Mechanisms of cGMP-dependent mesangial-cell relaxation: a role for myosin light-chain phosphatase activation

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Although the cGMP-dependent relaxation of contractile cells seems to depend on the ability of the cyclic nucleotide to interfere with intracellular calcium, this does not appear to be the only mechanism involved. The present experiments were designed to analyse alternative mechanisms, trying to test the hypothesis that cGMP could relax rat mesangial cells by activating myosin light-chain phosphatase (MLC-PP), with the subsequent dephosphorylation of myosin light chain (MLC). The effect of a cGMP analogue, dibutyl cGMP (dbcGMP), on angiotensin II (AII) and PMA-induced MLC phosphorylation (MLCP) was tested, in the presence of calyculin A (CA), an inhibitor of MLC-PP. MLCP was measured, after cell labelling with ³²P, by

immunoprecipitation. dbcGMP prevented the increased MLCP induced by AII or PMA, and this inhibition was blocked by CA. dbcGMP also increased the MLC dephosphorylation observed in cells incubated with AII and in which MLC kinase and protein kinase C activities were blocked. The AII-elicited increased intracellular calcium concentration was only partially inhibited by dbcGMP. These results suggest that the cGMP-induced mesangial-cell relaxation could be due, at least partially, to the stimulation of MLC-PP.

Key words: calyculin A, cyclic GMP, dibutyl cGMP, myosin light-chain kinase, myosin light-chain phosphorylation.

INTRODUCTION

The mechanisms involved in the regulation of smooth-muscle-cell contraction have been studied extensively [1,2]. The importance of calcium fluxes, inositol 1,4,5-triphosphate, the calcium-calmodulin complex and protein kinase C (PKC) as mediators of cell contraction [2–4], and the role of cyclic nucleotides as relevant second messengers of cell relaxation [5,6] have been demonstrated clearly. However, the molecular events that mediate cGMP-dependent cell relaxation have not been defined completely.

Myosin light chain (MLC) phosphorylation (MLCP) is the biochemical reaction that precedes cell contraction [2]. This phosphorylation seems to depend mainly on the activation of MLC kinase (MLCK), which phosphorylates specific amino acids in the MLC protein, but a role for PKC has also been proposed [7]. The dephosphorylation of MLC by a MLC phosphatase (MLC-PP) starts cell relaxation [8]. It has been proposed that cGMP may relax smooth-muscle or mesangial cells by interfering with the mechanisms that activate MLCK, particularly calcium, and it has been demonstrated that the increased intracellular calcium concentration induced by some contractile mediators may be blocked by cGMP [9,10]. However, this mechanism cannot completely explain the cell relaxation induced by this cyclic nucleotide, as the inhibition of the contraction induced by cGMP seems to be more relevant than its effects on calcium [11,12]. In consequence, alternative explanations must be sought in order to understand adequately

the intracellular events involved in cGMP-induced cell relaxation. In this respect, different possible mechanisms may be proposed, such as direct interference with MLCK or PKC or even activation of MLC-PP.

The present experiments were designed to test this last hypothesis in cultured mesangial cells. These cells share most of the contractile properties of vascular smooth-muscle cells, and the intracellular mechanisms responsible for cell contraction appear to be similar. However, the relaxation of mesangial cells observed in the presence of cGMP [13,14] has been less well studied, and it seems that most of the relaxing effects of this cyclic nucleotide depend on its ability to interfere with the calcium system [15,16]. We propose that the cGMP-induced relaxation of mesangial cells could be dependent, at least partially, on MLC-PP activation. Following a combined approach using the MLC-PP inhibitor, calyculin A (CA) [17], the study of cell contraction after MLCK and PKC blockade, and the direct measurement of intracellular calcium, the present results point to MLC-PP activation as one of the mechanisms involved in the development of cGMP-dependent mesangial-cell relaxation.

EXPERIMENTAL

Materials

The anti-platelet myosin antibody was provided kindly by Dr. Christine A. Kelley of the National Institutes of Health (Bethesda, MD, U.S.A.). Collagenase, PMSF, leupeptin, stauro-

Abbreviations used: MLC, myosin light chain; MLCP, MLC phosphorylation; MLCK, MLC kinase; PKC, protein kinase C; MLC-PP, MLC phosphatase; dbcGMP, dibutyl cGMP; AII, angiotensin II; CA, calyculin A; PCSA, planar cell-surface area; KRH, Krebs/Ringer/Hepes; ML-9, 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine.

