Increase in Na^+/K^+ pump numbers *in vivo* in healthy volunteers taking oral lithium carbonate and further upregulation in response to lithium *in vitro*

I. J. ANTIA, C. E. DORKINS, A. J. WOOD & J. K. ARONSON

MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE

- 1 We have measured [^3H]-ouabain binding to lymphocyte membranes in eight healthy volunteers before and after they had taken lithium carbonate for 14 days in doses which maintained the serum lithium concentration in the range 0.5–1.0 mmol l^{-1} .
- 2 There was a statistically significant increase in the [^3H]-ouabain binding capacity of the lymphocyte membranes (reflecting the number of Na^+/K^+ -ATPase molecules) after 14 days of lithium administration *in vivo*. This suggests that a failure to increase pump numbers after similar exposure to lithium *in vivo* in patients with manic-depressive psychosis is a primary abnormality associated with the disease.
- 3 *In vivo* lithium administration did not alter the normal adaptive (upregulatory) response of lymphocyte Na^+/K^+ -ATPase to standard pharmacological challenges, involving *in vitro* incubation for 3 days with lithium chloride (8 mmol l^{-1}) or sodium ethacrylate (1 $\mu\text{mol l}^{-1}$).
- 4 We have previously found that there is an impaired response of the Na^+/K^+ -ATPase to these *in vitro* stimuli in patients with manic-depressive psychosis, and our present data suggest that this abnormality is attributable to the disease itself and not to *in vivo* lithium therapy.
- 5 The data also suggest that the increase in *in vivo* Na^+/K^+ pump activity which we have previously described in healthy volunteers after 21 days of lithium administration is at least partly due to an increase in Na^+/K^+ pump numbers.

Keywords lithium lymphocytes manic-depressive psychosis Na^+/K^+ pumps

Introduction

Despite the wide use of lithium in the treatment of affective disorders its mode of action is still unknown. In recent years there have been attempts to link its therapeutic action with abnormalities of the membrane-bound Na^+/K^+ -ATPase (Na^+/K^+ pump) in patients with affective disorders in studies both *in vitro* (Hesketh *et al.*, 1978; Naylor *et al.*, 1977) and *in vivo* (Wood *et al.*, 1989a,b).

Several types of human cells can increase the number of their membrane-bound Na^+/K^+ pumps in response to certain biochemical and pharmacological stimuli. Lithium is one of the agents which can elicit this response (Boardman *et al.*, 1975), and other stimuli include foetal calf serum (Lamb *et al.*, 1976), ethacrynic acid (Lamb

& Newton, 1973), ouabain (Barve *et al.*, 1970), and a low external concentration of potassium (Boardman *et al.*, 1974).

We have previously shown that *in vitro* incubation of lymphocytes for 72 h in lithium (1–8 mmol l^{-1}) causes an increase in the number of Na^+/K^+ pumps in the cell membrane (Jenkins *et al.*, 1991; Rapeport *et al.*, 1986), and we have attributed this upregulation to a primary effect of lithium as an inhibitor of the inositol-1-phosphatase (Jenkins *et al.*, 1991). The upregulator response to other stimuli is also blocked by inhibition of protein synthesis (Lamb & Newton, 1973; Oh *et al.*, 1987), suggesting that it depends, at least in part, on *de novo* synthesis of protein. Evidence from studies using the

stimulus of lowering the extracellular concentration of potassium (Ameen *et al.*, 1992) suggests that upregulation of this kind in lymphocytes also depends on a slowing of the rate of internalization of Na^+/K^+ pumps.

We have also previously shown that when the lymphocytes of patients with manic-depressive psychosis are incubated with lithium chloride or sodium ethacrylate their Na^+/K^+ pumps do not manifest the usual upregulatory response (Wood *et al.*, 1991). Others have shown that the upregulatory response is also absent in patients with manic-depressive psychosis when the stimulus to upregulation is a reduction in the extracellular concentration of potassium (Naylor & Smith, 1981).

