Cell density modulates the decrease of cytosolic free Ca^{2+} induced by atrial natriuretic hormone, *S*-nitroso-*N*-acetylpenicillamine and 8-bromo cyclic GMP in cultured rat mesangial cells

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Cyclic GMP-elevating agents, including atrial natriuretic hormone and NO-generating vasodilators, decrease cytosolic free Ca^{2+} levels in mesangial cells. We have investigated the role of cell density as a modulator of the decrease in cytosolic free Ca^{2+} induced by the cyclic GMP (cGMP)-elevating vasodilators atrial natriuretic peptide (99–126) [ANP (99–126); 'atriopeptin 28'] and the NO-generating vasodilator *S*-nitroso-*N*-acetylpenicillamine (SNAP), in cultured rat mesangial cells. Increasing cell density was significantly correlated with the decrease in cytosolic free Ca^{2+} induced by ANP (99–126) or SNAP. Moreover, this effect was independent of the cells' proliferative status. ANP (99–126) and SNAP induced greater fold stimulation of cGMP accumulation in high-density cells, but the levels of cGMP elicited by high concentrations of ANP (99–126) or SNAP were similar in high- and low-density cells. 8-Bromo cGMP was more effective in decreasing cytosolic free Ca^{2+} in high- than in low-density cells, suggesting that the greater effectiveness of ANP (99–126) and SNAP was, in part, due to greater effectiveness of endogenous cGMP in high-density cells. The results document that cell density, but not proliferative status, plays an important role in the modulation of intracellular Ca^{2+} dynamics in rat mesangial cells by atriopeptins, NO-generating vasodilators and cGMP.

INTRODUCTION

The most clearly established pharmacological effects of atrial natriuretic hormone (ANH) are, on the one hand, vasorelaxation and, on the other, natriuresis and diuresis [1]. In the physiological setting, ANH is thought to influence cardiovascular homeostasis, in part, by modulating vascular resistance and renal function [2,3].

The renal glomerulus is a prominent target for ANH, as demonstrated by the increase in the glomerular ultrafiltration coefficient induced by the peptide [4] and by the presence of specific receptors for ANH in glomerular mesangial and epithelial cells [5,6]. Mesangial cells are thought to play an important role in regulating the patency of glomerular capillaries, and, indirectly, the glomerular surface for ultrafiltration. These cells contain ultrastructural elements commonly associated with a contractile apparatus. Moreover, the cultured mesangial cells undergo a shape change in response to pressor hormones [7–9], and recent studies by two groups of workers have shown that ANH opposes this effect, consistent with the notion that the hormone may be an important modulator of mesangial cell contractility *in vivo* [10,11].

We have reported that ANH inhibits the incorporation of thymidine and the proliferation of mesangial cells and that this action is mimicked by 8-bromo cGMP [12,13]. The inhibition of thymidine incorporation induced by ANH in mesangial cells has been confirmed [14] and extended to cultured vascular smooth-muscle cells [15]. Thus inhibition of vascular-cell proliferation represents an additional, recently discovered, biological activity of ANH that appears to be mediated, at least in part, by cGMP.

The ability of ANH to decrease cytosolic free Ca^{2+} in cultured mesangial cells has been documented by direct measurement, using the fluorescent indicator fura-2 [16,17]. During the course of earlier studies, we noted that cell density influences the effect of ANH on Ca^{2+} dynamics in mesangial cells. Furthermore, the

importance of cell density in the modulation of a variety of cell functions and characteristics, including differentiation, responsiveness to hormones and extracellular-matrix synthesis, among others, is supported by several recent studies [18–20]. The present studies were therefore designed to investigate, in detail, the effects of cell density on the decrease in cytosolic free Ca^{2+} and cGMP accumulation induced by ANH and the NO-generating vasodilator *S*-nitroso-*N*-acetylpenicillamine (SNAP), and to define further the relationship between the decrease in cytosolic free Ca^{2+} and the increase in cGMP in rat mesangial cells.

