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Concanavalin A causes an increase in sodium permeability and intracellular sodium content of pig lymphocytes

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1. The 3 mV depolarization of pig lymphocytes observed within 2½ min of treatment with concanavalin A [Felber & Brand (1983) *Biochem. J.* **210**, 885–891] is dependent on the presence of high extracellular [Na^+]. 2. The concanavalin A-induced changes in membrane potential at high and low extracellular [Na^+] are quantitatively explained by an increase in the electrogenic permeability coefficient for Na^+ (P_{Na}). This rises from a negligible value in resting cells to around 4% of the permeability coefficient for K^+ or Cl^- in stimulated cells. 3. Concanavalin A induces a 4 mM increase in the Na^+ content of pig lymphocytes. This increase in intracellular [Na^+] is not due solely to stimulation of electrogenic Na^+ influx resulting from the rise in P_{Na}. 4. Thus concanavalin A stimulates both an electrogenic pathway for Na^+ influx, resulting in a small depolarization of the plasma membrane, and a non-electrogenic Na^+ influx pathway, resulting in a rise in intracellular [Na^+].

Depolarization of human peripheral lymphocytes (Shapiro *et al.*, 1979) and pig mesenteric lymphocytes (Felber & Brand, 1982*b*, 1983) occurs within minutes of exposure to the mitogen concanavalin A. In the present paper we investigate the causes of this depolarization.

The pig lymphocyte plasma-membrane potential ($\Delta\psi_p$) has been fully characterized previously (Felber & Brand, 1982*a*). It consists of an electrogenic component, $\Delta\psi_{ATPase}$, owing to activity of the ($Na^+ + K^+$)ATPase and an ionic component, $\Delta\psi_{ion}$, owing to electrogenic ion fluxes. The change in lymphocyte $\Delta\psi_p$ could be due to alteration of either or both of these components.

An alteration in $\Delta\psi_{ion}$ may be caused by changes in the electrogenic permeability of the plasma membrane to K^+, Na^+, Cl^- or other ions. In the preceding paper (Felber & Brand, 1983) we have shown that concanavalin A does not change the electrogenic K^+ permeability of pig lymphocytes. Thus the pig cell depolarization cannot be attributed to a decrease in electrogenic K^+ flux. An increase in electrogenic Na^+ permeability could account for the drop in $\Delta\psi_p$ and may also be compatible with a rise in cytosolic [Na^+], which has been observed both in human peripheral lymphocytes (Segel *et al.*, 1979) and in mouse thymocytes (Averdunk, 1976) after treatment with mitogen.

In the present paper we show that the depolarization of pig lymphocytes (Felber & Brand, 1982*b*, 1983) is not caused by a change in $\Delta\psi_{ATPase}$, but is dependent on the presence of high extracellular [Na^+]. From this we conclude that concanavalin A induces a rise in the electrogenic Na^+ permeability coefficient (P_{Na}) of pig lymphocytes. We also demonstrate that although concanavalin A causes a rise in the intracellular Na^+ content of pig lymphocytes, this rise cannot be accounted for solely by an electrogenic Na^+ influx resulting from the rise in P_{Na}.

Abbreviations used: $\Delta\psi_p$, difference in electrical potential across the plasma membrane (positive outside), given a positive sign throughout the present paper; $\Delta\psi_{ATPase}$, transmembrane difference in electrical potential due to activity of electrogenic ($Na^+ + K^+$)ATPase; $\Delta\psi_{ion}$, transmembrane difference in electrical potential due to passive ion fluxes; P_K, P_{Na} and P_{Cl}, electrogenic permeability coefficient for K^+, Na^+ or Cl^- respectively: $TPMP^+$, methyltriphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Materials

$^{22}NaCl$ was from Amersham International, Amersham, Bucks., U.K. Sources of other reagents are described in the preceding paper (Felber & Brand, 1983).

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Preparation of pig mesenteric lymphocytes and mouse thymocytes

For details of these preparations, see the preceding paper (Felber & Brand, 1983).

