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Regulation of Mg^{2+} uptake in isolated rat myocytes and hepatocytes by protein kinase C

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A large Mg^{2+} cell uptake against concentration gradients is stimulated in collagenase-dispersed rat myocytes by carbachol and in hepatocytes by carbachol or vasopressin. The signalling pathway(s) responsible for this stimulation of Mg^{2+} uptake was investigated by using various activators or inhibitors of protein kinase C (PKC) and by correlating Mg^{2+} uptake with cell PKC activity and cAMP content. In both cell preparations, the direct stimulation of PKC by diacylglycerol analogs or phorbol esters reproduce the same pattern of Mg^{2+} uptake as that induced by carbachol or vasopressin. These data indicate that the activation of PKC is responsible for a stimulation of Mg^{2+} uptake by myocytes or hepatocytes, whereas increase in cAMP in these cells stimulates Mg^{2+} release.

Mg^{2+} transport; Protein kinase C; Hepatocyte; Myocyte

1. INTRODUCTION

In spite of the major role of Mg^{2+} in regulating metabolic pathways, channel activities and cell bioenergetics, the regulation of cell Mg^{2+} homeostasis is far less understood than that of other major cations.

As a consequence of hormonal stimulation, dynamic changes in cell Mg^{2+} content or concentration have been observed in various organs such as liver [1–4], hearts [5–7] and isolated cells [4,6,8–12].

We have recently reported that hormones or agonists which increase intracellular cAMP produce a large Mg^{2+} efflux from perfused hearts or livers and from collagenase-dispersed myocytes or hepatocytes [4,6,7]. A major mobilization of Mg^{2+} could be demonstrated upon the direct addition of nanomolar concentrations of cAMP to digitonin-permeabilized hepatocytes or to isolated rat liver mitochondria [13]. Hence, most of the observable Mg^{2+} efflux from those cells upon increase of cAMP can be accounted for by a redistribution of Mg^{2+} from the mitochondrial pool to the cytosol, followed by a plasma membrane Mg^{2+} efflux which may or may not be activated by cAMP.

By contrast, the mechanism of Mg^{2+} uptake by cells remains unclear in spite of recent efforts by various laboratories. Evidence for a Na^+–Mg^{2+} exchanger has been clearly documented in the literature [14,15], and a novel P-class ATPase inwardly transporting Mg^{2+} has recently been reported in bacteria [16,17].

Less data are available on the modulation of this uptake. Using different experimental approaches, Murphy et al. [1] and Bond et al. [2] observed an increase in cell Mg^{2+} both in vivo and in vitro upon stimulation with vasopressin or carbachol. More recently, we observed a large Mg^{2+} cell uptake against concentration gradients in myocytes after addition of carbachol [4], and in hepatocytes after addition of carbachol and vasopressin [6].

In this work we have investigated the signalling pathway(s) responsible for the observed cell Mg^{2+} uptake induced by those hormones and agonists in hepatocytes and myocytes. By using various activators and inhibitors of protein kinase C (PKC), we correlate modulation of Mg^{2+} uptake with PKC activity and with cAMP content. These correlations have permitted us to provide evidence of an involvement of PKC in the regulation of cell Mg^{2+} uptake, but not release, in both myocytes and hepatocytes.

Abbreviations: HEPES, 4-(2-hydroxymethyl)-1 piperazine ethane sulphonic acid; PIPES, piperazine-*N*-*N*′-bis[2-ethane-sulfonic acid]; EGTA, ethylene glycol-bis(*b*-aminoethyl ether) *N*,*N*,*N*′,*N*′,-tetraacetic acid; EDTA, ethylenediamine-tetraacetic acid; FCCP, carbonyl-cyanide *p*-(trifluoromethoxy-)phenylhydrazone; AA, atomic absorbance spectroscopy; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol (C18:1, [*cis*]-9/C2:0); PKC, protein kinase C; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol (C18:0/C20:4, [*cis*,*cis*,*cis*,*cis*]-5,8,11,14); PDBU, phorbol 12,13-dibutyrate; TPA, phorbol 12-myristate 13-acetate; H7,1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