MATERIALS AND METHODS

Materials used in these experiments were obtained from the following sources: rat ANH [atrial natriuretic peptide (99–126); ANP (99–126)] (Calbiochem or Peninsula), fura-2 and fura-2 acetoxymethyl ester (AM) (Molecular Probes), fetal bovine serum and RPMI 1640 (GIBCO), 2'-*O*-succinyl[^{125}I]iodotyrosine methyl ester) cGMP (NEN/Dupont), specific antibody against cGMP (Kew Scientific), fatty-acid-free BSA, Hepes and 8-bromo-cGMP (Sigma). SNAP was synthesized as described previously [12].

Rat mesangial cells were isolated and cultured in RPMI 1640 medium further supplemented with 20% (v/v) heat-inactivated fetal bovine serum plus 10 μg of insulin/ml, 10 μg of transferrin/ml and 10 ng of selenium/ml, as previously described [12,17,21]. For experimental purposes, cells were used between subcultures 7 and 13. Unless otherwise mentioned, cells were seeded at densities ranging from $\sim 1 \times 10^3$ cells/cm² to $\sim 1 \times 10^5$ cells/cm² and cultured for 3 days in 20%-serum-containing medium. On day 3, cells were treated with fresh culture medium supplemented with 5% fetal bovine serum and used the next day. Experiments using mitogenically quiescent cells were performed with cells that were cultured for 3 days in serum-containing

Abbreviations used: ANH, atrial natriuretic hormone; cGMP, cyclic GMP; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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medium, followed by culture in serum-free medium for 2 days. Thus these cells were used on day 5 of culture.

The measurement of cytosolic free Ca^{2+} , by using fura-2, was performed on a Perkin-Elmer model LS-5 or a Shimadzu model RF5000U fluorescence spectrophotometer in continuously superfused cells, as previously described in detail [21,22]. The composition of the superfusion medium was as follows (in mM): 135 NaCl, 5 KCl, 1 Na_2HPO_4 , 0.5 MgSO_4 , 1.8 CaCl_2 , 10 glucose, 10 Hepes, 0.025 % fatty-acid-free BSA, pH 7.4.

cGMP was measured by radioimmunoassay in cell extracts obtained by treating the cells for ~ 10 min with 0.1 M-HCl. Radioimmunoassay was performed by the acetylation modification of Brooker *et al.* [23]; 50 % displacement of radioiodinated cGMP ligand was obtained by ~ 5 fmol of non-radioactive cGMP.

Cell number was determined by detaching adherent cells with trypsin/EDTA and counting in a haemocytometer.

Statistical comparisons were performed by the unpaired two-tailed Student's *t* test or by analysis of variance, followed by Fisher's protected least-significant difference test.

RESULTS

Effect of mesangial-cell density and time in culture on ANP (99–126)-induced decrease in cytosolic free Ca^{2+}

The purpose of these experiments was to investigate the relationship between cell density and the decrease of cytosolic free Ca^{2+} induced by ANP (99–126). The results given in Fig. 1

Fig. 1. Time course of the decrease in cytosolic free Ca^{2+} induced by ANP (99–126) (a) or SNAP (b) in cells at low density (□) or high density (■).

Seeding densities on day 0 were $\sim 1 \times 10^3$ cells/ cm^2 (low density) or $\sim 1 \times 10^5$ cells/ cm^2 (high density). Cultures were used on day 4, and at that time cell densities for cultures defined as 'low density' were in the range 1.8×10^3 – 5.4×10^3 cells/ cm^2 , whereas for those at high density they were in the range 7.0×10^4 – 15.8×10^4 cells/ cm^2 . Results are from a representative experiment from a total of at least 5 such experiments.

depict the time course of the effect of ANP (99–126) (Fig. 1a) or SNAP (Fig. 1b) on cytosolic Ca^{2+} in low- versus high-density mesangial cells. The densities of cultures defined as having low density were in the range 1.8×10^3 – 5.4×10^3 cells/ cm^2 , whereas those defined as having high density were in the range 7.0×10^4 – 15.8×10^4 cells/ cm^2 . Cytosolic free Ca^{2+} in resting cells was not significantly different in low- as compared with high-density cultures (92 ± 10 nM versus 88 ± 6 nM). Moreover, the kinetics of the decrease in Ca^{2+} induced by ANP (99–126) or SNAP were similar in low- and high-density cells (Fig. 1). Cytosolic free Ca^{2+} decreased to its final new value in about 3–4 min and remained stable for the duration of the experiments.