Determination of [^3H]TPMP$^+$ accumulation by pig lymphocytes

Cells were routinely pre-incubated for 30min at 37°C at a concentration of $(2–6)\times10^7$ cells·ml^{-1} either in standard medium (RPMI 1640/10mM-Hepes, pH 7.4) or in a basic salts medium {0.4mM-$Ca(NO_3)_2$, 0.4mM-$MgSO_4$, 11mM-glucose, 1.0mM-Na_2HPO_4 and 10mM-Hepes, pH 7.4, with 8mM-K^+, 118mM-Cl^- and Na^+ and choline$^+$ in various proportions where $[Na^+ + \text{choline}^+] = 115$mM}. RPMI contains 8mM-$K^+$, 111mM-$Cl^-$ and 113mM-Na^+. Further procedures are described in the preceding paper (Felber & Brand, 1983).

Determination of $^{22}Na^+$ accumulation by pig lymphocytes

Cells were pre-incubated as described above. Each experiment, performed in triplicate, was started by mixing 500μl of cells with 500μl of medium in an Eppendorf centrifuge tube with appropriate additions. These were: 2.0μCi of ^{22}NaCl/ml (0.4μM) and 0.8μCi of [^{3}H]inulin/ml (56μM). Other additions are described in the Figure legends. Tubes were centrifuged at defined times and treated as described in the preceding paper (Felber & Brand, 1983).

An extracellular pellet volume ([^{3}H]inulin-permeable space) was calculated for each sample. Parallel determination of the ^3H_2O-permeable space was performed to allow calculation of the intracellular volume (^3H_2O space minus [^{3}H]inulin space). The procedure was as described above except that cells were incubated with 2.0μCi of ^{22}NaCl/ml (0.4μM) and 1.5μCi of ^3H_2O/ml. Accumulation ratios were calculated by dividing Na^+ spaces corrected for extracellular space by the intracellular volume.

Measurement of intra- and extra-cellular Na^+

The intracellular Na^+ content of pig cells was assayed by $^{22}Na^+$ accumulation as described above. This technique could be used in pig cells, as $^{22}Na^+$ equilibrates within 5min owing to very rapid $^{22}Na^+/Na^+$ exchange (Fig. 1). Extracellular Na^+ was measured by assaying relevant supernatants in a Perkin–Elmer atomic absorption spectrophotometer 380.

Results

In our recent study (Felber & Brand, 1982*a*) we used TPMP$^+$ to characterize the pig lymphocyte resting potential. We found that $\Delta\psi_p$ can be resolved into two components, $\Delta\psi_{ion}$ and $\Delta\psi_{ATPase}$. $\Delta\psi_p$ of pig resting lymphocytes can be described by a modified constant field equation (Goldman, 1943; Hodgkin & Katz, 1949) as follows:

$$\Delta\psi_p = \Delta\psi_{ATPase} + \boldsymbol{RT}/F\log\left(\frac{P_K[K^+]_i + P_{Cl}[Cl^-]_e + P_{Na}[Na^+]_i}{P_K[K^+]_e + P_{Cl}[Cl^-]_i + P_{Na}[Na^+]_e}\right) \quad (1)$$

where $\boldsymbol{RT}/F = 61.5$ at 37°C. The ratio of permeability coefficients P_K/P_{Cl} is approx. 1.0 and P_{Na} is very much smaller than P_K in the resting lymphocyte (Felber & Brand, 1982*a*).

The depolarization of $\Delta\psi_p$ that we observe on treatment of pig cells with concanavalin A (Felber & Brand, 1982*b*, 1983) could be explained either by a drop in $\Delta\psi_{ATPase}$ or by a change in P_{Na}, P_{Cl} or the permeability coefficient for another ion such as Ca^{2+} or Mg^{2+}.

Effect of concanavalin A on $\Delta\psi_{ATPase}$

First we considered the possibility that concanavalin A may decrease $\Delta\psi_{ATPase}$. This was measured as the difference in potential between control and ouabain-treated cells (Felber & Brand, 1982*a*). We found, however, that $\Delta\psi_{ATPase}$ was similar in magnitude in control and stimulated cells (Table 1). Moreover concanavalin A does not greatly affect ouabain-inhibitable $^{86}Rb^+$ uptake (Fig. 8 of Felber & Brand, 1983). Thus concanavalin A must be affecting some factor contributing to $\Delta\psi_{ion}$.

