9	38.5	1.73	1.63
A23187	65.0	62.6	2.31	2.26

attribute the hyperpolarization of mouse thymocytes by concanavalin A to activation of the Ca^{2+} -dependent K^+ channel after a rise in cytoplasmic free $[\text{Ca}^{2+}]$. Since pig cells do not hyperpolarize when stimulated by concanavalin A either cytoplasmic free $[\text{Ca}^{2+}]$ does not rise in response to mitogen in these cells or the Ca^{2+} -activated K^+ channel is in a different functional state. We tested the latter possibility by looking at the effect of quinine and the Ca^{2+} ionophore A23187 on $\Delta\psi_p$ and Rb^+ accumulation by pig resting cells. Quinine substantially lowered $\Delta\psi_p$ (Table 1) and also decreased Rb^+ influx (Fig. 5). A23187 did not significantly alter $\Delta\psi_p$ (Table 1) but did slightly decrease Rb^+ accumulation (Fig. 5).

Thus pig cells have a quinine-sensitive K^+ conductance that cannot be further activated by increasing free cytoplasmic $[\text{Ca}^{2+}]$.

In the presence of FCCP quinine still markedly decreased Rb^+ uptake (Table 1). However, under these uncoupled conditions A23187 no longer decreased Rb^+ accumulation (Table 1). The drop in Rb^+ accumulation seen on addition of A23187 in the absence of uncoupler (Fig. 5) may therefore be due to the deleterious effect of the ionophore on $\Delta\psi_m$ (S. M. Felber & M. D. Brand, unpublished work).

To consolidate our results using TPMP $^+$ we have employed the fluorescent probe bis-oxonol to enable us to make a direct comparison between the effects of quinine and A23187 on $\Delta\psi_p$ of mouse resting thymocytes and $\Delta\psi_p$ of pig resting cells. Figs. 6(a) and 7 show that quinine depolarizes both mouse and pig cells, whereas A23187 induces a hyperpolarization of mouse cells as reported by Tsien *et al.* (1982) but has no significant effect on pig cells. In the presence of quinine, A23187 did not hyperpolarize mouse thymocytes (Fig. 6b), thus confirming that the response of $\Delta\psi_p$ to ionophore in these cells is mediated by a Ca^{2+} -activated K^+ channel.

Fig. 6. Effect of A23187 and quinine on mouse thymocyte $\Delta\psi_p$ measured with bis-oxonol

Fluorescence was measured as described in the Experimental section. Additions were 167 nM-bis-oxonol, 1.8×10^6 mouse thymocytes/ml, 0.5 mM-quinine and 50 nM-A23187 at the points indicated. An upward deflection of the trace indicates a depolarization.

Our results from both radiolabelled tracer and fluorescent probe studies confirm the presence of a Ca^{2+} -activatable K^+ conductance in the mouse thymocyte plasma membrane and demonstrate that such a channel also exists in pig lymphocytes. However, the channel is only partly activated in mouse resting thymocytes, whereas in pig unstimulated cells it is virtually fully active.

Hence we would not expect any rise in free cytoplasmic $[\text{Ca}^{2+}]$ (resulting from mitogen action) to be accompanied by an increased K^+ conductance and hyperpolarization of $\Delta\psi_p$ in pig cells.

Why do pig cells depolarize on treatment with mitogen?

The experiments described above show why pig cells do not hyperpolarize after concanavalin A treatment, but do not explain why they depolarize. We looked to see if the depolarization could also be accounted for by an alteration in K^+ permeability, this time a decrease. Table 1 shows that concanavalin A causes depolarization of $\Delta\psi_p$ under conditions both of total inhibition of the Ca^{2+} -dependent K^+ channel (plus quinine) and of maximal activation (plus A23187). The effect of concanavalin A on pig cells is therefore independent of the degree of activation of this channel. Ouabain, an inhibitor of the Na^+ pump, markedly reduces Rb^+

influx into pig cells (Fig. 8). We assume that the remaining ouabain-insensitive K^+ influx rate reflects the electrogenic K^+ permeability of the cells. We found that concanavalin A has no effect on ouabain-insensitive Rb^+ influx into pig cells within 30 min (Fig. 8). Thus the depolarization of $\Delta\psi_p$ induced by concanavalin A in pig lymphocytes cannot be attributed to a decrease in total electrogenic K^+ permeability.

