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Isoproterenol-induced Mg^{2+} uptake in liver

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Isoproterenol increased the Mg^{2+} content of hepatocytes after injection into rats or after addition to collagenase-dispersed hepatocytes. cAMP also the increased cellular Mg^{2+} content of isolated hepatocytes. This effect was prevented by staurosporine. Phorbol ester had no effect on the Mg^{2+} content of isolated hepatocytes, and after injection of isoproterenol into rats, protein kinase C of liver was not affected. It was concluded that isoproterenol induced long-term Mg^{2+} influx via the activation of protein kinase A which can be inhibited by staurosporine.

Magnesium influx; Isoproterenol; cAMP; Protein kinase C; Rat liver

1. INTRODUCTION

When isolated liver was perfused with Mg^{2+}-free medium, β-adrenergic stimuli caused a transient efflux of Mg^{2+} [1–3] which was dependent on extracellular Na^+ and inhibited by amiloride [2] as was Na^+/Mg^{2+} antiport from other cells [3,4]. Activation of Mg^{2+} efflux from hepatocytes by β-adrenergic stimulation was also demonstrated by $^{28}Mg^{2+}$ loss when the cells were incubated at an extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) of 1.2 mM [5].

In isolated Mg^{2+}-loaded thymocytes, cAMP activated Na^+/Mg^{2+} antiport up to a maximal level [6]. Hence, β-adrenergics stimulate Mg^{2+} efflux via Na^+/Mg^{2+} antiport. On the other hand, an increase of Mg^{2+} content in rat liver by about 20% was found 4 h after injection of 3 mg/kg isoproterenol [7] or 1 and 2 days after injection of 65 mg/kg isoproterenol [8], indicating net Mg^{2+} influx.

The present experiments were undertaken to clarify the obviously different effects of isoproterenol on Mg^{2+} transport in hepatocytes.

2. MATERIALS AND METHODS

2.1. *In vivo experiments*

Male Wistar rats weighing 200 g were subcutaneously injected with (−)-isoproterenol hydrochloride (Sigma). At various times after injection, as indicated in Fig. 1, or after injection of various isoproterenol doses, as indicated in Fig. 2, livers were taken under Nembutal anesthesia (50 mg/kg s.c.), frozen in liquid nitrogen, freeze-dried and powdered in a plastic mortar. Powdered liver was ashed in the Plasma Processor 200-E (Technics, Munich, Germany). The ash was dissolved in 0.1 N HCl and Mg^{2+} was measured by atomic absorption spectrophotometry (Philips, SP 9).

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2.2. *In vitro experiments*

2.2.1. Preparation of hepatocytes

Isolated perfused rat livers were dispersed by a two-step procedure of Ca^{2+} removal, followed by collagenase (Sigma, type I) treatment according to Seglen [9]. The isolated hepatocytes were filtered through two layers of gauze, washed twice in prewarmed (37°C) Na^+ medium by centrifugation at 44 g for 1 min and purified from damaged cells and cell debris by Percoll centrifugation. Na^+ medium contained (in mM): 125 NaCl, 15 $NaHCO_3$, 5 KCl, 1 KH_2PO_4, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 1 adenosine, 20 HEPES-NaOH, pH 7.4.

For Percoll centrifugation, 1 vol. cells was suspended in 4 vols. Na^+ medium and mixed 1:1 (v/v) with 70% Percoll (Pharmacia) in Na^+ medium and centrifuged at 1,500 × *g* for 5 min. The sedimented hepatocytes (95% viable by Trypan blue exclusion) were resuspended in incubation medium and taken for measuring Mg^{2+} influx.

2.2.2. Mg^{2+} influx

The isolated hepatocytes were suspended in prewarmed (37°C) incubation medium containing (in mM): 125 NaCl, 15 $NaHCO_3$, 5 KCl, 1 KH_2PO_4, 2.4 $CaCl_2$, 0.9 $MgCl_2$, 5 glucose, 1 adenosine, 50 g/l bovine serum albumin (Serva, fraction V), 20 HEPES-NaOH, pH 7.4. Cell concentration amounted to 2–3% (v/v) corresponding to 1–2×10^6 cells/ml. Cell suspensions were gassed with 95% O_2/5% CO_2. At the beginning of incubation and after various times as indicated, 1 ml aliquots of the cell suspensions were centrifuged (1 min at 44 × *g*). The supernatant was sucked off and 1 ml 5% (w/v) trichloroacetic acid (TCA) was added to the pellet. After homogenisation and centrifugation, Mg^{2+} concentration of the TCA extract was measured by atomic absorption spectrophotometry . Protein content of the TCA precipitates was measured with the Pierce BCA Protein Assay [10]. Mg^{2+} influx was calculated from the increase of cellular Mg^{2+} content.