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2. MATERIALS AND METHODS

Hearts or livers were obtained from fed male Sprague-Dawley rats (220–250 g) and anesthetized by intraperitoneal injection of phentobarbital (0.1 ml/100 g body wt.). The hearts were removed and myocytes prepared as previously described [18]. In different rats, livers were perfused in situ and collagenase-dispersed hepatocytes were

prepared according to the Seglen procedure [19]. Both myocytes and hepatocytes were washed 3-times with a buffer containing (mM): 120 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 12 $NaHCO_3$, 10 NaHEPES, pH 7.2 (at 37°C in presence of O_2:CO_2, 95:5) and maintained in this buffer until used. The cell viability (71 ± 3% and 90 ± 2% SD for myocytes and hepatocytes, respectively; n=7) was measured by Trypan blue exclusion test and did not significantly increase during 3–4 h following isolation.

Myocytes and hepatocytes were incubated at 37°C, at the concentration of 100 and 250 μg protein/ml, respectively, in the reaction mixtures previously described but containing no added Mg^{2+}. The total contaminant Mg^{2+} in the buffer was measured using atomic absorbance spectrophotometry (AA) and ranged between 15–20 μM. 5 min after the addition of the cells, norepinephrine (NE), carbachol, vasopressin (VP) or a modulator of PKC activity (either OAG, SAG, TPA, PDBU, H7 or staurosporine) were added to the incubation mixture. At the times indicated, aliquots of the reaction mixture were withdrawn, the cells sedimented in microfuge tubes and the Mg^{2+} content measured in the supernatant by AA.

To estimate the total cell Mg^{2+} content, hepatocytes were sedimented through an oil layer (dibutyl-phthalate:dioctyl-phthalate, 2:1 by volume). The pellets were digested overnight in 1 ml 10% HNO_3 and the Mg^{2+} content was measured by AA in known aliquots of the acid extract.

The Mg^{2+} content of intracellular compartments was measured in hepatocytes before and after stimulation with 20 μM TPA. After treatment with TPA cells were transferred for the incubation in a buffer containing (mM): 100 KCl, 5 NaCl, 10 glucose, 12 $NaHCO_3$, 1.2 KH_2PO_4, 2 EGTA, 1 $CaCl_2$, 10 NaHEPES, pH 7.2, at 37°C with O_2:CO_2 95:5. The free Ca^{2+} concentration of this buffer, estimated using the Fabiato program [20], ranged between 150–170 nM, whereas the Mg^{2+} content, measured by AA was 15–20 μM. The cells were permeabilized in 3 min by adding 80 μg/ml digitonin. 2 μg/ml FCCP and 10 μg/ml A23187 were sequentially added to the permeabilized hepatocytes to stimulate Mg^{2+} release from different intracellular compartments. At selected times after these additions, cells were withdrawn, sedimented through an oil layer and digested overnight in 1 ml 10% HNO_3. The Mg^{2+} content was measured by AA in the acid extract.

For PKC assay, aliquots of hepatocytes were sedimented in microfuge tubes and the cell pellets were extracted in 400 μl of 50 mM Tris/HCl, pH 7.2, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% b-mercaptoethanol, 1% nonionic detergent Nonidet P-40. 25 μl aliquots of cell extract were diluted into a final volume of 100 μl containing 10 mM $MgCl_2$, 0.3 mg/ml BSA, 18 mM PIPES/KOH, pH 6.6, 100 μM [^{32}P]γATP, 8 μM PKC substrate peptide, and tested for PKC activity in presence or absence of 1 μM PMA, 170 μg/ml phosphatidylserine, 0.5 mM $CaCl_2$ for 3 min at 30°C. The assay medium was acidified as reported in [21] and spotted onto P81 cellulose squares, which were processed according to the procedure of Kempe et al. [22].

For measuring cAMP cell content, aliquots of reaction mixtures were withdrawn and sedimented at 10 000 × g for 1 min. The pellets were resuspended in 60 μl 0.1 N HCl and stored overnight at 4°C before freezing at −80°C [23]. The cell cAMP content was measured in 25 μl of the acid extract by radioimmunoassay [24].

Protein was measured by the Bradford procedure [25].

