THE EFFECT OF Ca^{2+}-MOBILISING HORMONES ON THE Na^+–K^+ PUMP IN ISOLATED RAT LIVER HEPATOCYTES

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1. Introduction

In addition to their effect on liver glucose metabolism [1], α-agonists are known to alter the movements of ions through the hepatocyte plasma membrane. In most species, the activation of hepatocyte α-adrenoreceptors leads to a net loss of K^+ from the cells [1,2]. This effect is believed to be due to a receptor-mediated rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) which in turn stimulates the Ca^{2+}-dependent K^+ channels located in the plasma membrane [3,4].

However, in isolated rat liver cells this net loss of K^+ is entirely absent and instead, application of α-agonists and other agents which are thought to increase $[Ca^{2+}]_i$ (e.g., applied ATP, Ca^{2+} ionophore A23187) [4,5] results in a net uptake of K^+. This uptake has been attributed to an activation of the Na^+–K^+ pump [4]. These results confirm the hypothesis and show that α-adrenergic stimulation of the Na^+–K^+ pump is dependent on the presence of Ca^{2+}. Depleting the hepatocytes of their Ca^{2+} activates the Na^+–K^+ pump and, at the same time, blocks the stimulatory effect of α-agonists and A23187. It is proposed that the activation of the Na^+–K^+ pump by noradrenaline and other Ca^{2+}-mobilising agents is the result of a displacement of an inhibitory pool of Ca^{2+} located on the internal face of the plasma membrane in the microenvironment of the pump.

2. Materials and methods

Hepatocytes were isolated from the livers of female Wistar rats as indicated in [3], then equilibrated at 37°C and pH 7.40 for 30–60 min in a modified Eagle's solution containing: (mM) – NaCl, 116; KCl, 5.6; $CaCl_2$, 1.8; $MgCl_2$, 1.23; NaH_2PO_4, 0.52; $NaHCO_3$, 25; and (mg/l) – amino acids, 805; vitamins, 8.1; glucose, 2000; L-glutamine, 292; phenol red, 10. This medium was supplemented with 2% albumin (FV, Sigma) and gassed with 5% CO_2 in O_2. The cells were then divided, some being kept in the above medium (control cells) and some being placed in a Ca^{2+}-free medium containing 100 μM EGTA (Ca^{2+}-depleted cells) for 30–180 min. In some experiments the Na^+ content of both control and Ca^{2+}-depleted cells was increased by omitting K^+ from the media.

The Na^+ content was determined by incubating the cells (5–10 mg dry wt/ml) with $^{22}Na^+$ (0.5 μCi/ml) to isotopic equilibrium (30 min, see [6]). The external $[K^+]$ was measured using a K^+-sensitive electrode as indicated in [4]. The Na^+–K^+ pump activity was determined from either $^{42}K^+$ or $^{86}Rb^+$ influx. Control and Ca^{2+}-depleted cells (5–10 mg/ml) were incubated with the tracer (1 μCi/ml) for 90 s in the absence or in the presence of 1 mM ouabain. The ouabain was added 6 min before $^{42}K^+$ or $^{86}Rb^+$. Noradrenaline, ATP and the Ca^{2+} ionophore A23187 (applied in 10 μl ethanol which by itself had no effect) were added at the same time as $^{42}K^+$ or $^{86}Rb^+$. When the α-agonist phenoxybenzamine was used, it was applied 6 min before markers and at the same time as the ouabain. All the solutions contained 5 μM propranolol to block β-adrenoreceptors.

At the end of all experiments, i.e., 90 s after the addition of agents 0.1 ml samples were centrifuged through an oil phase supplemented with a washing solution (NaCl, 150 mM; EGTA, 2 mM; pH 7.4) for counting of $^{42}K^+$ or $^{86}Rb^+$. In the case of $^{22}Na^+$, the cell samples were centrifuged 120 s after the addition of noradrenaline and the washing solution contained

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traces of [^{3}H]inulin to estimate $^{22}Na^+$ trapped in the extracellular space of the pellet [6]. The viability of cells was checked before and after each experiment by their ability to exclude trypan blue (0.4%) and was ~92%. The dry weight of the cell samples was estimated as in [4].

3. Results

Fig.1 shows that noradrenaline (5 μM) in the presence of the β-blocker propranolol (5 μM) stimulates the ouabain-sensitive K^+ influx in rat hepatocytes from a basal level of 5 to an activated level of 9 nmol . mg dry wt^{-1} . min^{-1}. This response was blocked by the α-antagonist phenoxybenzamine (50 μM). Neither propranolol nor phenoxybenzamine altered the Na^+–K^+ pump activity and noradrenaline did not affect the ouabain-resistant K^+ influx. The response to the hormone was dose-dependent with an EC_{50}-value of ~0.1 μM. Fig.1 also shows that other agents which in common with α-agonists, are thought to increase $[Ca^{2+}]_i$, such as ATP (10 μM) and the Ca^{2+} ionophore A23187 (2.5 μM corresponding to a cell concentration of ~100–200 μmol/l) also activated the pump.