2.2.3. Protein kinase C

For determination of protein kinase C (PKC), 1 g freshly removed liver was homogenized at 0°C with 9 ml homogenization buffer which contained (in mM): 25 Tris-HCl pH 7.5, 250 suerose, 5 EDTA, 10 EGTA, 50 2-mercaptoethanol, and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 120 × *g* for 5 min at 0°C, and the 120 × *g* supernatant was centrifuged at 100,000 × *g* for 1 h at 0°C. The 100,000 × *g* supernatant represented the cytosolic fraction.

The 120 × *g* sediment was suspended in 8 ml homogenization buffer plus 0.3% Triton X-100 and incubated for 1 h at 0°C (membrane fraction) [11].

Cytosolic fractions were diluted 1:10 with homogenization buffer, membrane fractions were diluted 1:10 with homogenization buffer plus 0.3% Triton X-100. From both dilutions, 25 μl were taken for the determination of PKC activity by the protein kinase C assay system (phosphorylation of histone type IIIS) according to the instructions of the producer (Amersham, code RPN 77).

3. RESULTS AND DISCUSSION

To measure the rate of Mg^{2+} efflux, short-term experiments were done [1,2,5]. Therefore, we measured the time course of Mg^{2+} content of livers from rats injected with 3 mg/kg isoproterenol. As shown in Fig. 1, isoproterenol induced Mg^{2+} uptake which reached a maximum 6 h after injection.

In order to ascertain whether the effect of isoproterenol on Mg^{2+} content is dose-dependent, we tested the effect of various isoproterenol doses on Mg^{2+} uptake. As shown in Fig. 2, all used doses of isoproterenol induced Mg^{2+} uptake.

Mg^{2+} uptake in vivo was measured in freeze-dried livers [7,8]. Therefore, we tested whether the isoproterenol-induced loss of hepatic glycogen had simulated the increase in hepatic Mg^{2+}. In control livers, glycogen content amounted to 20 g/kg dry weight, and 4 h after 3 mg/kg isoproterenol, glycogen was no longer detectable (data not shown). Hence, the isoproterenol-induced loss of glycogen (2% of dry weight) had not caused the elevated Mg^{2+} content (13%) 4 h after isoproterenol injection. Hence, there must be an induction of net Mg^{2+} uptake in hepatocytes by isoproterenol.

In experiments with isolated hepatocytes net uptake of Mg^{2+} was induced by stimulation of PKC [5] and it was postulated that Mg^{2+} efflux is caused by β-adrenergics and Mg^{2+} influx by PKC [5,12].

β-agonist-induced Mg^{2+} efflux from isolated hepatocytes was terminated within 8 min [5] and from perfused liver within 15 min [2] whereas isoproterenol-induced Mg^{2+} uptake in vivo continued for much longer time periods (Fig. 1).

Therefore, we investigated the time course of Mg^{2+} content in isolated hepatocytes after addition of isoproterenol and dibutyryl cAMP (dbcAMP).

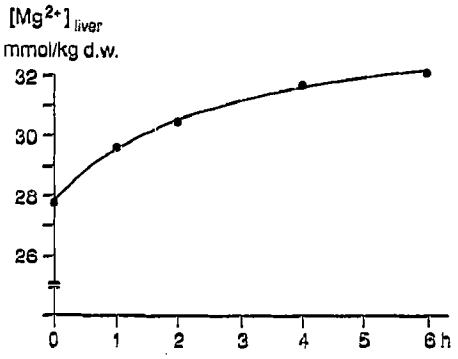

Fig. 1. Time course of Mg^{2+} uptake in rat liver after s.c. injection of 3 mg/kg isoproterenol. Mean of 3 rats for each time point.

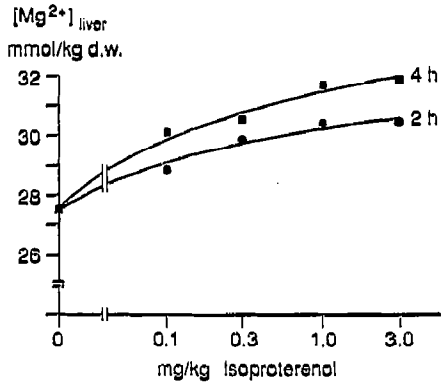

Fig. 2. Dose-dependency of Mg^{2+} uptake in rat liver after s.c. injection of isoproterenol. ●, 2 h after injection; ■, 4 h after injection. Mean of 4 rats.