2.1. *Chemicals*

Collagenase CLS-II (for myocytes isolation) and Collagenase CLS-I (for hepatocytes preparation) were from Worthington (Freehold, NJ, USA). H7 was from Seikagaku America Inc. (St. Petersburg, FL, USA). Staurosporine was from Kamiya Biomedical Co. (Thousand Oaks, CA, USA). Dibutyl-phthalate and dioctyl-phthalate were from Aldrich Chemical Co. (Milwaukee, WI, USA). [^{32}P]ATP was from Amersham (Arlington Heights, IL, USA). PKC substrate peptide was from Gibco (Grand Island, NY, USA). [^{125}I]Adenosine 3′-5′-*Sc*-phosphoric acid-2′-OS-TME for cAMP radioimmuneassay was from NEN-Du Pont (Wilmington, DE, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

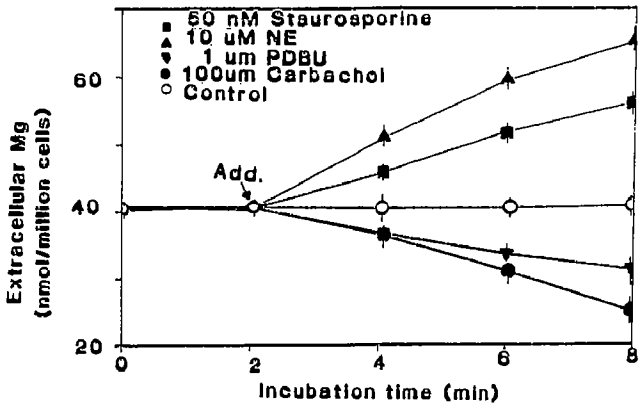

Fig. 1. Magnesium movements in collagenase-isolated myocytes. Myocytes were prepared and incubated as reported in Materials and Methods. Where indicated, 10 μM NE, 50 nM staurosporine, 1 μM PDBU or 100 μM carbachol was added. A similar amount of incubation buffer was added to the control sample. Magnesium content in the supernatant was measured by AA (see Materials and Methods). Data are means ± SEM of 4 different experiments.

3. RESULTS

Fig. 1 shows the results of an experiment where the Mg^{2+} content of supernatants was measured after collagenase-dispersed myocytes were stimulated with various agents and the cells were rapidly sedimented at the times indicated. As previously reported, [4] the addition of norepinephrine (NE) results in a large and time-dependent Mg^{2+} efflux. A qualitatively similar efflux was observed after stimulation with staurosporin, an inhibitor of PKC [26]. By contrast, the addition of carbachol stimulates Mg^{2+} uptake by the myocytes. Stimulation of Mg^{2+} uptake is also observable in the presence of PDBU, a specific activator of PKC [27,28].

Table I confirms the involvement of PKC in the modulation of Mg^{2+} uptake. Myocytes incubated in the presence of the diacylglycerol analogs OAG and SAG or in the presence of the phorbol esters TPA and PDBU, agents directly stimulating the activity of PKC [26–30],

Table I

Magnesium uptake and release by collagenase-dispersed myocytes after modulation of protein kinase C activity

Agent	Incubation time (min)		
	0	4	6
	Extracellular Mg^{2+} content (nmol Mg^{2+}/10^6 cells)		
Control	40.50 ± 0.45	40.64 ± 0.57	40.68 ± 0.68
TPA (80 nM)	40.54 ± 0.59	33.65 ± 0.16	30.80 ± 0.67
PDBU (1 μM)	40.63 ± 0.58	35.53 ± 0.44	32.34 ± 0.49
OAG (20 μM)	40.24 ± 0.32	35.20 ± 0.35	33.24 ± 0.37
SAG (20 μM)	40.88 ± 0.64	35.68 ± 0.48	32.52 ± 0.68
H7 (50 μM)	40.68 ± 0.26	49.06 ± 0.36	53.98 ± 0.58

The data are means ± SEM of 4 different preparations.