We have now studied the effect of lithium administration *in vivo* on the number of Na^+/K^+ pumps in the lymphocytes of healthy volunteers and the subsequent effects of lithium and ethacrylate *in vitro* on the upregulatory response of the Na^+/K^+ pumps. The purpose of this study was to explore further the mechanism of the adaptive responses to lithium *in vivo* and *in vitro* in healthy volunteers and thereby to understand more clearly the demonstrated abnormalities in patients with manic-depressive psychosis.

The results suggest that, in healthy volunteers, lithium upregulates the number of Na^+/K^+ pumps in lymphocytes *in vivo*, and that further *in vitro* upregulation is possible when the cells are later incubated with lithium or ethacrylate. This in turn suggests that the failure of the lymphocytes of patients with manic-depressive psychosis to upregulate *in vitro* in response to lithium and ethacrylate is associated with the predisposition to the disease and not with drug therapy.

Methods

Choice of volunteers and administration of lithium

We studied eight healthy male volunteers (mean age 30 years, range 26–40) and screened them to exclude a personal or close family history of affective illness or other clinical disorders associated with altered Na^+/K^+ -ATPase activity (including hypertension and renal impairment). None of the volunteers was taking medication at the time of the study.

The volunteers took oral lithium carbonate BP, 800 mg each evening for 2 weeks. None of them complained of any adverse effects. Serum lithium concentrations were measured by flame photometry on days 4, 8 and 15 in samples taken 12 h after the previous dose of lithium.

Lymphocyte preparation and in vitro incubation

Venous blood for the studies was taken 1 week before the start of lithium administration and on the morning after the last dose of lithium. Lymphocytes were prepared by a modification of the method of Böyum (1968), similar to that used by Rapeport *et al.* (1986). Briefly, venous blood (90 ml) was collected from an antecubital vein through a 21-gauge butterfly needle and anticoagulated with sodium heparin. The whole blood was diluted

with RPMI 1640 (3:1) and layered on to Ficoll/sodium diatrizoate (Histopaque, Sigma). The lymphocytes were separated by centrifugation at 250 g at room temperature for 30 min. Platelets were removed by repeated washing with RPMI 1640 and slower centrifugation (75 g).

An aliquot of lymphocytes was used for measuring Na^+/K^+ pump numbers before *in vitro* incubation (Day 0). The rest of the lymphocytes were incubated in RPMI 1640 containing 10% dialysed foetal calf serum (Globe Pharm) with penicillin (100 iu ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$). The incubation continued for 72 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 . These experiments were carried out in the presence and absence of lithium chloride (8 mmol l^{-1}) or sodium ethacrylate ($1 \mu\text{mol l}^{-1}$). At the end of the incubation period (Day 3) the cells were collected by centrifugation and resuspended in a potassium-free solution (Aronson *et al.*, 1977) for measuring Na^+/K^+ pump numbers.

Measurement of Na^+/K^+ -ATPase pump numbers by [^3H]-ouabain binding

We measured the number of lymphocyte Na^+/K^+ pumps by measuring the B_{max} of binding of [^3H]-ouabain to the cells (Boon *et al.*, 1984b). We incubated the cells ($1\text{--}2 \times 10^6$ per tube) for 3 h in triplicate at 37°C in potassium-free solution containing 25 nmol l^{-1} [^3H]-ouabain. Non-specific binding was measured in the presence of non-radioactive digoxin ($5 \mu\text{mol l}^{-1}$). The incubation was terminated by centrifugation at 1000 g for 5 min at 4°C . The cell pellets were washed in ice-cold potassium-free solution, then disrupted by incubation for 30 min in 1 mol l^{-1} NaOH. The amount of [^3H]-ouabain bound was measured by counting an aliquot of cell lysate in a liquid scintillation counter using Ecoscint-A (National Diagnostics, Manville, New Jersey, USA) as scintillant. We have previously shown that this method of measuring the B_{max} of [^3H]-ouabain binding gives results which are comparable with the values of B_{max} obtained from binding curves generated over a wide range of ouabain concentrations (Boon *et al.*, 1984b).