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sporine, PMA, CA, dibutyl cGMP (dbcGMP), angiotensin II (AII), dithiothreitol, ML-9 [1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine] and MLC standard were purchased from Sigma (St. Louis, MO, U.S.A.). Penicillin was obtained from Laboratories Level SA (Barcelona, Spain). Streptomycin sulphate was obtained from Antibioticos SA (Madrid, Spain). RPMI 1640, Hanks balanced salt solution and fetal calf serum were obtained from Flow Laboratories (Basingstoke, Hants, U.K.). [32 P]Orthophosphate (200 mCi/mmol) was purchased from Amersham International (Little Chalfont, Bucks, U.K.), Pansorbin was from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). Low-molecular-mass standard was from Pharmacia Biotech (Uppsala, Sweden). All the other reagents were of the highest commercially available grade.

Mesangial-cell culture

Kidneys were removed under ether anaesthesia from Wistar rats weighing 100–150 g. The glomeruli were isolated by successive mechanical sieving (150 and 50 μ m) and were treated with collagenase type IA, plated in plastic cultured flasks and incubated as described previously [13]. The culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (1 mM), penicillin (0.66 μ g/ml) and streptomycin sulphate (60 μ g/ml) and was buffered with Hepes, pH 7.2. Culture media were changed every 2 days. Studies were performed in primary cultures on days 20–22, at which time epithelial cells were no longer detected in the culture flasks. The identity of the cells was confirmed by morphological and functional criteria [13]. Cell toxicity was assessed by means of the Trypan Blue dye-exclusion test (in every case, exclusion was over 97%).

Experimental design for the analysis of mesangial-cell contraction

In the first group of experiments, cells were incubated at room temperature with dbcGMP at different concentrations for 10 min, and 10 nM AII was added to the cells for 30 min without removing dbcGMP from the incubation media. Cell contraction was measured by analysing the MLCP or by measuring the changes in planar cell-surface area (PCSA) observed in the cells. Similar experiments were performed with dbcGMP (1 μ M) plus AII (10 nM), but in cells preincubated for 10 min with 2 nM CA. Adequate controls, with buffer, AII, dbcGMP and CA were also performed. The second group of experiments had the same experimental design, but PMA (0.3 μ M), a direct activator of PKC [18], was used instead of AII. In the third group of experiments, cells were incubated for 30 min with 10 nM AII. Then 50 μ M ML-9, a MLCK inhibitor [19], and 100 nM staurosporine [20] were added for 15 min, and finally incubated for 15 min with 1 μ M dbcGMP. After the complete incubation period of 60 min, MLCP was measured.

Measurement of cell contraction

As mentioned previously, cell contraction was evaluated by analysing MLCP and, in some cases, by measuring the changes in PCSA. Phosphorylation of MLC was determined after immunoprecipitation and SDS/PAGE protein separation, as reported previously [21,22]. Mesangial cells were labelled with 50 μ Ci/ml [32 P]orthophosphate for 3 h before being incubated under the conditions detailed above. After finishing the different incubations, the reactions were stopped by freezing the cells in ethanol at -70°C . After protein solubilization, the supernatants were saved, incubated with human anti-platelet myosin antibody at 4°C for 90 min, and Pansorbin was used to precipitate

the immuno-linked MLC; the inter-assay variability of the immunoprecipitation procedure was under 8%. This fraction was separated by SDS/PAGE, the gel was frozen and exposed for 24 h to Kodak XR-5 X-ray film, the phosphorylated MLC was identified on the autoradiographs, and its absorbance was measured by videodensitometry (Bio-Rad, Richmond, CA, U.S.A.). Densitometric signals were in the range where the relationship between signal intensity and the amount of phosphorylated protein was linear. Samples for the measurement of MLCP were processed in duplicate, and a mean value for both densitometric signals was calculated and expressed in arbitrary density units. These data were corrected for the [32 P]orthophosphate radioactivity on the day of the study (taking day 1 as a reference), as well as for the protein content from each sample (by the Bradford method). Under these conditions, the maximal interassay variability between control samples was 15%.

For measurement of the changes in PCSA, mesangial cells grown in conventional plastic culture flasks, maintained at room temperature, were observed under phase contrast with a photomicroscope. Serial photographs were taken under the experimental conditions cited above and 10–18 cells were analysed per photograph. PCSA was determined by computer-aided planimetric techniques. Measurements were performed by two different investigators in a blind fashion [21]. The maximal intra-observer and inter-observer variations were 3 and 5%, respectively.