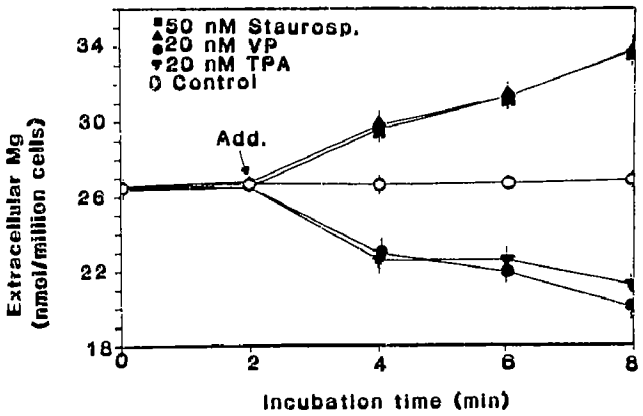

Fig. 2. Magnesium movement in collagenase-dispersed hepatocytes. Hepatocytes were prepared and incubated as reported in Materials and Methods. Where indicated, 10 μM NE, 50 nM staurosporine, 20 nM TPA or 20 nM VP was added. A similar amount of incubation buffer was added to the control sample. Magnesium content in the supernatant was measured by AA. Data are means ± SEM of 4 different experiments.

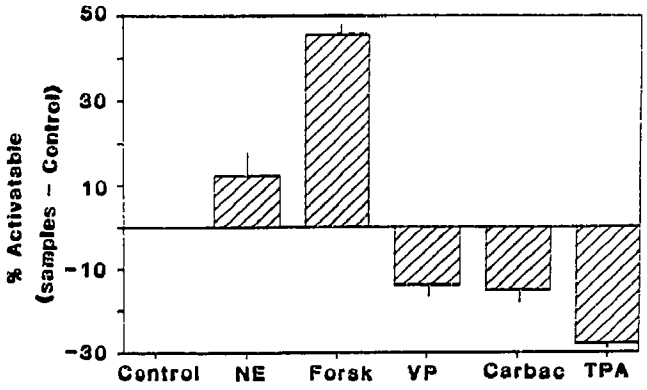

Fig. 4. PKC activation in collagenase-dispersed hepatocytes. Hepatocytes were stimulated for 5 min with 10 μM NE, 50 μM forskolin, 20 nM VP, 100 μM carbachol or 20 nM TPA. Activatable PKC is expressed as percent increase or decrease of activation with respect to basal level. Data are means ± SEM of 3 different preparations.

accumulate Mg^{2+} from the medium similar to that observed in the presence of carbachol. By contrast, the addition of the PKC inhibitor H7 [31] stimulates a Mg^{2+} efflux from myocytes quantitatively similar to that observed with norepinephrine (Fig. 1) or forskolin (not shown).

Fig. 2 shows a similar pattern of Mg^{2+} uptake and release in isolated hepatocytes when stimulated by 10 μM NE, 50 nM staurosporin, 20 nM TPA and 10 μM vasopressin. Also in this cell model, the stimulation of PKC by diacylglycerol analogs or its inhibition by H7 results in the same direction of Mg^{2+} movements as those observed in myocytes (data not shown).

In addition to activating PKC, carbachol and vasopressin have been shown to slightly decrease cAMP level in cardiac and liver cells, respectively [32,33]. This effect may be partly attributed to activation of G_i protein and partly to some cross-talk and modulation between cAMP and PKC [30,34]. Therefore, we measured both the cAMP level and the PKC activity to ascertain which of these intracellular signals was involved in the regulation of Mg^{2+} uptake. Fig. 3A shows that hepatocytes incubated immediately after isolation (not shown), or after 2 h incubation without additions, show a slight increase in cAMP (15–20%) when stimulated with H7 and staurosporin. Under identical conditions (not shown), NE induces a 40% cAMP increase. These cells show a decrease in cAMP of 15–20% with respect to controls when stimulated with vasopressin, carbachol, TPA, OAG or SAG (Fig. 3A). By contrast, hepatocytes pre-incubated during the same 2 h with phorbol ester show no decrease in cAMP level when stimulated with carbachol, VP, TPA, PDBU or SAG (Fig. 3B) but an increase in cAMP level when stimulated with H7, OAG or staurosporine.