Statistical analyses

The lithium concentrations are presented as mean (range). The data are presented as mean (s.d.) or as median (range) when expressed as percentage change in binding site numbers. The results were analysed by Student's paired *t*-tests or by Mann-Whitney or Wilcoxon signed rank tests as appropriate.

Ethical approval

The study was approved by the Oxford Psychiatric Sector Research Ethics Committee and was carried out with the informed consent of the volunteers.

Results

By the fourth day of lithium administration the mean serum lithium concentration was 0.62 (range 0.50–0.83)

mmol l⁻¹. On days 8 and 15 it was 0.63 (range 0.50–0.93) and 0.63 (range 0.47–1.00) mmol l⁻¹ respectively.

Na⁺/K⁺ pump numbers before and after in vivo lithium administration

There was a significant increase in the number of [³H]-ouabain binding sites in lymphocyte membranes after 2 weeks of *in vivo* administration of lithium. The data are shown in Figure 1. During lithium administration the mean number of binding sites (measured *ex vivo*, prior to *in vitro* incubation) increased from 66500 (9078) to 80548 (14736) sites/cell ($P = 0.04$).

In vitro upregulatory responses to lithium and ethacrynate

The upregulatory responses of the Na⁺,K⁺-ATPase in lymphocytes which were exposed to lithium or ethacrynate *in vitro* are shown in Figure 2. Before *in vivo* lithium administration the numbers of Na⁺/K⁺ pumps on the lymphocytes increased significantly in response to both lithium and ethacrynate *in vitro*. The median increase was 13.8 (2.8–29.6)% after *in vitro* stimulation with lithium and 11.9 (–0.19–24.4)% after *in vitro* stimulation with ethacrynate. After the oral administration of lithium for 2 weeks there were similar significant increases in the numbers of lymphocyte Na⁺/K⁺ pump sites in response to stimulation by both lithium and ethacrynic acid *in vitro*; the median increase was 12.9 (3.0–46.9)% for lithium and 9.3 (–0.66–18.6)% for ethacrynate. There were no differences in the magnitudes of the *in vitro* upregulatory responses to either lithium or ethacrynate after oral (*in vivo*) lithium administration for 14 days.

For comparison, we have also shown in Figure 2 some of the results of our previous study of the *in vitro*

Figure 1 [³H]-ouabain binding to lymphocytes after lithium administration *in vivo* for 14 days. The results are expressed as sites per cell and are depicted as mean and standard deviation ($n = 8$). There was a statistically significant difference between the two groups ($P = 0.04$).

Figure 2 The effect of incubation *in vitro* with (a) lithium and (b) ethacrynate on [³H]-ouabain binding to the lymphocytes of volunteers before (○) and after (●) lithium administration ($n = 8$). For comparison our previous results from lithium-treated patients are also shown (□, $n = 9$). The results are expressed as the percentage change in [³H]-ouabain binding compared with control incubations. The horizontal bars represent the median values.

upregulatory responses of lymphocytes from patients with manic-depressive psychosis. The patients were euthymic and were taking long-term oral lithium treatment at the time of the study (Wood *et al.*, 1991). The lymphocytes from these patients did not increase their Na⁺/K⁺ pump numbers in response to either lithium or ethacrynate *in vitro*, in an identical experiment.

Discussion

The effect of in vivo lithium administration on lymphocyte Na⁺/K⁺ pumps

The results of this study show that the Na⁺/K⁺ pumps in the lymphocyte membranes of healthy volunteers are increased in number after 14 days of *in vivo* lithium administration.

Our finding that lithium administration *in vivo* causes an increase in lymphocyte Na⁺/K⁺ pump numbers in healthy volunteers extends our previous findings of similar upregulation *in vitro* (Jenkins *et al.*, 1991; Rapeport *et al.*, 1986). It is also consistent with our previous finding that the activity of the Na⁺/K⁺ pump *in vivo* increased in the erythrocytes of healthy volunteers after 21 days of *in vivo* lithium administration (Wood *et al.*, 1989b).