Measurement of cytosolic free calcium concentration

Cytosolic free calcium concentration was measured by the fura-2 method, as described [23]. For that purpose, cells were plated on to 12-mm glass coverslips in 24-well culture dishes. After removing the culture medium, cells were washed with Krebs/Ringer/Hepes (KRH) solution (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2 mM CaCl_2 , 6 mM glucose and 25 mM Hepes, pH 7.4) and then incubated with 5 mM fura-2 acetoxymethyl ester in KRH with 2% BSA (40 min, 37°C). Thereafter, coverslips were removed from the plate and placed in fresh KRH at room temperature. Cells were incubated under the different conditions selected (see Figure legends). Fluorescence measurements were performed by placing the glass coverslips in a diagonal position inside a standard 1-cm² cuvette containing 1.5 ml of KRH. Rapid mixing of the agents added was achieved by continuous stirring with a magnetic stirring bar placed at the bottom of the cuvette inside a 5-mm-long piece of plastic tubing, which prevented the movement of the coverslip during stirring. The cuvette was placed in a fluorimeter (Perkin Elmer LS50B) for continuous recording at room temperature of the fluorescence signals at excitation and emission wavelengths of 340 and 500 nm, respectively. At the end of each measurement, 0.1% Triton X-100 and 10 mM EGTA were added sequentially to obtain F_{max} and F_{min} respectively. Cytosolic free calcium concentration was calculated according to the method of Grynkiewicz et al. [24], assuming a K_d for fura-2–calcium interaction at room temperature of 225 nM.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. and the number of experiments is shown in every case (see Figure legends). As the number of experiments was under 10 and they were performed with a paired design, Friedman's ($n > 2$) or Wilcoxon's ($n = 2$) tests were used to compare the value distributions. $P < 0.05$ was considered statistically significant.

RESULTS

As shown in Figure 1, dbcGMP prevented the increased MLCP induced by AII, with a dose-dependent pattern. This prevention was maximal from 1 μ M dbcGMP (Figure 1). The cGMP analogue also prevented the changes in PCSA induced by AII, with a similar pattern to that observed for MLCP (Figure 2). dbcGMP alone (1 μ M) only slightly decreased the phosphorylation of MLC ($92 \pm 5\%$ versus control values, $n = 3$) in mesangial cells.

In order to gain insights into the mechanisms responsible for the dbcGMP blockade of AII effects, different experiments were performed. First, the ability of dbcGMP to modulate the AII-induced changes in intracellular calcium was tested. The main results of these experiments are included in Figure 3. The rapid and significantly increased cytosolic free calcium concentration elicited by AII was partially prevented by the cGMP analogue. Moreover, the sustained calcium increase observed in the presence of AII disappeared after dbcGMP incubation. Secondly, the possible relaxing effect of dbcGMP was studied in cells incubated with PMA, a direct activator of PKC. As in the case of AII, dbcGMP also blocked the increased phosphorylation of the regulatory unit of myosin induced by PMA [cells incubated with 0.3 μ M PMA, $198 \pm 6\%$ ($P < 0.05$ versus control values); cells pretreated with 1 μ M dbcGMP and then incubated with PMA, $135 \pm 9\%$; values are expressed as a percentage of control MLCP, $n = 3$], and prevented the reduction of PCSA induced by this PKC agonist [cells incubated with 0.3 μ M PMA, $83 \pm 3\%$ ($P < 0.05$ versus initial values); cells pretreated with 1 μ M dbcGMP and then incubated with PMA, $95 \pm 3\%$; values expressed as a percentage of the initial PCSA, $n = 5$]. Third, the possible role of

Figure 1 Effect of different concentrations of dbcGMP on the AII-induced changes in MLCP in cultured rat mesangial cells

Cells were preincubated for 10 min at room temperature with dbcGMP, and then 10 nM AII was added for 30 min. (Upper panel) Results of an immunoprecipitation experiment. C, control cells; AII, cells incubated with 10 nM AII; 0.1, 1, 10, cells incubated with 10 nM AII and increasing concentrations (μ M) of dbcGMP. (Lower panel) Results shown are the means $\pm$ S.E.M. of the densitometric analysis of three different experiments, corrected for the protein content and the labelled orthophosphate activity, and expressed as a percentage of control values (% C). The control values, considered as 100%, corresponded to a MLCP of 1.49 ± 0.11 c.p.m./ μ g of total protein. Only data of cells incubated with 10 nM AII are included in this lower panel. * $P < 0.05$ versus the other groups.