The results depicted in Fig. 2(a) indicate the correlation between cell density and the decrease in cytosolic Ca^{2+} induced by ANP (99–126). Fig. 2(b) depicts similar experiments using SNAP. With either ANP (99–126) or SNAP as the stimulatory agent, an excellent correlation was found between the increase in cell density and the decrease in cytosolic free Ca^{2+} .

In the experiments described above, cells of random age in culture were used to obtain cultures of different densities. Thus it was conceivable that culture age and not cell density was the independent variable. To investigate this aspect, we evaluated the effect of ANP (99–126) on cytosolic free Ca^{2+} in cultures having cell densities that were not significantly different from each other but that had been cultured for 3, 7 or 10 days. Although there was a tendency towards a greater effect of ANP (99–126) on cytosolic free Ca^{2+} in the 10-day-old cultures as compared with that in the 3-day-old cultures, this difference did not reach statistical significance (Table 1). Nevertheless, to eliminate fully culture age as a potential variable, most subsequent experiments were performed with cultures of identical age (4 days old) but differing cell densities.

The differential decrease in cytosolic free Ca^{2+} elicited by ANP (99–126) in high- versus low-density cells could conceivably be

Fig. 2. Relationship between cell density and decrease in cytosolic free Ca^{2+} induced by ANP (99–126) (100 nM, a; correlation coefficient = 0.70, $P < 0.001$) or SNAP (100 μM , b; correlation coefficient = 0.82, $P < 0.001$).

Each point represents the result of a single experiment.

Table 1. Relationship between decrease in cytosolic Ca²⁺ induced by ANP (99–126) (100 nM) and age in culture, at similar cell densities

Results are means $\pm$ S.E.M. ($n = 5$). Final cell densities are given as no. of cells ($\times 10^{-4}$)/cm². To achieve similar cell densities at different days in culture, cells were initially seeded at different densities. Thus 10-day-old cultures were seeded at $\sim 3 \times 10^3$ cells/cm², 7-day-old cultures at $\sim 1 \times 10^4$ cells/cm² and 3-day-old cultures at $\sim 4 \times 10^4$ cells/cm². Neither final cell densities nor decrements in cytosolic Ca²⁺ in each experimental category were significantly different from each other ($P > 0.05$).

Time in culture (days)	Final cell density	Percentage decrease in Ca ²⁺
3	3.39 $\pm$ 0.06	23.3 $\pm$ 4
7	3.81 $\pm$ 0.22	27.3 $\pm$ 3
10	3.78 $\pm$ 0.13	33.2 $\pm$ 3

the expression of different amounts of fura-2 loading in the cells used at different cell densities, although all experiments were performed with cells loaded in the presence of 4 μ M-fura-2/AM. To investigate this possibility, high-density cells were loaded for 1 h in the presence of 1 μ M-, 5 μ M-, 10 μ M- or 20 μ M-fura-2/AM. The percentage decrease in cytosolic free Ca²⁺ elicited by 100 nM-ANP (99–126) under these different loading conditions was 20 $\pm$ 1.5 %, 24.6 $\pm$ 7.4 %, 24.6 $\pm$ 5.1 % and 21 $\pm$ 4.1 % (mean $\pm$ S.E.M., $n = 3$) respectively, indicating that differential loading of fura-2 could not have accounted for the differences in Ca²⁺ decrements occurring in high- compared with low-density cells.

Effect of cell density on the concentration–response relationship for the decrease in cytosolic free Ca²⁺ induced by ANP (99–126) or SNAP

The next experiments were performed to determine the effect of cell density on the ANP (99–126)-induced decrease in cytosolic Ca²⁺ as a function of ANP (99–126) concentration. In view of the hypothesis that cGMP may be an important mediator of the actions of atriopeptins, we also investigated the effect of the NO-generating vasodilator SNAP, an agent that stimulates soluble guanylate cyclase activity, on cytosolic free Ca²⁺.

