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Effect of the pyridoindole antioxidant stobadine on sodium handling of renal Na,K-ATPase in rats with streptozotocin-induced diabetes

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Abstract Overload of reactive oxygen species during diabetes is known to impair cellular homeostasis and to promote deterioration of membrane function in the organism. The aim of the present study was to examine the effect of dietary supplementation with the pyridoindole atioxidant stobadine on functional properties of the renal Na, K-ATPase in diabetic rats. After 16 weeks of streptozotocin-induced diabetes (single intravenous dose of streptozotocin; 55 mg/kg), a significant inhibition (by 35%–42%) of the enzyme was observed throughout the range of NaCl 2–100 mmol/l, probably as an event of altered functional properties of Na,K-ATPase, suggested by the 42% decrease of the V_{max} value. Administration of 0.05% (w/w) stobadine in the diet dramatically improved the function of renal Na,K-ATPase in diabetic rats with regard to sodium handling, as suggested by significant stimulation (by 104%–77% in accordance with increasing concentration of NaCl) of the enzyme over the whole NaCl concentration range investigated. This stimulatory effect was accompanied by an increase of V_{max} value to the level of non-diabetic rats on standard diet. In conclusion, stobadine was found to antagonise the negative effects of diabetes on the renal Na,K-ATPase, preserving its normal function in regulation of intracellular homeostasis of Na^+ and K^+ ions.

Key words Sodium pump • Diabetes • Kidney

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Introduction

Diabetes mellitus is characterised by a series of complications affecting several organs, including the kidney. The development and progression of diabetic nephropathy is dependent on glucose and many other factors. Altered sodium homeostasis is also a consistent finding in diabetes, as both insulin-dependent and non-insulin-dependent diabetic patients have a significant increase of total exchangeable sodium [1, 2]. Considerable attention has been focused on abnormalities of renal sodium handling in diabetes, since the kidney is the main regulator of body salt and water homeostasis. One of the crucial systems involved in maintaining the sodium balance is Na,K-ATPase, which utilises energy derived from hydrolysis of ATP for transmembraneous transport of Na^+ and K^+ ions against their respective concentration gradients. The development of diabetic nephropathy, involving changes in Na,K-ATPase, is closely correlated with the duration and magnitude of hyperglycaemia.

Studies in the streptozotocin (STZ)-induced diabetic rat model showed highly time-dependent changes in basal Na,K-ATPase activity. After a period of six weeks, an increase both in activity and expression of Na,K-ATPase was observed; however, after 12 weeks of diabetes the activity as well as the expression of the enzyme were significantly decreased [3].

Among multiple cellular consequences of hyperglycaemia, oxidative stress induced by reactive oxygen species has been considered an important pathway linking various diabetic complications. Beneficial effects of various antioxidants have been described [4]. Recent studies using the pyridoindole antioxidant stobadine, which can act as potent scavenger of peroxyl radicals both in aqueous and lipid phases [5], showed that this substance ameliorated kidney status and function in streptozotocin-induced diabetic rats [6, 7]. The present study was

designed to investigate the influence of the pyridoindole antioxidant stobadine on properties of Na,K-ATPase in isolated plasmalemmal membranes from kidneys of rats suffering from chronic (16 weeks) STZ-induced diabetes. On studying the function of the enzyme, we focused on the response of Na^+-binding sites to diabetes and to the treatment by stobadine.

Materials and methods

This study was approved by the ethics committee of the Institute and performed in accordance with Principles of Laboratory Animals Care (NIH publication 83–25, revised 1985) and Slovak law regulating animal experiments (Decree 289, part 139, 9 July 2003). The animals were of monitored conventional quality and came from the Breeding Facility of the Institute of Experimental Pharmacology Dobra Voda (Slovak Republic). Experimental diabetes was induced in 8-week-old male Wistar rats, weighing 200–230 g, by a single intravenous dose of streptozotocin (STZ; 55 mg/kg). STZ was dissolved in 0.1 mol/l citrate buffer pH 4.5. The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Ten days after STZ administration, animals with plasma glucose level higher than 15 mmol/l were considered diabetic and were included in the study. Age-matched rats receiving a single dose of 0.1 mol/l citrate buffer served as controls.