Fig. 4 shows measurements of PKC activity of iso-

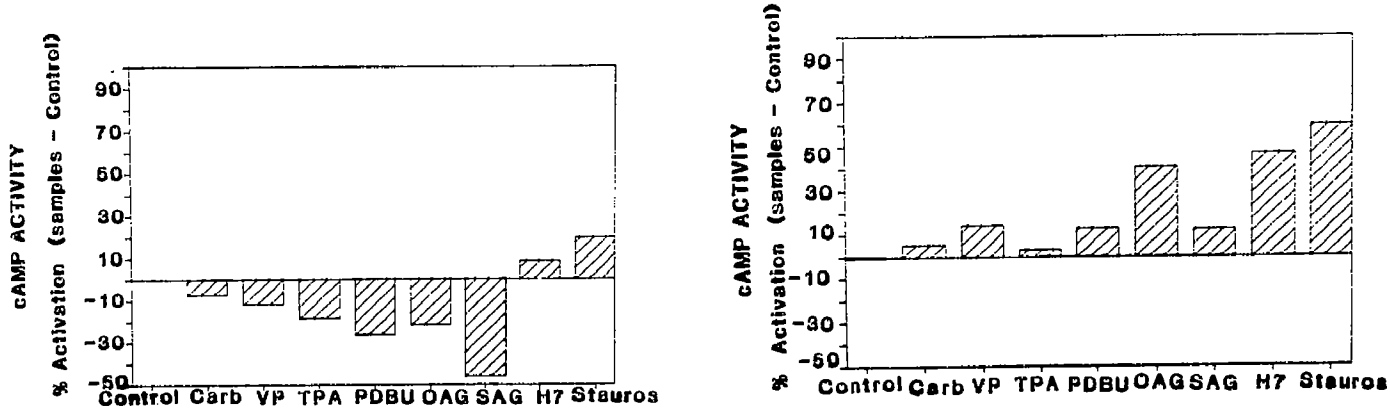

Fig. 3. Intracellular level of cAMP in hepatocytes pre-incubated for 2 h in the absence (A) or in presence (B) of phorbol ester, and stimulated by different agents. Isolated hepatocytes were pre-incubated for 2 h in the absence (A) or presence (B) of 350 μM TPA. Cells were washed and incubated for 5 min in the presence of either 100 μM carbachol, 20 nM VP, 20 nM TPA, 1 μM PDBU, 30 μM H7, 50 nM staurosporine, 20 μM OAG or 20 μM SAG. cAMP content was measured by radio-immunoassay (see Materials and Methods). cAMP movements are expressed as percent increase or decrease of cellular cAMP amount with respect to the basal level. Data are means ± SEM of 4 different experiments.

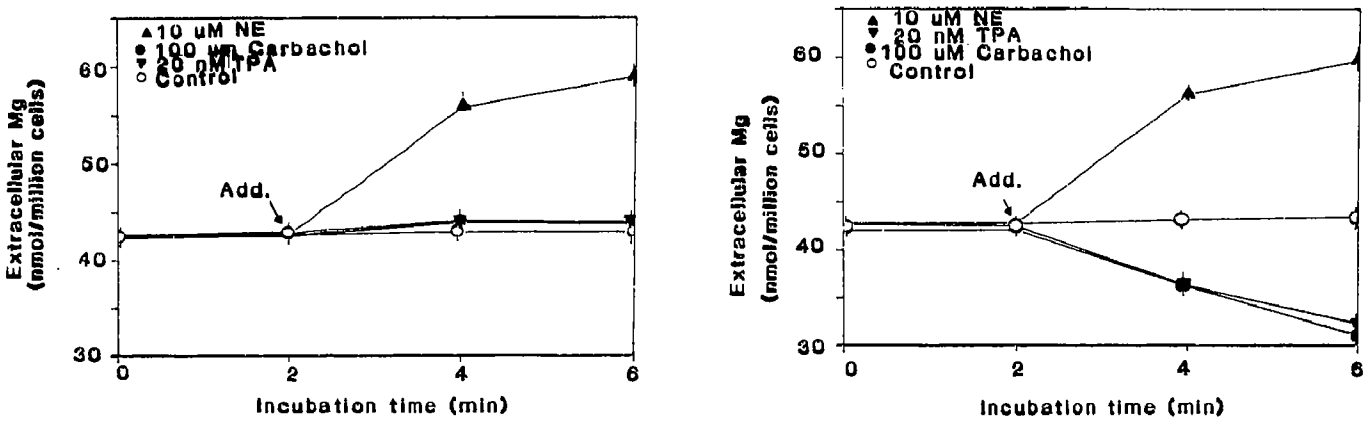

Fig. 5. Magnesium movement in isolated myocytes pre-incubated for 2 h in the presence (A) or absence (B) of 250 μM TPA. Collagenase-isolated myocytes were pre-incubated for 2 h in the presence (A) or absence (B) of 250 μM TPA as reported in Materials and Methods. The cells were then stimulated with 10 μM NE, 100 μM carbachol and 20 nM TPA and the magnesium amount in the supernatant was measured by AA. Data are means ± SEM of 5 different experiments.