The densities of cultures defined as low or high were as given above; moreover, the cultures at both cell densities were 4 days old. There were important differences in ANP (99–126)- or SNAP-induced effects at different cell densities, as shown in Fig. 3. Fig. 3(a) depicts the concentration–response relationships for the ANP (99–126)-induced decrease in cytosolic free Ca²⁺ in low- or high-density cultures. The effective threshold concentration of ANP (99–126) was ~ 0.1 nM in both low- and high-density cultures. This value is similar to that found in our previous work [17]. However, between 1 nM- and 100 nM-ANP (99–126), the peptide dose-dependently decreased cytosolic Ca²⁺ in high-density cells, whereas no dose-dependency was observed in low-density cells. Similarly, as shown in Fig. 3(b), SNAP was also more effective in high- than in low-density cultures.

The rate of proliferation of mesangial cells tends to decrease as the cell density increases [12]. Thus the possibility existed that the difference between high- and low-density cells reflected differences in their proliferative status. To explore this aspect, we compared the effect of ANP (99–126) (100 nM) in high- versus low-density mesangial cells that were made quiescent by a 48 h incubation in serum-free medium. Under these conditions, the atrial peptide induced a 62.5 $\pm$ 9.5 % ($n = 4$) decrease in cytosolic free Ca²⁺ in high-density cells and a 22.8 $\pm$ 8.3 % ($n = 4$) decrease in low-density cells ($P < 0.02$). Thus the differential effect of ANP

Fig. 3. Dose–response relationship of the decrease in cytosolic free Ca²⁺ induced by ANP (99–126) (a) or SNAP (b) in low-density cells (□) or high-density cells (■)

Results are means $\pm$ S.E.M. ($n = 5–11$). Two-factor analysis of variance indicated a highly significant difference between the data at low- versus high-density cultures [for ANP (99–126)-induced effect, $F = 18.8$, $df = 1$, $P < 0.001$; for SNAP-induced effect, $F = 21.1$, $df = 1$, $P < 0.001$]; * indicates $P < 0.05$ relative to control values lacking ANP (99–126) or SNAP.

(99–126) in low- versus high-density cells appeared to be independent of the cells' proliferative status.

Effect of cell density on cGMP accumulation elicited by ANP (99–126) or SNAP

In light of the proposed involvement of cGMP in the effects of ANP (99–126) and SNAP, we investigated possible differences in cGMP accumulation in low- versus high-density mesangial cultures. Fig. 4 depicts the time courses of ANP (99–126)- and SNAP-induced cGMP accumulation in low- or high-density mesangial cells and, as shown, these time courses were not different in cultures of differing cell densities. That is, cGMP reached a peak after 2 min of incubation in the presence of either agonist in both low- and high-density cells. Thus a 2 min incubation time was chosen for subsequent concentration–response experiments.

The results presented in Fig. 5 provide the concentration–response relationship of ANP (99–126)- and SNAP-induced cGMP accumulation in low- or high-density mesangial cultures. High-density cells appeared to be more sensitive to the stimulatory effect of ANP (99–126) or SNAP, as demonstrated by the finding that there was a clear dissociation between the lowest concentration of ANP (99–126) or SNAP which induced significant cGMP accumulation in high- as compared with low-density cells. At the highest concentration tested, ANP (99–126) increased cGMP to a slightly lower value in high-density than in

Fig. 4. Time course of increase in cGMP accumulation induced by ANP (99-126) (a) or SNAP (b) in low-density (□) or high-density (■) cultures

Also shown are control experiments for low-density (△) or high-density (▲) cultures. Results are means $\pm$ S.E.M. ($n = 4$).

low-density cells, whereas SNAP elicited similar increments in high- and low-density cultures.