Na^+ dependence of $\Delta\psi_p$ depolarization induced by concanavalin A

The Na^+ contribution to $\Delta\psi_{ion}$ is negligible in the resting lymphocyte and thus $\Delta\psi_p$ (56mV) is far from the diffusion potential for Na^+ (−34mV). An increase in P_{Na} would cause a depolarization of $\Delta\psi_p$. Therefore an increase in P_{Na} in stimulated cells such that the contribution of Na^+ to $\Delta\psi_p$ becomes significant may account for the drop in $\Delta\psi_p$ observed in the preceding paper (Felber & Brand, 1983). If this is the case the size of the Na^+ contribution to $\Delta\psi_p$ in concanavalin A-treated cells will vary with extracellular $[Na^+]$ ($[Na^+]_e$). At low $[Na^+]_e$, as $[Na^+]_i/[Na^+]_e$ approaches 8, it will become insignificant as the equilibrium potential for Na^+ approaches $\Delta\psi_p$. We should therefore be able to demonstrate a dependence of the drop in $\Delta\psi_p$ on $[Na^+]_e$.

Cells were pre-incubated for 30min in basic salts media of different $[Na^+]$. Concanavalin A was then added and $\Delta\psi_p$ and $[Na^+]_e$ were measured after 15min. We found that concanavalin A depolarized cells to the same extent (around 2.8mV) in standard RPMI medium and in high Na^+ medium (Table 2). In 15mM-Na^+ medium the depolarization observed was smaller in magnitude (1.0mV). In 1.5mM-Na^+

Table 1. *Effect of concanavalin A on* $\Delta\psi_{ATPase}$

The [^{3}H]TPMP$^+$ accumulation ratio was measured at 15 min in the presence of 5 μM-FCCP as described in the Experimental section. $\Delta\psi_p$ was calculated from the Nernst equation as in Felber & Brand (1982*a*). $\Delta\psi_{ATPase}$ is the difference in potential between control and ouabain-treated cells. Reagents were: 0.2 mM-ouabain; 10 μg of concanavalin A/ml. Results are means of seven experiments ± S.E.M.

		$\Delta\psi_p$ (mV)			*P* (Student's paired *t* test)	
Medium	Mitogen	Control	+Ouabain	$\Delta\psi_{ATPase}$	$\Delta\psi_p$ (control versus ouabain)	$\Delta\psi_{ATPase}$ (with mitogen versus without mitogen)
RPMI (118 mM-Na^+)	—	58.8 ± 1.1	55.4 ± 1.0	3.4	<0.01	—
RPMI (118 mM-Na^+)	Concanavalin A	56.0 ± 1.4	52.4 ± 1.1	3.6	<0.001	Not significant
High Na^+ (127 mM-Na^+)	—	56.0 ± 1.0	52.4 ± 1.0	3.6	<0.01	—
High Na^+ (127 mM-Na^+)	Concanavalin A	53.3 ± 1.2	51.2 ± 1.6	2.1	<0.05	Not significant

Table 2. *Effect of extracellular* [Na^+] *on size of depolarization*

The [^{3}H]TPMP$^+$ accumulation ratio was measured at 15 min in the presence of 5 μM-FCCP as described in the Experimental section. $\Delta\psi_p$ was calculated from the Nernst equation as in Felber & Brand (1982*a*).Concanavalin A was used at 10 μg/ml. The size of the depolarization expected in the different [Na^+] media was calculated from eqn. (1) taking into account the changes in $[Na]_i$ under each condition. Pig lymphocyte intracellular [K^+] and [Cl^-] are 136 mM and 30 mM respectively (Felber & Brand, 1982*a*). Pig lymphocyte intracellular [Na^+] measured in RPMI medium is 29 mM (Fig. 1). Results are means ± S.E.M. for the numbers of experiments shown in parentheses. *P* values are for control versus concanavalin A.