Discussion

In the present paper we show that concanavalin A depolarizes pig lymphocytes by 3 mV but, in contrast, hyperpolarizes mouse thymocytes. The difference is caused by the resting state of the Ca^{2+} -activated K^+ channel. In unstimulated mouse thymocytes the channel is only partly activated, and a rise in cytoplasmic free $[\text{Ca}^{2+}]$ is able to increase K^+ permeability and hence cause hyperpolarization (Tsien *et al.*, 1982). In unstimulated pig lymphocytes the channel is fully activated, so any rise in cytoplasmic free $[\text{Ca}^{2+}]$ does not alter K^+ fluxes through the channel.

The Ca^{2+} -activated K^+ channel has been well-characterized in the red cell (Lew & Ferreira, 1978). An increase in K^+ permeability triggered by a rise in cytosolic $[\text{Ca}^{2+}]$ has also been observed in some

Fig. 7. Effect of quinine and A23187 on pig lymphocyte $\Delta\psi_p$ measured with bis-oxonol

Fluorescence was measured as described in the Experimental section. Additions were 67 nM-bis-oxonol, 1.8×10^6 pig lymphocytes/ml, 0.5 mM-quinine and 50 nM- and 100 nM-A23187 at the points indicated. An upward deflection of the trace indicates a depolarization.

vertebrate and invertebrate neurons (Meech, 1978) in β -cells of the pancreas (Atwater *et al.*, 1979) and in liver (Weiss & Putney, 1978). Different activation states of the Ca^{2+} -dependent K^+ channel have previously been reported in red cells and liver cells from different species (Lew & Ferreira, 1978; Burgess *et al.*, 1981). We cannot say why the channel is activated differently in mouse and pig lymphocytes. It could be that resting cytoplasmic free $[\text{Ca}^{2+}]$ is different, that the channel is sensitive to Ca^{2+} in a different concentration range or that other effectors are at different concentrations or have different affinities for the channel.

Since the changes in $\Delta\psi_p$ in mouse and pig cells that we and others see differ in direction it seems improbable that any particular change in $\Delta\psi_p$ is itself an important general mitogenic signal in lymphocytes.

If a rise in cytoplasmic free $[\text{Ca}^{2+}]$ is the important primary signal for lymphocyte mitogenesis (see Metcalfe *et al.*, 1980) then hyperpolarization of $\Delta\psi_p$ will occur as a secondary response only in those cells with the Ca^{2+} -dependent K^+ channel in an activatable form. Lym-

Fig. 8. Effect of concanavalin A on Rb^+ uptake into pig lymphocytes in the presence and absence of ouabain. The $^{86}\text{Rb}^+$ accumulation ratio was measured as described in the Experimental section. ●, Control; ○ and □, +2.5 μg of concanavalin A/mg; ■ and □, +0.2 mM-ouabain. The results are means of four experiments. The difference in $^{86}\text{Rb}^+$ accumulation ratio between control and concanavalin A-treated cells is significant at $P < 0.05$ in the absence of ouabain and insignificant in the presence of ouabain (Wilcoxon's signed rank test).

phocytes that do not respond by hyperpolarizing, such as pig cells (the present work) and human cells (Shapiro *et al.*, 1979), do in fact slightly depolarize on treatment with mitogen. In the present paper we have only considered $\Delta\psi_p$ changes in relation to alteration in K^+ fluxes. We have shown that depolarization of pig cells cannot be attributed to a mitogen-induced decrease in electrogenic K^+ permeability. Clearly the depolarization of pig cells is caused by a change in some other factor contributing to $\Delta\psi_p$. We continue this line of investigation in the following paper and show that the depolarization is caused by an increase in electrogenic Na^+ permeability (Felber & Brand, 1983).

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