The above-mentioned experiments dealing with cGMP accumulation were performed in cells not loaded with fura-2. To verify the potential confounding effect of fura-2 loading on the accumulation of cGMP, we preincubated mesangial cells for 1 h in the absence or presence of 1–20 μ M-fura-2/AM and found that the pretreatment had no significant effect on either basal cGMP or that increased by ANP (99-126) (results not shown). It should also be noted that neither ANP (99-126) nor SNAP significantly increased cyclic AMP in mesangial cells, as measured by radio-immunoassay (results not shown), and thus both vasodilators were found to increase cGMP selectively in mesangial cells.

Effect of 8-bromo cGMP on cytosolic Ca^{2+} levels in low- versus high-density cultures

A mechanism that could explain differential effectiveness of ANP (99-126) or SNAP on cytosolic free Ca^{2+} is differential sensitivity of the cells to cGMP. Thus the purpose of these experiments was to compare the effect of 8-bromo-cGMP on cytosolic free Ca^{2+} in high- versus low-density cells. The time course of the effect of 8-bromo cGMP on cytosolic Ca^{2+} in low- and high-density cells is given in Fig. 6. As indicated, there was no difference in the kinetics of the cytosolic Ca^{2+} decrease induced by the cGMP analogue in high- versus low-density cells. Fig. 7 depicts the relationship between cell density and the decrease in cytosolic free Ca^{2+} induced by 8-bromo-cGMP. Similar to the results described above, we found a significant correlation between cell density and decrements in cytosolic free

Fig. 5. Concentration-response relationship for the increase in cGMP accumulation induced by ANP (99-126) or SNAP in low-density cells (□) or high-density cells

Results are means $\pm$ S.E.M. ($n = 4$); * indicates $P < 0.05$ relative to control levels in the absence of ANP (99-126) or SNAP.

Fig. 6. Time course of the decrease in cytosolic free Ca^{2+} induced by 8-bromo cGMP (8-Br-cGMP) in low-density (□) or high-density (■) cells

Results are from a representative experiment from a total of at least five such experiments.

Ca^{2+} . To dissect the role of cell density versus culture age, we repeated these experiments in cells of identical age but at high or low density. Thus 8-bromo cGMP (100 μ M) decreased cytosolic free Ca^{2+} by $23 \pm 5\%$ and $6.4 \pm 3.3\%$ in high- and low-density 4-day-old cultures respectively, with a statistically significant difference between these values ($P < 0.05$).

Fig. 7. Relationship between cell density and decrease in cytosolic free Ca^{2+} induced by 8-bromo cGMP ($100 \mu\text{M}$)

Correlation coefficient = 0.73, $P < 0.001$. Each point represents the result of a single experiment.

DISCUSSION

The cellular mechanisms of action underlying the effects of ANH in cells are poorly understood. The hormone induces the accumulation of cGMP in mesangial cells, and this increase, in turn, appears to mediate many of the peptide's biological effects, as demonstrated by the ability of NO-generating vasodilators or cGMP analogues to mimic the actions of ANH. A second category of vasoactive agent, endothelium-derived relaxing factor (EDRF), which also appears to be linked to cGMP as the transducing signal, was recently identified as either NO or a closely related substance [24,25]. Thus NO-generating vasodilators, e.g. SNAP, can serve as EDRF-like agents. We have used ANH and SNAP to investigate the relationship between, on the one hand, cell density and, on the other, Ca^{2+} dynamics and cGMP accumulation in rat mesangial cells. Thus the principal novel finding in the present work is that responsiveness of mesangial cells to ANH, SNAP or cGMP can be modulated by the cell density.