	$\Delta\psi_p$ (mV)		Depolarization	*P* (Student's paired *t* test)	Predicted depolarization assuming $P_{Na} = 0.04\,P_K$ (mV)
Medium	Control	+ Concanavalin A	(mV)		
RPMI (118 mM-Na^+)	58.8 ± 1.1 (7)	56.0 ± 1.4 (7)	2.8	<0.001	2.8
High Na^+ (127 mM-Na^+)	56.0 ± 1.0 (7)	53.3 ± 1.2 (7)	2.7	<0.01	3.2
Low Na^+ (15 mM-Na^+)	57.5 ± 2.4 (6)	56.5 ± 2.4 (6)	1.0	<0.1	0.5
'Na^+ free' (1.5 mM-Na^+)	60.2 ± 3.9 (3)	59.8 ± 1.5 (3)	0.4	Not significant	0.1
'Na^+ free' + Na^+ (14 mM-Na^+)	57.5 ± 3.3 (3)	55.3 ± 3.1 (3)	2.2	<0.1	0.3

medium it was effectively abolished but could be restored by adding back Na^+ to 14 mM for 15 min. These results, presented in Table 2, demonstrate that the pig $\Delta\psi_p$ response to concanavalin A is dependent on the presence of high extracellular [Na^+] and therefore suggest that the mitogen induces a rise in P_{Na}.

How much does P_{Na} *change?*

We have previously reported that the ratio of P_K to P_{Cl} in pig lymphocytes is about 1.0 (Felber & Brand, 1982*a*). By using eqn. (1) we can calculate that the 2.8 mV drop in $\Delta\psi_p$ seen in RPMI and high-Na^+ medium (Table 2) is consistent with an increase in P_{Na} from a negligible value in the resting cell to around 4% of P_K and P_{Cl} in the stimulated cell.

Taking a value for P_{Na}/P_K of 0.04 we can predict quantitatively, by using eqn. (1), the effect of varying $[Na^+]_e$ on the depolarization of $\Delta\psi_p$. Since intracellular [Na^+] ($[Na^+]_i$) alters under each condition it was necessary to take these changes into account. Therefore in parallel experiments we measured the $[Na^+]_i$ of pig cells after incubation in media of different [Na^+]. We found that $[Na^+]_i$ drops from 33.5 mM in control medium (127 mM-Na^+) to 9.9 mM and 4.1 mM in cells incubated for 45 min in media containing 15 mM- and 1.5 mM-Na^+ respectively. After incubation of cells in 1.5 mM-Na^+ for 45 min Na^+ was added back to 14 mM. $[Na^+]_i$ was measured in these cells 15 min later to be 26 mM. The other intracellular ion concentrations required for substitution into eqn. (1) are given in the legend to Table 2. Where appropriate we have assumed that the Na^+ lost from cells is replaced by equimolar concentrations of K^+. Such a correction, however, only slightly alters the values of predicted depolarization in low-Na^+ and 'Na^+-free' media calculated without taking other ion movements into account. As shown in Table 2 a value of 0.04 for P_{Na}/P_K in concanavalin A-treated cells is compatible with all our experimental results.

Thus we conclude that concanavalin A causes a drop in $\Delta\psi_p$ of pig lymphocytes, which reflects a rise in P_{Na} from a negligible value in resting cells to 4% of P_K and P_{Cl} in stimulated cells.

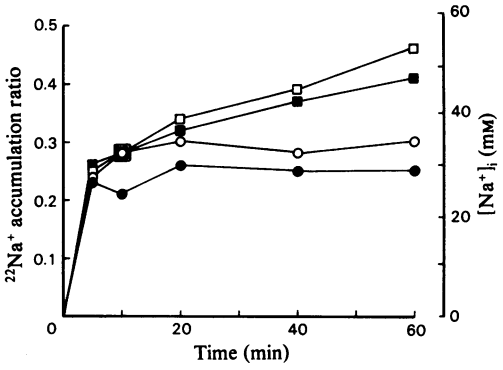

Fig. 1. *Effect of ouabain and concanavalin A on Na^+ uptake into pig lymphocytes*

The $^{22}Na^+$ accumulation ratio was measured as described in the Experimental section. ●, Control; ○ and □, +10 μg of concanavalin A/ml; ■ and □, +0.2 mM-ouabain.