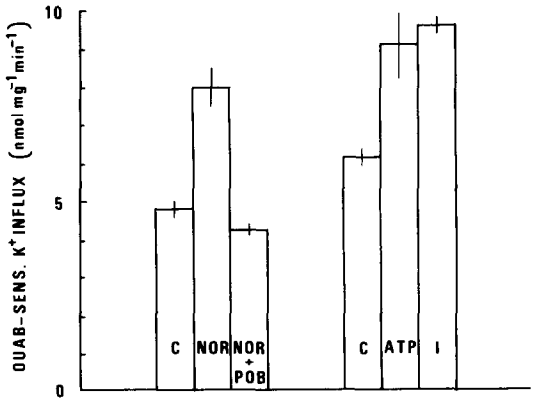

Fig.1. Application of noradrenaline (NOR) (5 μM, in the presence of the β-blocker propranolol (POB) at 5 μM), ATP (10 μM) and A23187 (2.5 μM applied in 10 μl ethanol) stimulates the ouabain-sensitive $^{86}Rb^+$ nflux (nmol . mg dry wt^{-1} . min^{-1}). Cell suspension (1 ml) contained 5–10 mg dry wt. When used, the α-antagonist phenoxybenzamine (50 μM) was added 6 min before noradrenaline. Cell samples (0.1 ml) were centrifuged through an oil phase 90 s after the addition of the tracer and the agent mentioned. Mean of 5–18 values ± SE of a mean.

Table 1
Effect of noradrenaline (5 μM, in the presence of 5 μM propranolol) on $[Na^+]$ of isolated rat hepatocytes and on $[K^+]$ of their bathing fluid

	Control	Noradrenaline	Noradrenaline + propranolol
$[Na^+]_i$ (nmol/mg)	30.8 ± 1.23	19.4 ± 0.83	29.2 ± 0.56
$[K^+]_o$[a] (mM)	6.001 ± 0.124	5.956 ± 0.123	–

[a] SEM for $[K^+]$ were high because control bathing fluids (before the addition of noradrenaline) had $[K^+]$ values of 5.681–6.703 mM. However, the effect of noradrenaline was highly significant ($P < 0.01$) when compared to paired controls (see [4])

Each measurement was made 120 s after the addition of noradrenaline and the values given are the means ± SEM (n = 6–8)

The α-receptor-mediated activation of the Na^+–K^+ pump results in a net decrease in internal $[Na^+]$ content of the hepatocytes and decrease in external $[K^+]$. Ionophore A23187 caused similar decreases in $[Na^+]_i$ and $[K^+]_o$ (not shown). Analysis of the time courses of the net movements of Na^+ and K^+ calculated from the $[Na^+]$ and external $[K^+]$ showed that the Na^+–K^+ pump was maximally activated by noradrenaline at 30 s and returned to its basal level within 3–5 min.

As the stimulatory effect of the hormones noradrenaline and ATP was mimicked by the Ca^{2+} ionophore A23187, the rôle of Ca^{2+} in activating by the Na^+–K^+ pump was investigated. Cells were incubated in low-Ca^{2+} media as in section 2. In rat hepatocytes this treatment depletes internal Ca^{2+} stores [7] and does not alter the integrity of plasma membrane (unpublished). Fig.2 illustrates that depleting the cells of their Ca^{2+} resulted in an activation of the Na^+–K^+ pump. This increase in activity (~60%) was similar to that caused by maximal doses of noradrenaline and ATP. Fig.2 also shows that in Ca^{2+}-depleted cells, noradrenaline was no longer able to stimulate the Na^+–K^+ pump. Similar results were observed with the Ca^{2+} ionophore A23187. These results suggest that α-agonists or externally applied ATP activate the Na^+–K^+ pump by a mechanism which is dependent on the presence of Ca^{2+}.