Isolated hepatocytes expressed an Mg^{2+} content of 48.5 nmol/mg protein (Fig. 3), compared to 64.5 nmol Mg^{2+}/mg protein of rat liver in vivo. This difference is caused by loss of Mg^{2+} during the isolation procedure. After reincubation, isolated hepatocytes took up Mg^{2+}. Mg^{2+} uptake was stimulated by isoproterenol and dbcAMP. After 90 min of reincubation 100 μM isoproterenol and 100 μM dcAMP caused an increase of Mg^{2+} content in hepatocytes by 7.6% and 8.4% (Fig. 3). The increase in hepatic Mg^{2+} 90 min after isoproterenol injection was in the same range (Fig. 1). Thus, the effects of isoproterenol on liver Mg^{2+} in vivo may represent a direct effect of isoproterenol on hepatocytes and not an indirect effect by secondary events, e.g. alteration of hepatic blood flow or release of other hormones.

During the first 10 min of reincubation of isolated hepatocytes, Mg^{2+} content was not significantly changed (Fig. 3). Probably, during this phase there was a small β-adrenergic-induced efflux of hepatic Mg^{2+}

Table I

Effect of dbcAMP and stauroporine on Mg^{2+} uptake in collagenase-dispersed hepatocytes. Values (Δ Mg^{2+}) represent Mg^{2+} content after 60 and 90 min incubation minus Mg^{2+} content at zero time which amounted to 48.3 nmol/mg protein

	Δ Mg^{2+} (nmol/mg protein)	
	60 min	90 min
Control	6.3±0.7	7.5±0.8
Staurosporine, 10^{-7} M	6.5±0.6	7.3±0.9
dbcAMP, 10^{-4} M	10.2±1.2[a]	12.2±1.4[a]
dbcAMP, 10^{-4} M + staurosporine, 10^{-7} M	6.0±0.6	7.4±0.7

Mean ± S.E.M. of 4 experiments in quadruplicates. Significant difference between different groups by unpaired Student's t-test. [a] $P<0.05$.

Table II

Effect of phorbol ester (PMA) on Mg^{2+} uptake in collagenase-dispersed hepatocytes. See legend to Table I

	Δ Mg^{2+} (nmol/mg protein)	
	60 min	90 min
Control	6.0	7.6
Staurosporine, 10^{-7} M	5.9	7.5
PMA, 10^{-6} M	6.0	7.6
PMA, 10^{-6} M + staurosporine, 10^{-7} M	6.0	7.5

Mean of 3 experiments in quadruplicates. No significant differences.

Table III

Protein kinase C in cytosolic and membrane fraction of rat liver at various time points after injection of 3 mg/kg isoproterenol

Time after injection	Cytosol (pmol ^{32}P/mg prot·min)	Membranes (pmol ^{32}P/mg prot·min)
Control	863±70	143±2
1	903±58	137±3
2	788±63	137±4
4	842±90	159±8

Mean ± S.E.M. of 3 rats at each time point. No significant differences to control (uninjected rats) by unpaired Student's *t*-test.

[1,2,5] which could not be detected by measuring cellular Mg^{2+} content.

From the effects of isoproterenol on Mg^{2+} content in vivo and in vitro it may be concluded that β-adrenergics and cAMP have a biphasic effect on Mg^{2+} transport, immediately inducing a short-term efflux and thereafter a long-term influx of Mg^{2+}. The mechanism of the first Mg^{2+} efflux phase induced by cAMP has already been investigated. It is produced by Mg^{2+} release from mitochondria [5] and activation of Na^+/Mg^{2+} antiport [2]. The second Mg^{2+} influx phase by cAMP may be caused by various mechanisms:

1. There may be a monodirectional control system by which cAMP and protein kinase A (PKA) may enhance the activity of PKC [13]. This mechanism was recently demonstrated for cultured renal LLC-PK_1 cells [14]. Thus, the second phase of cAMP would be mediated by PKC.
2. PKA and PKC may act synergistically by phosphorylating the same target protein which may enhance Mg^{2+} uptake by hepatocytes. A synergistic phosphorylation of the same protein by PKA and PKC was recently shown [15].
3. Mg^{2+} uptake is induced only by PKA which may have two separate effects, an early induction of net Mg^{2+} efflux and a late induction of net Mg^{2+} influx.