Figure 2 Effect of different concentrations of dbcGMP on the AII-induced changes in PCSA of cultured rat mesangial cells

Cells were preincubated for 10 min at room temperature with dbcGMP, and then 10 nM AII was added for 30 min. Results shown are the means $\pm$ S.E.M. of six different experiments, and are expressed as a percentage of initial PCSA. In cells incubated under control conditions (buffer only), the PCSA after 40 min of incubation, expressed as a percentage of initial values, was $98 \pm 3\%$. Only data of cells incubated with 10 nM AII are included. * $P < 0.05$ versus the other groups.

MLC-PP was evaluated by using CA. This phosphatase inhibitor completely blunted the blockade of MLCP observed in cells incubated with AII (Figure 4, left-hand panel) or PMA (Figure 5) and pretreated with dbcGMP. The intrinsic effect of CA is shown in the right-hand panel of Figure 4: the phosphatase inhibitor alone minimally modified MLCP in control cultured rat mesangial cells.

The possible importance of MLC-PP was analysed further. Figure 6 shows the effect of dbcGMP on the AII-induced MLCP of cultured rat mesangial cells, but after blocking PKC and MLCK with ML-9 and staurosporine. In these experiments, MLCP was first induced by AII, then (30 min later) kinases were blocked to be certain that the effects of dbcGMP were not dependent on an inhibition of these systems, and finally (15 min later) dbcGMP or buffer was added. As expected, AII induced a significantly increased phosphorylation of MLC after 60 min of incubation, and the presence of both kinase inhibitors significantly inhibited the AII-dependent increased phosphorylation. Even under these conditions, dbcGMP induced an additional reduction of MLCP.

DISCUSSION

The relaxation of mesangial and smooth-muscle cells induced by cGMP has been attributed to an effect of this nucleotide on intracellular calcium levels. Thus by interfering with calcium influx or by increasing calcium efflux, cGMP may block the calcium-coupled contractile responses [9,10]. However, controversies exist about the importance of calcium inhibition in the cGMP-dependent cell relaxation [11,12], and the present results point to alternative mechanisms of mesangial-cell relaxation, as dbcGMP prevented AII-elicited cell contraction while only partially inhibiting the changes in intracellular calcium. Of the mechanisms proposed to explain the relaxation observed in the presence of cGMP, the stimulation of Ca^{2+} -activated K^{+} channels, with the subsequent membrane hyperpolarization [16,25], has been one of the most widely studied. However, alternative mechanisms need to be proposed to explain completely the cGMP-induced mesangial-cell relaxation, and the

Figure 3 Effect of dbcGMP on the AII-induced changes in intracellular calcium in cultured rat mesangial cells (Upper panel) Two characteristic calcium experiments, with AII (left) or with dbcGMP + AII (right). The arrows shows the addition of AII. (Lower panel) The mean values of basal intracellular and maximal intracellular calcium concentrations (expressed as percentages of basal values) under the different experimental conditions. Cells were incubated for 10 min with 1 µM dbcGMP, and then 10 nM AII was added. Results shown are the means ± S.E.M. of five different experiments. **P* < 0.05 versus basal AII; ***P* < 0.05 versus basal dbcGMP and peak AII.

Figure 4 Effect of CA on the interaction of dbcGMP with AII on MLCP in cultured rat mesangial cells (Left-hand panels) The interactions of CA with AII and dbcGMP are shown. (Right-hand panels) The intrinsic effect of CA is included. Cells were preincubated for 10 min at room temperature with 2 nM CA, then 1 µM dbcGMP was added and finally a 30-min incubation with 10 nM AII was performed. (Upper-left panel) Results of an immunoprecipitation experiment. C, control cells; AII, AII-incubated cells; dbcGMP-AII, dbcGMP + AII-incubated cells; CA-dbcGMP-AII, CA + dbcGMP + AII-incubated cells. (Lower-left panel) Results shown are the means ± S.E.M. of the densitometric analysis of three different experiments, corrected for the protein content, and expressed as a percentage of control values (% C). The abbreviations are the same as in the upper-left panel. **P* < 0.05 versus the other groups. (Upper-right panel) Results of an immunoprecipitation experiment. CA, CA-incubated cells. (Lower right panel) Details are as for the lower-left panel. The abbreviations are the same as in the upper-right panel. **P* < 0.05 versus C; ***P* < 0.05 versus C and AII.

present experiments were designed to analyse the possible involvement of MLC-PP.