lated hepatocytes following the addition of agonists. The activity is measured in vitro as the amount of [^{32}P]ATP transferred from TPA-activated PKC to a specific substrate [22]. Under these conditions, cells whose PKC was not activated (cells incubated in the presence of NE and forskolin) show a major increase in activatable PKC. By contrast, the previous stimulation with carbachol, vasopressin and TPA resulted in a lower activability of PKC. Hence, the activation of PKC during the experimental protocol is represented in Fig. 4 as an inhibition and increase. Fig. 4 shows that, with respect to controls, hepatocytes increase activity in PKC when stimulated by carbachol, vasopressin or TPA, whereas there is a decrease in PKC activity when stimulated by forskolin. An identical pattern was observed in cells incubated for 2 h in the absence of phorbol ester. By contrast, no detectable levels of PKC activity were observed in control cells pretreated 2 h with phorbol ester (not shown).

Table II

Magnesium uptake and release in collagenase-dispersed hepatocytes after modulation of protein kinase C activity

Agent	Incubation time (min)	
	0	5
	Extracellular Mg^{2+} content (nmol $Mg^{2+}/10^6$ cells)	
after 120 min without TPA		
Control	26.90 ± 0.08	26.87 ± 0.24
Norepinephrine (10 μM)	26.43 ± 0.12	34.81 ± 0.15
Forskolin (50 μM)	26.64 ± 0.10	34.55 ± 0.10
Carbachol (100 μM)	26.51 ± 0.19	21.31 ± 0.58
Vasopressin (10 nM)	26.81 ± 0.23	20.61 ± 0.29
TPA (20 nM)	26.41 ± 0.35	19.54 ± 0.49
after 120 min with TPA		
Control	26.74 ± 0.30	27.40 ± 0.38
Norepinephrine (10 μM)	26.44 ± 0.23	36.65 ± 0.21
Forskolin (50 μM)	26.81 ± 0.23	37.23 ± 0.05
Carbachol (100 μM)	27.55 ± 0.23	27.67 ± 0.27
Vasopressin (10 nM)	27.16 ± 0.20	27.44 ± 0.26
TPA (20 nM)	27.60 ± 0.18	28.06 ± 0.13

The data are means ± SEM of 4 different experiments.

The involvement of PKC in Mg^{2+} cell uptake is confirmed by the experiments performed on myocytes pre-incubated for 90 min in the presence of 350 μM TPA in order to down-regulate the PKC [35]. During this treatment no significant changes in cell viability were observed. After 90 min the cells were washed to remove the phorbol ester, transferred to the reaction mixture and stimulated with NE (10 μM), carbachol (100 μM) and TPA (20 μM). The addition of NE (Fig. 5A) induces a Mg^{2+} efflux superimposable on that observed in control myocytes pre-incubated for 90 min in the absence of TPA (Fig. 5B) or in myocytes stimulated a few minutes after isolation (Fig. 1). By contrast, the Mg^{2+} uptake stimulated by carbachol or TPA disappears (Fig. 5A). Larger concentrations of TPA and carbachol (up to 100 nM and up to 500 μM, respectively) were also ineffective (not shown). Fig. 5B shows that control cells, incubated for 90 min in the absence of TPA, respond with Mg^{2+} release upon stimulation with NE and Mg^{2+} uptake upon addition of carbachol and TPA.

A similar behavior is reported in Table II for isolated hepatocytes. Cells pre-incubated for 120 min in the presence of 350 μM TPA extrude Mg^{2+} after addition of NE or forskolin, but they do not respond with Mg^{2+} uptake to the addition of carbachol, vasopressin and TPA. The level of cAMP in hepatocytes pre-incubated for 120 min in the absence or presence of phorbol ester did not significantly differ from the data reported in Fig. 3 (not shown).