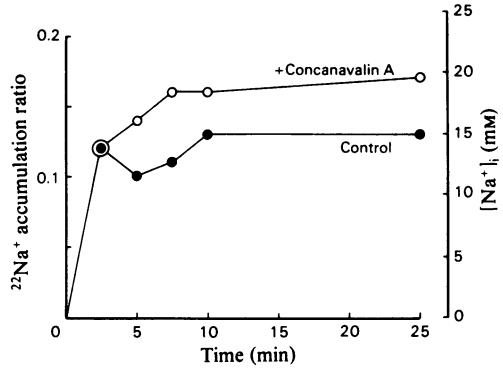

Fig. 2. *Effect of concanavalin A on Na^+ uptake into mouse thymocytes*

The $^{22}Na^+$ accumulation ratio was measured as described in the Experimental section. Concanavalin A (2.0 μg/ml) was present where indicated. The intracellular volume for mouse thymocytes was 116 ± 14 (mean ± S.D.) fl/cell.

Effect of concanavalin A on intracellular Na^+ content of pig and mouse lymphocytes

Averdunk (1976) has observed an increase in $[Na^+]_i$ on stimulation of mouse thymocytes with concanavalin A. Segel *et al.* (1979) have reported an increase in active Na^+ and K^+ transport on treatment of human lymphocytes with phytohaemagglutinin that is caused by a 6 mM increase in $[Na^+]_i$. We looked to see if concanavalin A causes a change in intracellular Na^+ content of pig lymphocytes similar to that reported in mouse thymocytes.

A time course of $^{22}Na^+$ uptake into pig lymphocytes is presented in Fig. 1. Steady-state $^{22}Na^+$ accumulation was attained within 5 min. Similar rapid $^{22}Na^+/Na^+$ exchange was reported by Lichtman *et al.* (1972) in rat thymocytes. Fig. 1 shows that ouabain, an inhibitor of the $(Na^+ + K^+)$ ATPase, does not slow down $^{22}Na^+$ influx into pig cells. Thus fast exchange must occur via an Na^+-linked carrier other than the ATPase. Ouabain did, however, cause a steady increase in $[Na^+]_i$ (Fig. 1) due to gradual collapse of the Na^+ gradient in the absence of uphill Na^+ transport. The effect of concanavalin A was to increase $[Na^+]_i$ from around 29 mM to 33 mM, a rise of about 4 mM-Na^+ (Fig. 1). This effect was also seen in the presence of ouabain (Fig. 1).

Fig. 2 shows that concanavalin A caused $[Na^+]_i$ to rise by 4 mM from around 15 mM to 19 mM in mouse thymocytes. This result is in agreement with those of Averdunk (1976).

Thus concanavalin A induces a very early rise in the Na^+ content of both pig lymphocytes and mouse thymocytes.

Discussion

In the present paper we show that concanavalin A causes both a rise in electrogenic Na^+ permeability (P_{Na}) and an increase in the intracellular Na^+ content of pig lymphocytes within 5 min. The increase in P_{Na} from a negligible value in resting cells to 0.04 of P_K and P_{Cl} in stimulated cells accounts for the 3 mV depolarization of $\Delta\psi_p$ that we have observed previously (Felber & Brand, 1982*a*, 1983). We have also observed a rise in the intracellular $[Na^+]$ of mouse thymocytes, in agreement with Averdunk (1976).

In the preceding paper (Felber & Brand, 1983) we concluded that since the pig lymphocyte and mouse thymocyte $\Delta\psi_p$ respond differently to concanavalin A, a change in $\Delta\psi_p$ cannot be an important general mechanism for signalling mitogenesis. However, a change in ion flux associated with alteration of $\Delta\psi_p$ could be a necessary component of the mitogenic signal. The hyperpolarization observed in mouse thymocytes on treatment with concanavalin A (Tsien *et al.*, 1982; Felber & Brand, 1983) reflects an increase in K^+ permeability. However, the change in K^+ flux in these cells seems to be only secondary to a rise in cytoplasmic $[Ca^{2+}]$, which stimulates a Ca^{2+}-dependent K^+ channel in the thymocyte plasma membrane. In contrast electrogenic Na^+ influx, associated with the depolarization of $\Delta\psi_p$ in pig cells, may play an important role in the activation of lymphocytes by mitogens.