The study of cell contraction was performed by analysing MLCP, as this phenomenon is considered to be the biochemical event that triggers the contractile process [2]. In the first experimental approach, two different contractile agonists, AII and PMA, were used in order to localize the place of action of cGMP as a cell-relaxing intracellular mediator. It is a well-recognized fact that phorbol esters, such as PMA, are able to induce cell contraction by a mechanism dependent on PKC

Figure 5 Effect of CA on the interaction of dbcGMP with PMA on MLCP in cultured rat mesangial cells

Cells were preincubated for 10 min at room temperature with 2 nM CA, then 1 μ M dbcGMP was added and finally a 30-min incubation with 0.3 μ M PMA was performed. (Upper panel) Results of an immunoprecipitation experiment. C, control cells; PMA, PMA-incubated cells; dbcGMP-PMA, dbcGMP + PMA-incubated cells; CA-dbcGMP-PMA, CA + dbcGMP + PMA-incubated cells. (Lower panel) Results shown are the means $\pm$ S.E.M. of the densitometric analysis of three different experiments, corrected for protein content, and expressed as a percentage of control values (% C). * P < 0.05 versus the other groups.

activation [18]. The fact that cGMP blocked the contractile effects of AII and PMA in a similar way strongly supports the contention that this cyclic nucleotide relaxes cells by acting after PKC activation. However, the possibilities of interfering with PKC are multiple, and additional experiments were needed to assess the specific mechanism involved in cGMP-induced cell relaxation. For this reason, experiments with CA were performed. CA is a rather specific inhibitor of phosphatases I and IIa [17], and it was expected that, in the instance that the relaxing effect of cGMP was mediated by MLC-PP activation, this effect would disappear in the presence of CA, as was the case.

However, all these results did not confirm definitively the proposed role for cGMP as a direct activator of MLC-PP, as CA did not block this phosphatase specifically. In fact, it has been proposed previously that the above-mentioned activation of the Ca^{2+} -activated K^+ channel could be also dependent on the activation of a specific phosphatase, which would modulate the channel activity [26]. For this reason, we tested the effect of cGMP in a system in which the activities of MLCK and PKC were previously blocked (see Figure 6). The pharmacological blockade was performed with staurosporine [20] and ML-9 [19]. In this situation, if the effects of cGMP were not completely dependent on interference with MLCK or PKC, it was expected that decreased MLCP would occur when cells were incubated with cGMP analogues, as turned out to be the case.

Taken together, the present results point to MLC-PP as one of the systems that may be modulated by cGMP in order to induce mesangial-cell relaxation. Two recent reports in rabbit ileum smooth muscle [19] and in rat aortic smooth-muscle cells [27] also support this contention. In consequence, it can be proposed that cGMP-dependent relaxation of mesangial cells is a complex phenomenon, which does not only include the interference with the intracellular calcium or the blockade of MLCK or PKC.

Figure 6 Effect of dbcGMP on MLCP of cultured rat mesangial cells in the presence of staurosporine and a myosin kinase inhibitor, ML-9, in cells previously contracted with AII

Cells were preincubated for 30 min with 10 nM AII, then 100 nM staurosporine + 50 μ M ML-9 were added for 15 min, and finally a 15-min incubation with 1 μ M dbcGMP was performed. (Upper panel) Results of an immunoprecipitation experiment. C, control cells; AII, AII-incubated cells; AII-M, AII + staurosporine + ML-9-incubated cells; AII-M-dbcGMP, AII + staurosporine + ML-9 + dbcGMP-incubated cells. (Lower panel) Results shown are the means $\pm$ S.E.M. of the densitometric analysis of three different experiments, corrected for the protein content, and expressed as a percentage of control values (% C). The legends are the same as in the upper panel. * P < 0.05 versus C; ** P < 0.05 versus C and AII; *** P < 0.05 versus AII and AII-M.

Also seems to be dependent on activation of MLC-PP, with the subsequent dephosphorylation of this myosin regulatory protein and cell relaxation.

Note added in proof (received 4 January 2000)

Recently, Surks et al. [28] have described the regulation of myosin phosphatase by a specific interaction of this enzyme with cGMP-dependent protein kinase I α .

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