Control and diabetic rats were randomly divided into two groups each. Eight control rats (group C) and 8 diabetic rats (group D) were fed a pelleted standard diet, while 4 control rats (group CS) and 8 diabetic rats (group DS) were fed a stobadine dipalmitate-enriched diet (0.05% w/w).

During the experiment, the animals were housed in groups of two in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (exchanged daily). All rats were allowed free access to food and drinking water. The animal room was air-conditioned and the environment was continuously monitored for the temperature of 23°±1° C and relative humidity of 40%–70%.

Plasma glucose levels were measured using the commercial glucose (Trinder) kit (Sigma, St. Louis, USA).

A plasmalemmal membrane fraction from the kidney was isolated according to standard procedures [8]. Protein concentrations were determined according to Lowry et al. [9], using bovine serum albumin as standard.

Sodium kinetics of Na,K-ATPase was estimated at a temperature of 37° C by measuring the hydrolysis of ATP by 10 μg plasmalemmal protein in the presence of increasing concentrations of NaCl in the range of 2–100 $mmol \cdot l^{-1}$. The total reaction volume was 0.5 ml. Plasmalemmal proteins were diluted into a reaction buffer containing 50 mM imidazole (pH 7.4), 4 mM $MgCl_2$, and 10 mM KCl. After 20 minutes of preincubation in substrate-free medium, the reaction was started by addition of ATP to 4 mM; after 20 minutes the reaction was stopped by addition of 0.3 ml ice-cold solution of 12% trichloroacetic acid. The liberated inorganic phosphorus was determined according to standard procedure [10]. In order to establish Na,K-ATPase activity, ATP hydrolysis that occurred in the sole presence of Mg^{2+} was subtracted.

Kinetic parameters were evaluated from the data by direct nonlinear regression. The significance of differences between the individual groups was determined by using ANOVA, Bonferroni test. A value of $p<0.05$ was regarded as significant.

Results

Streptozotocin-induced diabetes lasting 16 weeks resulted in a significantly lower body weight (56% less) compared to control rats (Table1). Dietary administration of stobadine to controls did not significantly alter the body weight. Similarly no significant difference was observed between diabetic animals fed with standard diet and with stobadine-enriched diet. Absolute kidney weight was not affected significantly by diabetes in animals fed either standard or stobadine-enriched diet. Stobadine itself induced a slight but statistically significant 13% increase of absolute kidney weight in control rats. However, the ratio of kidney weight to body weight was significantly increased as a consequence of diabetes: by 150% in animals fed standard diet (group D vs. C) and by 123% in stobadine-treated rats (group DS vs. CS). Administration of stobadine to controls as well as to diabetic animals did not affect the level of plasma glucose (Fig. 1).

Table 1 Influence of streptozotocin-induced diabetes and stobadine (STB) on weight parameters of rats measured at the end of the sixteenth week after onset of experimental diabetes (or mock induction in control animals). Control rats fed with standard diet (C), STZ-diabetic rats fed with standard diet (D), control rats fed with stobadine-enriched diet (CS) and STZ-diabetic rats fed with stobadine-enriched diet (DS). Values are mean (SEM)

	C (n=8)	D (n=7)	CS (n=4)	DS (n=8)
Body weight, g	443 (9)	193 (10)*	438 (16)	205 (7)*‡
Kidney weight, g	2.27 (0.08)	2.44 (0.10)	2.56 (0.08)*	2.66 (0.09)
(Kw/Bw) x 10^{-3}	5.12 (0.12)	12.79 (0.64)*	5.86 (0.05)*	13.05 (0.60)*‡

*$p<0.05$ vs. group C; ‡$p<0.05$ vs. group CS
Kw/Bw, ratio of kidney weight to body weight