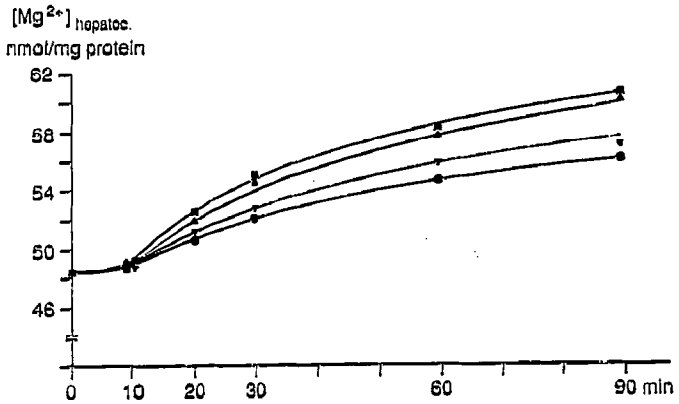

Fig. 3. Mg^{2+} uptake in collagenase-dispersed hepatocytes. ●, control; ▼, addition of 10 μM isoproterenol, ▲, addition of 100 μM isoproterenol; ■, addition of 100 μM dbcAMP. Mean of 6 experiments.

The following experiments were undertaken to discriminate between these possibilities.

As shown in Table I, Mg^{2+} uptake in isolated hepatocytes was stimulated by 0.1 mM dbcAMP and the cAMP-induced Mg^{2+} uptake was reduced to control values by 10^{-7} M staurosporine. Staurosporine alone was ineffective. This result may indicate that the long-term effect of cAMP may be caused by PKC. However, staurosporine may also have inhibited PKA, since this substance inhibits PKC and PKA with the same efficacy [16].

In order to see whether PKC is involved in the long-term cAMP-induced net Mg^{2+} uptake an analogous experiment was performed with phorbol ester. As shown in Table II, 10^{-6} M PMA (phorbol 12-myristate 13-acetate) had no significant effect on the Mg^{2+} content of isolated hepatocytes. In short-term experiments with isolated hepatocytes phorbol ester reduced $[Mg^{2+}]_o$ when the cells were incubated at extremely low $[Mg^{2+}]_o$ [12]. Hence, the amount of Mg^{2+} uptake induced by phorbol ester and PKC may be small and may not cause any significant increase in total cellular Mg^{2+} content. Also, the long-term increase in $^{28}Mg^{2+}$ influx by phorbol ester in S49 lymphoma cells was limited to a very small subcytoplasmic pool which amounted only to 2–3% of total cellular Mg^{2+} [17]. Since Mg^{2+} efflux from lymphoma cells was not affected by phorbol ester [17], the action of phorbol ester may result in a 2–3% increase of total cellular Mg^{2+} in lymphoma cells. If phorbol ester had the same effect on hepatocytes, this effect could not be determined by measuring total cellular Mg^{2+}.

However, in S49 lymphoma cells, Mg^{2+} fluxes may be differently regulated, because in these cells β-adrenergic agonist inhibited Mg^{2+} influx without affecting Mg^{2+} efflux [18], whereas in hepatocytes β-agonists induced Mg^{2+} efflux [1,2,5].

To additionally investigate the role of PKC in isoproterenol-stimulated Mg^{2+} uptake in hepatocytes, we measured the activity of PKC in the cytosolic and mem-

brane fractions of rat liver cells at various times after injection of isoproterenol in parallel to the increase of Mg^{2+} content. As shown in Table III, subcutaneous injection of 3 mg/kg isoproterenol did not affect PKC in the cytosolic and membrane fractions of liver cells. Hence, the isoproterenol-induced Mg^{2+} uptake in liver cells is not mediated via PKC.

From the results it can be concluded that the β-agonist-induced long-term Mg^{2+} uptake by hepatocytes is mediated via PKA, which can be inhibited by staurosporine. When the short-term effect of isoproterenol, resulting in an induction of Mg^{2+} efflux, is also taken into consideration, it can be assumed that isoproterenol has a biphasic effect on Mg^{2+} fluxes: induction of Mg^{2+} efflux within the first few minutes of action and thereafter induction of Mg^{2+} influx.

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