The lack of Mg^{2+} uptake following stimulation in hepatocytes treated for 2 h with phorbol ester was not due to changes in cell Mg^{2+} content occurring during incubation. Total Mg^{2+} content of the hepatocytes was 98 ± 5 and 97 ± 3 nmol Mg^{2+}/mg protein (n=4) in

hepatocytes incubated for 2 h in the presence and absence of phorbol ester, respectively.

4. DISCUSSION

The mechanism of Mg^{2+} transport across plasma membranes is best understood in bacteria where at least 3 different mechanisms have been described [16,17]. In mammalian systems, plasma membrane Mg^{2+} transport appears to involve Na^+ and/or other ion co-transport [14,15,36,37].

We have recently shown that a large Mg^{2+} uptake is induced by carbachol in myocytes and by vasopressin on hepatocytes [4,6]. These data are consistent with the observations of Bond et al. [2] showing, by electron probe microanalysis, an increase in cell Mg^{2+} content in livers in situ after addition of vasopressin. In S49 lymphoma cell, a stimulation of Mg^{2+} uptake has been described and found to not be regulated by cAMP but possibly by activation of PKC [38].

The data shown in this communication confirm that carbachol in myocytes, and carbachol and vasopressin in hepatocytes, stimulate cell Mg^{2+} uptake from the surrounding medium. The extent of Mg^{2+} uptake is significant and results in an increase in total cell Mg^{2+} of approximately 10% within 5 min. To acquire sensitivity in the measurements of Mg^{2+} with AA, the Mg^{2+} concentration in the reaction mixture surrounding the cell was decreased to 15–20 μM. Since the cytosolic Mg^{2+} concentrations are presumably much greater [39,40] this Mg^{2+} uptake appears to occur against large Mg^{2+} concentration gradients. However, a similar extent of Mg^{2+} uptake was measured in both cell types when carbachol and vasopressin were added to cells suspended in 1.2 mM Mg^{2+} (not shown).

Vasopressin, and to a lesser degree carbachol, result in enhanced activity of phospholipase C and D [41,42], leading to an accumulation of diacylglycerol and stimulation of PKC activity. They also activate Ca^{2+} mobilization through IP_3 formation, and to a lesser extent, inhibit cAMP formation by activating G_i proteins.

Using various PKC activators and inhibitors and designing experimental conditions where activation or inactivation of PKC can occur without concomitant changes in cAMP, this study shows a relationship between PKC activation and Mg^{2+} uptake. In both cardiac and liver cells the direct stimulation of PKC by diacylglycerol analogs reproduces the same pattern of Mg^{2+} uptake as that induced by carbachol or by vasopressin stimulation. By contrast, Mg^{2+} release occurs when PKC was inhibited by H7 or staurosporine. In all these conditions radioimmunoassay measurements of intracellular cAMP level show a 15–20% decrease of basal cAMP level. This decrease was not significantly modified by a prior incubation of the hepatocytes with 350 μM phorbol ester for 2 h (Fig. 3). Despite this decrease in cAMP levels, Mg^{2+} uptake (but not release) by hepatocytes or myocytes was fully prevented, indicating that the involvement of PKC rather than cAMP is mediating the uptake. In fact, the decrease in PKC activity observed in cells previously stimulated with carbachol, VP and TPA (Fig. 4) indicates that this pathway had been already activated by these agents and could not be further stimulated. Additionally, the 2 h pretreatment with phorbol ester completely abolished the stimulation of Mg^{2+} uptake by PKC both in control cells and NE-stimulated cells (data not shown). This abolition in PKC activity strongly correlates with the absence of Mg^{2+} uptake both in myocytes (Fig. 5A,B) and in hepatocytes (Table II) pre-incubated with phorbol ester.

In conclusion the data reported here indicate that both myocytes and hepatocytes are able to accumulate Mg^{2+}, even against large concentration gradients, using a mechanism whose regulation can be ascribed to changes in PKC activity rather than to fluctuations in cellular cAMP level. However the possibility that cAMP or other pathways, such as an increase in intracellular Ca^{2+} or the stimulation of G_i proteins, may cooperate and reverberate on the PKC levels cannot be ruled out.

Additionally, these experiments are not conclusive to indicate whether the action of PKC on cell Mg^{2+} uptake is through a transporter within the plasma membrane or subcellular organelle or both.

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