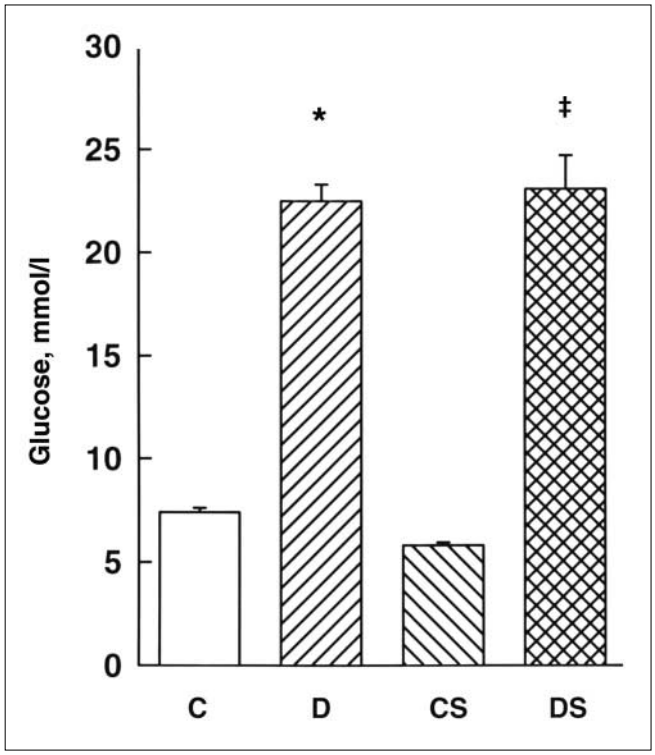

Fig. 1 Plasma levels of glucose at the end of experiment in control rats fed standard diet (C), in STZ-diabetic rats fed standard diet (D), in control rats fed stobadine-enriched diet (CS) and in STZ-diabetic rats fed stobadine-enriched diet (DS). Data represent means and SEM. *p<0.001 vs. group C; ‡p<0.001 vs. group CS

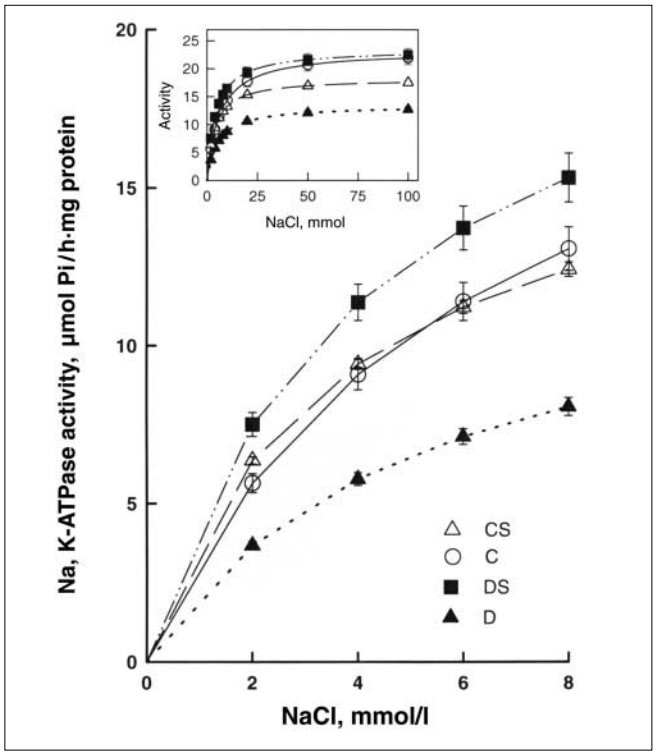

Fig. 2 Activation of renal Na,K-ATPase by cofactor Na^+. Effect of increasing concentrations of NaCl in control rats fed standard diet (C), STZ-diabetic rats fed standard diet (D), control rats fed stobadine-enriched diet (CS) and STZ-diabetic rats fed stobadine enriched-diet (DS). Detailed projection of activities in the presence of low concentrations of NaCl is shown. Insert, activation of the enzyme throughout the NaCl concentration range investigated