An increase in intracellular $[Na^+]$ has been observed in pig stimulated lymphocytes (the present work), mouse thymocytes (Averdunk, 1976; the present work) and human peripheral lymphocytes (Segel *et al.*, 1979). The increase in P_{Na} has so far

only been seen in pig cells (the present work). It is, however, possible that the depolarization of human peripheral lymphocytes on treatment with concanavalin A or phytohaemagglutinin (Shapiro *et al.*, 1979) reflects a similar change in Na^+ permeability. Furthermore the large hyperpolarization seen in mouse thymocytes may well prevent such a change from being detected in these cells.

An important question is whether the increase in electrogenic Na^+ permeability seen in pig cells is associated with a Na^+ influx large enough to account for the rise in intracellular $[Na^+]$ of 4 mM. Assuming that none of the Na^+ is extruded from the cell via active Na^+ transport pathways, we can calculate the maximum possible increase in cytosolic $[Na^+]$ that will result from a rise in P_{Na} from a negligible value to 0.04 of P_K within 5 min by using the following equation adapted from Katz (1966):

$$\text{Influx}_{Na} = \frac{0.04 \cdot \text{influx}_K \cdot [Na^+]_e}{[K^+]_e} \qquad (2)$$

We have measured the rate of K^+ influx (influx_K) as the ouabain-insensitive Rb^+ influx rate in Fig. 8 of the preceding paper (Felber & Brand, 1983). If there is any electroneutral contribution to this ^{86}Rb uptake we will overestimate influx_K and hence overestimate the magnitude of any rise in $[Na^+]_i$ caused by the electrogenic Na^+ permeability. By using a value for the pig lymphocyte cell volume of 178 femtolitres (Felber & Brand, 1982*a*) influx_K is 52 amol·min^{-1}·cell^{-1}. $[K^+]_e$ and $[Na^+]_e$ are 8 mM and 113 mM respectively. Substituting into eqn. (2) we can calculate a value for influx_{Na} of 29 amol·min^{-1}·cell^{-1}. Thus within 5 min the intracellular $[Na^+]$ would rise by a maximum of only 0.84 mM. To account for a rise in $[Na^+]_i$ of 4 mM within 5 min of concanavalin A treatment P_{Na} would have to increase to at least 20% of P_K and P_{Cl}. However, by using eqn. (1) we can calculate that this would depolarize $\Delta\psi_p$ by around 12 mV, which does not happen. Clearly the increase in P_{Na} that we observe does not by itself cause the rise in $[Na^+]_i$ of 4 mM. Concanavalin A must also stimulate a non-electrogenic Na^+-linked pathway in pig lymphocytes perhaps identical with that which allows the rapid $^{22}Na^+/Na^+$ exchange seen in Fig. 1. Clearly more work is required to characterize the nature of Na^+ influx pathways in the lymphocyte.

Deutsch *et al.* (1981) have shown that mitogen-induced DNA synthesis in human peripheral blood lymphocytes assayed on day 3 is dependent on the presence of extracellular Na^+ for the first few hours of stimulation. It is likely that two events so far apart in time may not be directly related. However, the results are consistent with the possibility that an early Na^+ influx plays a significant role in the activation of lymphocytes.

Observations in other cell types perhaps suggest a more general role for Na^+ permeability changes in the mechanism of cell activation. The rapid depolarization of $\Delta\psi_p$ seen after fertilization of sea-urchin eggs is largely dependent on increased membrane permeability to Na^+ and Ca^{2+} (Chambers & de Armendi, 1979). A requirement for extracellular Na^+ suggests that an influx of Na^+ may be an essential step in the activation of stimulus–secretion coupling in the human neutrophil exposed to concanavalin A or immune complexes (Korchak & Weissmann, 1980). Serum stimulates the rate of Na^+ influx, measured by $^{22}Na^+$ distribution, in mouse 3T3 cells (Rozengurt & Mendoza, 1980).

In the present paper we have established that concanavalin A stimulates a small Na^+ influx in pig lymphocytes due to a rise in electrogenic Na^+ permeability and also promotes Na^+ influx via another non-electrogenic pathway, which raises cytosolic $[Na^+]$ by 4 mM. Either or both of these events may be important in lymphocyte activation by mitogens.

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