The effect of Ca^{2+}-depletion on the Na^+–K^+ pump activity did not result from an increase in internal $[Na^+]$. This could have occurred if the reduction in the electrochemical gradient for Ca^{2+} had promoted a net

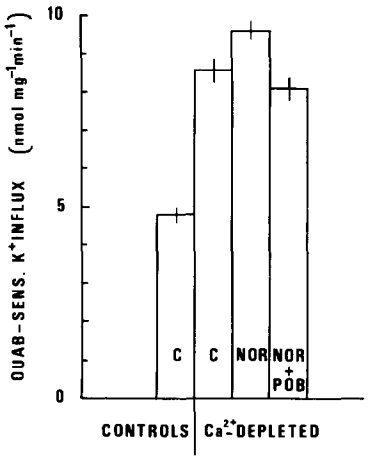

Fig.2. Effect of Ca^{2+} depletion on the Na^+–K^+ pump and on the α-mediated increase of the Na^+–K^+ pump activity. Experimental was as in fig.1 except that Ca^{2+}-depleted cells were pre-incubated for 30–180 min in a Ca^{2+}-free media supplemented with 100 μM EGTA to deplete internal Ca^{2+} stores. Ca^{2+} depletion increased the Na^+–K^+ pump activity and blocked the increase provoked by 5 μM noradrenaline.

influx of Na^+ mediated for example by a Na^+/Ca^{2+} exchange mechanism. However the $[Na^+]$ of Ca^{2+}-depleted cells, 37.7 ± 1.3 nmol/mg (n = 24) was not different from that found in control cells, 37.1 ± 1.0 nmol/mg (n = 24). Moreover, when cells were enriched in Na^+ (78.7 ± 1.3 nmol/mg, n = 18) by pre-incubation in K^+-free media so that the Na^+–K^+ pump activity was increased to ~16 nmol/mg, noradrenaline continued to be able to stimulate the Na^+–K^+ pump. In the Na^+-enriched cells, Ca^{2+}-depletion was also able to increase the pump activity and to greatly reduce the stimulatory effect of noradrenaline.

4. Discussion

Noradrenaline via α-adrenoreceptors, externally applied ATP and the Ca^{2+} ionophore A23187 lead to a stimulation of the Na^+–K^+ pump in isolated rat liver cells incubated with Ca^{2+}. The EC_{50}-value of the response to noradrenaline was 0.1 μM. It occurred without apparent delay, was maximal within the first 30 s and was complete within 3–5 min following the addition of noradrenaline. This is in keeping with other physiological responses to α-agonists in rat hepatocytes such as the stimulation of glycogen phosphorylase and net Ca^{2+} efflux both of which have similar time courses [8,9]. In contrast, the α-adrenoreceptor agonist isoprenaline (0.05–0.2 μM) had no apparent effect on the Na^+–K^+ pump in these cells (G. M. B., unpublished).

The Na^+–K^+ pump is an intrinsic protein which spans the plasma membrane. It is stimulated by cytosolic Na^+, ATP and external K^+ and inhibited by cytosolic ADP and Ca^{2+} [10]. During α-activation, we have found that $[Na^+]_i$ and $[K^+]_o$ were decreased (table 1); cytosolic concentrations of ATP and ADP are not substantially altered [11]. As $[Ca^{2+}]_i$ increases [12] it is possible that the effect of hormones and the Ca^{2+} ionophore is mediated via the plasma membrane itself. However, these results also imply that the mechanism by which these agents alter the Na^+–K^+ pump activity is dependent on Ca^{2+}. This is based on the observations that:

(i) The response is mimicked by the Ca^{2+} ionophore A23187;

(ii) Ca^{2+}-depletion stimulates the Na^+–K^+ pump;

(iii) The effect of noradrenaline and the Ca^{2+} ionophore disappears in the absence of Ca^{2+}.

A possible explanation is that the binding of noradrenaline and ATP to their respective receptors or the introduction of A23187 into the membrane lipids could transiently displace Ca^{2+} tightly bound to the membrane in the microenvironment of the Na^+–K^+ pump. If this Ca^{2+} normally had an inhibitory effect on the Na^+–K^+ pump, its influence could thus be removed.

This hypothesis has been reinforced by our observation (unpublished) that noradrenaline triggers a release of Ca^{2+} fro[illegible]gh affinity binding sites in isolated rat liver plasma membranes. This displacement of Ca^{2+} could thus be part of a membrane coupling mechanism which follows the occupation of receptors by the hormones and which precedes the activation of the Na^+–K^+ pump. In [13] α-agonists and Ca^{2+}-free media were also reported to stimulate the Na^+–K^+ pump in isolated plasma membranes of other tissues [13]. This hypothesis could also explain the apparent contradiction that Ca^{2+} depletion mimicks the effects of hormones which use Ca^{2+} as an intracellular messenger to exert their effect on the cell metabolism. The stimulation of α-adrenoreceptors mobilises Ca^{2+} from internal stores [14]. If this hypothesis is valid, the internal face of plasma membrane could also participate in the rise of $[Ca^{2+}]$.

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