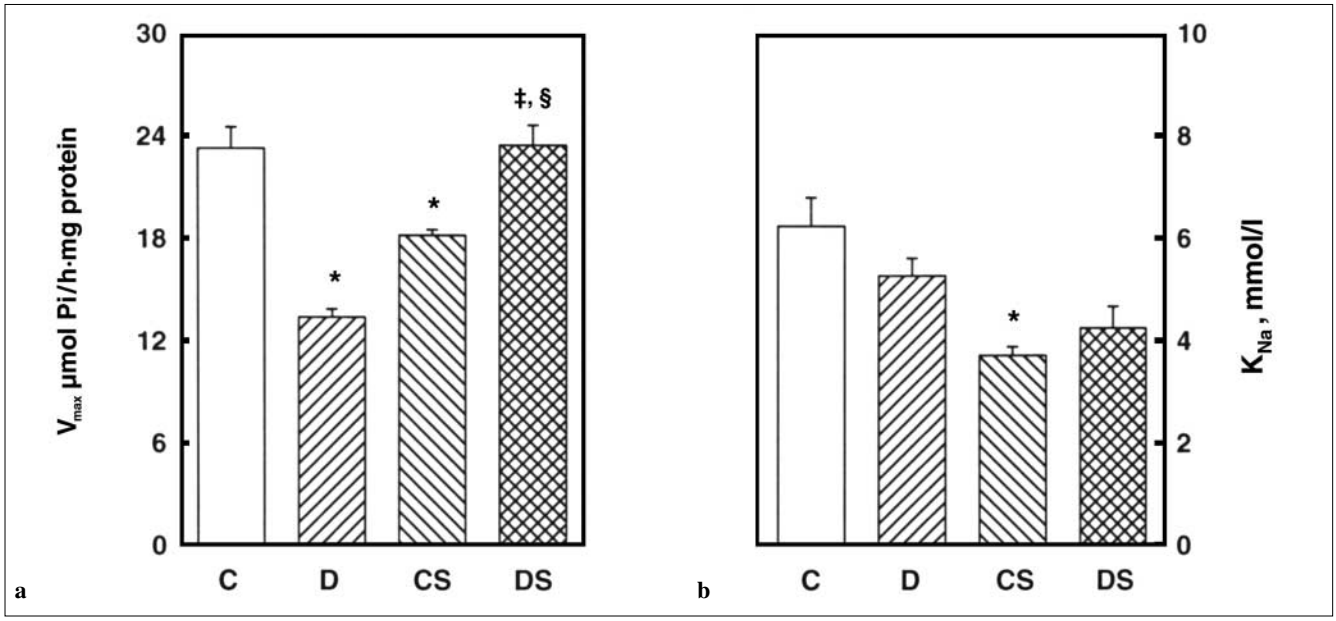

Fig. 3a, b *Kinetic parameters of Na,K-ATPase activation by cofactor Na^+ in kidney. Parameters were evaluated at the end of the sixteenth week after onset of experimental diabetes (or mock induction) by nonlinear regression of data presented in Fig. 2.* **a** Maximal velocities (V_{max}) of enzyme. **b** K_{Na} values representing the concentrations of NaCl necessary for half-maximal activation of the enzyme. *C*, control rats fed standard diet; *D*, in STZ-diabetic rats fed standard diet; *CS*, control rats fed stobadine-enriched diet; *DS*, STZ-diabetic rats fed stobadine-enriched diet. Data represent means and SEM. *p<0.001 vs. group C; ‡p<0.001 vs. group D; §p<0.005 vs. group CS

Kinetic properties of renal Na,K-ATPase in animals fed standard diet

When activating the enzyme with increasing concentration of NaCl, we observed a significantly decreased enzyme activity in the kidneys of diabetic animals in comparison with controls. At the lowest concentration of NaCl investigated (2 mmol/l), the decrease represented 35%. With increasing concentration of the cofactor, the effect gradually increased and in the presence of the highest concentration of NaCl investigated (100 mmol/l) the enzyme activity was reduced by 42% (Fig. 2). Nonlinear regression analysis revealed that diabetes depressed the V_{max} value by 42% and did not significantly affect the K_{Na} value (Fig. 3).

Influence of stobadine on renal Na, K-ATPase in nondiabetic rats

Increasing concentrations of NaCl induced a dual effect on the Na,K-ATPase activity in nondiabetic rats fed with stobadine-enriched diet (Fig. 2). In the presence of the lowest concentration of NaCl (2 mmol/l), we observed in the CS group a stimulation of the enzyme amounting to 12% in comparison with the C group. In the presence of 6 mmol/l NaCl, the enzyme activity was similar to that in rats fed standard diet. At higher sodium concentrations, a gradual deactivation started, which reached its maximum (by 20%) in the presence of 100 mmol/l NaCl (Fig. 2). Evaluation by nonlinear regression resulted in statistically significant decrease of both investigated kinetic parameters in the CS group. The V_{max} value was lower by 22% and K_{Na} by 41% (Fig. 3).