Neither the results of our present ouabain binding experiment nor our previous *in vivo* Na^+/K^+ pump activity experiment (Wood *et al.*, 1989b) is consistent with the earlier results of Naylor *et al.* (1977). Those investigators demonstrated a small fall in Na^+/K^+ pump-mediated potassium uptake into erythrocytes *ex vivo* after lithium treatment, but no change in Na^+/K^+ -ATPase activity, measured as the rate of hydrolysis of ATP. This discrepancy between studies may either be explained by a difference between the results of *ex vivo* and *in vivo* methods (Boon *et al.*, 1986), or might reflect the fact that Naylor *et al.* (1977) studied non-nucleated erythrocytes which might have different adaptive responses to lithium compared with nucleated cells.

The mean increase in Na^+/K^+ pump numbers during *in vivo* lithium administration in the present study was 21%. By contrast, the rate constant for Na^+/K^+ flux activity in erythrocytes in the *in vivo* study of Wood *et al.* (1989b) was doubled after 21 days of lithium *in vivo*. Although this difference might simply represent a difference between erythrocytes and lymphocytes, it suggests that only part of the increase in Na^+/K^+ pump activity in response to lithium *in vivo* is attributable to an increase in pump number and that there is probably also an increase in the activity of individual pump sites.

These results in healthy volunteers can be contrasted with those in patients taking lithium (long-term *in vivo* exposure). In patients taking lithium the *in vitro* activity of Na^+/K^+ -ATPase activity was increased (Naylor *et al.*, 1974) and was significantly higher than in patients not taking lithium (Hokin-Neaverson *et al.*, 1976). Furthermore, there was a correlation between the magnitude of the increase in enzyme activity and the therapeutic response to lithium (Johnston *et al.*, 1980). However, there were no demonstrated changes in pump numbers in the earlier work (Naylor *et al.*, 1981). This is consistent with our previous finding that the lymphocytes of patients with manic-depressive psychosis are unable to upregulate their pump numbers in response to lithium *in vitro* (in contrast to healthy volunteers) (Wood *et al.*, 1991). Thus, a synthesis of past and present data suggests that patients with manic-depressive psychosis fail to increase their Na^+/K^+ pump numbers in response to an upregulatory stimulus, but might be able to increase their unit activity (measured previously as *in vitro* phosphatase activity).

An inability of the lymphocytes of manic-depressive patients to upregulate their pump numbers would also explain our previous finding (Wood *et al.*, 1991) that there was no difference in ouabain binding sites between unmedicated controls and patients taking long-term (*in vivo*) lithium treatment. However, this difference might also have been explained by different durations of lithium treatment (14 days in the volunteers vs at least 6 months in the patients).

The fact that the lymphocytes of patients with manic-depressive psychosis seem not to be able to change their pump numbers in response to standard stimuli, such as lithium and ethacrynate, might also explain why changes in pump activity seen *in vivo* lithium administration are apparently inconsistent (Wood *et al.*, 1989a). A likely explanation for this apparent discrepancy is an altered mechanism for the regulation of pump activity in these patients, in whom the capacity to alter pump numbers is

reduced. However, there is currently no experimental evidence which might support such a hypothesis.

In vitro upregulation of Na^+/K^+ pump numbers after lithium administration

In this study we have found that when the lymphocytes of healthy volunteers were incubated with lithium and sodium ethacrynate *in vitro* (before any *in vivo* lithium administration) the numbers of Na^+/K^+ pumps increased significantly. The same response occurred when the volunteers had taken oral lithium for 14 days. This is not unexpected; the cells were exposed to an extracellular lithium concentration of about 1 mmol l^{-1} *in vivo* and to a concentration of 8 mmol l^{-1} *in vitro*, and we have previously shown that the response of lymphocyte Na^+/K^+ pumps to upregulation by lithium is dose-responsive at least up to 8 mmol l^{-1} (Jenkins *et al.*, 1991). However, it is possible that this additive effect might also reflect different mechanisms of response to *in vivo* and *in vitro* lithium exposure.