Influence of stobadine on renal Na,K-ATPase in diabetic rats

In the diabetic group of rats (DS), administration of stobadine augmented the response of Na,K-ATPase to increasing concentrations of NaCl. In the presence of the lowest concentration of NaCl (2 mmol/l), the stimulation was 104%. With increasing concentrations of the cofactor, the stimulatory effect gradually decreased to 77%, as observed in the presence of 100 mmol/l NaCl (Fig. 2). Nonlinear regression analysis showed that administration of stobadine to diabetic rats resulted in a 75% increase of V_{max}, while the K_{Na} value was not altered significantly (Fig. 3).

Discussion

In rats suffering STZ-induced diabetes for 16 weeks, significantly lower body weight and higher relative kidney weight were observed in comparison to healthy controls. These changes are in agreement with data published previously, reporting a time-dependent reduction of weight gain in diabetic rats compared to control animals, during the development of diabetes. In acute (7-day) STZ-diabetes, this kind of reduction represented 10% [11]. Similarly, chronic STZ-diabetes lasting 4–12 weeks induced a 35%–45% reduction in weight gain [12–14]. Further prolongation of diabetes to 16–32 weeks was associated with approximately 50% reduction in body weight gain [15, 16]. Our observation of a 56% decrease of body weight gain in groups of diabetic animals in comparison to healthy controls, after 16 weeks of diabetes, fits well with previously published data. In the present study, administration of the pyridoindole antioxidant stobadine did not eliminate the diabetes-induced reduction of body weight gain, which is in agreement with previous findings [6, 7, 15]. The apparent increase of relative kidney weight in diabetes seems to be a consequence of lower body weight of diabetic animals, as the absolute kidney weight was not influenced significantly. Stobadine treatment did not affect significantly glycaemic state either control or diabetic rats, which is in agreement with previous data of an 8-month experiment [6, 15].

Studies in the streptozotocin-induced diabetic rat model showed that Na,K-ATPase was affected widely in the organism, e.g. in vascular smooth muscle [11], the aorta [12], heart [17–20], nerves [21], retina [22] and also in the kidney [3, 13, 14, 23]. Controversial data have been published concerning the response of renal Na,K-ATPase to complications induced by diabetes during the time course of its development. In the early phase of diabetes (up to week 6), a stimulatory effect on the enzyme was documented [3, 23]. Longer duration of diabetes was followed by a significant functional depression of renal Na,K-ATPase [3, 13, 14]. Since Na,K-ATPase is the main system responsible for the transport of excessive sodium out of the cell, in the present study we tried to elucidate the molecular principles, especially the sodium handling by Na,K-ATPase, in response to 16 weeks of STZ-diabetes. Our findings support and broaden the previously reported knowledge about deactivation of renal Na,K-ATPase due to long-term diabetes. In previous measurements, the enzyme activity was estimated at certain conditions in which the concentration of NaCl exceeded the intracellular level of Na^+ [3, 13, 14]. Our data provide evidence that under the diabetic state the activity of Na,K-ATPase is strongly decreased over a wide range of Na^+ concentrations, i.e. also in the presence of lower concentrations of Na^+ which are of physiological relevance. The unchanged K_{Na} and simultaneously depressed V_{max} values point to the possibility of a non-competitive inhibition of the enzyme by an endogenous inhibitor bound in the vicinity of the Na-binding site as a consequence of diabetes. The role of such a hypothetical inhibitor may be identical to a digitalis-like substance which was found to be elevated in STZ-diabetic rats and decreased cardiac Na,K-ATPase activity [24, 25].

An increased level of this substance also inhibited renal Na,K-ATPase [26]. This substance of relatively good solubility was found to be freely circulating in the blood [24]. In our experiment, the action of such an inhibitor seems to be unlikely, since it should be washed out during preparation of the plasmalemmal membrane fraction.

The second potential explanation for the loss of enzyme activity as a consequence of STZ-induced diabetes may be linked to the lack of insulin, which is a potential stimulator of Na,K-ATPase [27, 28]. In the normally functioning pancreas, C-peptide co-secreted with insulin also improves renal function by stimulating Na,K-ATPase activity [29] and its deficiency may also contribute to the decreased enzyme activity. According to the long-lasting time course of our experiment during which the expression of the Na,K-ATPase molecule may have been altered, a third plausible explanation may concern the decrease in the number of enzyme molecules, as suggested by the markedly lowered V_{max}. This proposal is supported also by previous studies documenting that after 12 weeks of diabetes the activity as well as the expression of the enzyme were significantly decreased [3]. Regardless of the mechanism responsible for the observed effect, during long-lasting diabetes Na,K-ATPase was less active throughout the investigated sodium concentration range, resulting in deteriorated transport of excessive sodium out of the cell.

Among numerous currently discussed hypotheses dealing with the development of diabetic complications, oxidative stress has been identified as a potential early initiating mechanism, by which the high glucose environment may lead to cellular injury in diabetes [4, 30]. Oxidative damage of Na,K-ATPase has been demonstrated in several tissues including brain [31], heart [32] and kidney [33]. Therefore, we investigated the effect of the pyridoindole antioxidant stobadine on diabetes-induced changes of Na,K-ATPase. Stobadine was shown previously to scavenge hydroxyl, peroxyl and alkoxyl radicals, to quench singlet oxygen, to repair oxidised amino acids and to preserve oxidation of SH groups by one-electron donation. These effects originated from its ability to form a stable nitrogen-centered radical on indole nitrogen. Consequently, stobadine was able to diminish lipid peroxidation and protein impairment under oxidative stress (reviewed in [34]).

Chronic toxicity studies (180 days) in diabetic animals after 6 and 8 months diabetes [6, 7] demonstrated no toxic effect of stobadine dipalmitate (7.07–70.07 mg/kg p.o. daily) on the clinical, haematological and pathological status of rats either during drug administration or in the following 2-month recovery period [35–37]. Stobadine significantly reduced total proteinuria, albuminuria and enzymuria in streptozotocin-induced diabetic rats [6]. In our experiment, when stobadine was supplemented in the diet of control rats, the functional properties of renal Na,K-ATPase were changed, as shown by decreased K_{Na} and V_{max} values. This behaviour may be again interpreted by two different mechanisms. The first probable explanation is the mechanism of uncompetitive inhibition, suggesting the presence of an inhibitor interacting with the complex of the enzyme with already bound sodium ions. Due to the rather high lipophilicity of stobadine (partition ratio in octanol/water at pH 7.4 is 3.72 [38]) and its relatively high chemical stability [34], it is possible that stobadine remaining in plasmalemmal membranes also after the isolation procedure inhibited renal Na,K-ATPase. The second probable explanation for the stobadine-induced effect on renal Na,K-ATPase in control rats is the expression of a new type of Na,K-ATPase with better affinity to sodium, as suggested by lowered K_{Na} value, and a reduced amount of the enzyme in tissue, as suggested by markedly lowered V_{max}. Regardless of the mechanism responsible for the observed effect, after long-term administration of stobadine to control rats, Na,K-ATPase seems to transport the excessive sodium with limited efficiency, especially in the presence of elevated concentration of intracellular Na^+.

On the other hand, administration of stobadine to diabetic rats dramatically improved the function of renal Na,K-ATPase with respect to sodium-handling. The dosage of stobadine (0.05% w/w) used in our experiments was effective in protecting diabetic tissues against oxidative stress [6, 7, 15]. Evaluation of sodium kinetic parameters revealed a profound increase in V_{max}, suggesting that suppression of diabetes-induced damage in the kidney by stobadine was followed by an increase in the amount of active Na,K-ATPase molecules. This conclusion is supported by the observations of other authors [13] using different antioxidants in preserving diabetes-induced complications. Restoration of Na,K-ATPase function by administration of γ-linolenic acid to diabetic rats was accompanied by increased expression of enzyme molecules [13]. However, the affinity of the enzyme for sodium remained unaffected, as documented by a statistically insignificant decrease of K_{Na} value.

In summary, our findings show that administration of stobadine to diabetic rats antagonises the negative effects of diabetes on renal Na,K-ATPase, thus enabling the enzyme to preserve its normal function in regulation of intracellular homeostasis of Na^+ and K^+ ions.

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