By contrast, we have previously shown that the Na^+/K^+ pumps in the lymphocytes of euthymic patients with a history of manic-depressive psychosis do not upregulate in response to either lithium or ethacrynate *in vitro* (Wood *et al.*, 1991 and see Figure 2). This finding was independent of whether the patients were taking concurrent long-term oral lithium treatment. This previous study suggested that the failure of patients' lymphocytes to upregulate was associated with the predisposition to the disease rather than with lithium therapy, but we were unable to rule out a possible effect of previous lithium treatment in the patients who were not taking lithium at the time, nor an effect of other previous drug therapy. However, our current results in healthy volunteers taking lithium support our view that a failure to upregulate Na^+/K^+ pump numbers is not primarily associated with lithium administration *in vivo*, and therefore supports our hypothesis that this may be a primary abnormality associated with a tendency to develop manic-depressive disorder.

Our comparison of patients with healthy volunteers might have been complicated by the effects of the previous medication given to patients with manic-depressive psychosis. It is not known for how long the many adaptive processes which occur during drug administration persist after drug withdrawal. However, in our studies of drug-free patients and of patients taking lithium (Wood *et al.*, 1991), the subjects had been on a stable regimen of lithium alone or on no psychotropic drug for at least 3 months. Furthermore, there was no correlation between the drug-free period and the adaptive responses in drug-free patients. We therefore believe that previous medication is unlikely to have affected our comparison of patients with healthy volunteers.

It is possible that other factors may have caused the differences between individuals whose cells were capable of upregulation and those whose cells were not. For example, there was a difference in the ages of the volunteers in this study and the lithium-treated patients in our previous study (Wood *et al.*, 1991), and the adaptive changes could have been affected by age. However, whilst this might change the magnitude of the

response, since Na^+/K^+ -ATPase activity falls with increasing age (Boon *et al.*, 1984a) it is unlikely that a small difference in the age ranges (26–40 years in the volunteers vs 23–68 years in the patients) would lead to the complete abolition of a clear upregulatory response.

Several hormones are also known to increase the numbers of Na^+/K^+ pumps *in vitro* (see Ameen *et al.*, 1992), but the fact that our *in vitro* incubations were carried out in artificial media after washing of the cells reduces the likelihood that plasma-borne factors would have significantly affected our results.

There is experimental evidence that the mechanisms whereby lithium and ethacrynate cause *in vitro* upregulation of Na^+/K^+ pump numbers are different: an increase in intracellular sodium concentration for ethacrynate (Boardman *et al.*, 1974) and inhibition of the inositol-1-phosphatase for lithium (Jenkins *et al.*, 1991). Thus, our previous finding that the lymphocytes of patients with manic-depression failed to respond to *both* of these stimuli suggests that there is a final common pathway involved in the different upregulatory mechanisms and that the defect in patients with manic-depression is in

that common pathway. Our current results suggest that neither of these independent mechanisms is affected by the administration of lithium *in vivo* for 14 days in healthy volunteers.

In conclusion, we have found that the number of Na^+/K^+ pumps increased in the lymphocytes of healthy volunteers taking lithium for 14 days. These data suggest that the increase in the activity of Na^+/K^+ pumps seen *in vivo* in our previous study in healthy volunteers is due in part to an increase in pump numbers. After exposure to lithium *in vivo* lymphocytes were able to increase their Na^+/K^+ pump numbers further when they were incubated *in vitro* with lithium or ethacrynate for 72 h. These results also support our view that the failure of the lymphocytes of patients with manic-depressive psychosis to upregulate *in vitro* in response to standard stimuli is not due to any effect of drug administration, and might be of importance in the predisposition to development of the disorder.

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