Previous studies from our laboratory showed that atriopeptins dose-dependently decreased resting Ca^{2+} in cultured mesangial cells and that the cells were highly sensitive to ANP (99–126), with a concentration as low as 0.1 nM eliciting a significant decrease in cytosolic free Ca^{2+} [17]. The present experiments disclose a significant correlation between increasing cell density and decreasing cytosolic Ca^{2+} induced by ANP (99–126). That these results are unlikely to be the expression of differences in cGMP accumulation induced by ANP (99–126) in high- versus low-density cultures is indicated by the finding that cGMP levels were somewhat lower in high- than in low-density cells, both in the resting condition and in cells stimulated with ANP (99–126). Furthermore, these experiments are unlikely to be related to cyclic AMP metabolism, since neither ANP (99–126) nor SNAP increases cyclic AMP in mesangial cells. On the other hand, the fold stimulation of cGMP accumulation induced by ANP (99–126) in high-density cells was clearly greater than that in low-density cells. It is not known whether cGMP-mediated events are influenced by the concentration of cGMP, or whether the fold increase in cGMP may be the relevant determinant. If cells were sensing the fold stimulation of cGMP elevation rather than its absolute concentration, greater fold stimulation might be expressed as greater cGMP-dependent responses, including that

of the decrease in cytosolic free Ca^{2+} , and this finding could, in part, explain the greater decrease in cytosolic free Ca^{2+} elicited by ANP (99–126) in high-density cells.

The NO-generating vasodilator drug SNAP also decreased cytosolic free Ca^{2+} as a function of concentration. Similarly to ANP (99–126), SNAP was more effective in high- than in low-density cells. These findings are consistent with the notion that ANP (99–126) and SNAP decrease cytosolic Ca^{2+} , at least in part, by convergent mechanisms. SNAP also induced similar accumulation of cGMP in high-density as compared with low-density cells. However, similar to that elicited by ANP (99–126), the fold stimulation of cGMP accumulation induced by SNAP was also considerably greater in high-density than in low-density cells.

The greater effectiveness of 8-bromo cGMP in decreasing cytosolic free Ca^{2+} in high-density cells suggests that the enhanced effectiveness of ANP (99–126) or SNAP may be the expression of increased sensitivity of the cells to cGMP. Recent studies demonstrating differences in cGMP-dependent protein kinase activity with increasing subculture in aortic smooth-muscle cells [26] lend support to the notion that cGMP-dependent protein kinase activity may be modulated by culture conditions and could therefore contribute to the expression of differential sensitivity to cGMP. Another potential mechanism underlying the current observations is cell-density-dependent alteration of processes that occur after activation of cGMP-dependent protein kinase activity. Further work will be necessary to distinguish between mechanisms involving cGMP-dependent protein kinase activity and post-kinase events.

Comparison of the concentration–response relationships for the decrease in cytosolic Ca^{2+} and the increase in cGMP induced by ANP (99–126) or SNAP indicates a discrepancy between these effects. For instance, whereas 0.1 nM ANP (99–126) significantly decreased cytosolic Ca^{2+} in both high- and low-density cells, the same concentration of ANP (99–126) did not elicit a significant increase of cGMP in either low- or high-density cells. Furthermore, the failure of concentrations of ANP (99–126) greater than 0.1 nM to decrease Ca^{2+} in low-density cells, in the face of increasing cGMP, indicates that the decrease in Ca^{2+} induced by ANP (99–126) in low-density cells is uncoupled from the increase in cGMP. Similarly, we have also observed a dissociation between SNAP-induced cGMP accumulation and decreased cytosolic Ca^{2+} in low-density cells. For instance, whereas concentrations of SNAP of $1 \mu\text{M}$ or greater significantly decreased cytosolic Ca^{2+} , the lowest concentration of SNAP eliciting significant cGMP accumulation was $100 \mu\text{M}$. Although these findings are consistent with the notion that both ANP (99–126) and SNAP can decrease cytosolic free Ca^{2+} by a cGMP-independent mechanism, we cannot rule out the possibility that these agents may have increased cGMP in a segregated cellular compartment, and that we were unable to measure a small increase in the face of high basal cGMP levels. Relevant to this issue are the findings that, in hepatic cells, guanylate cyclases appear to be associated with several distinct morphological structures [27]; therefore, a similar compartmentation of guanylate cyclases in mesangial cells could be a potential explanation for the current observations.

The increased effectiveness of cGMP-elevating agents in high-density cells may also be relevant to the regulation of cell proliferation, in view of the finding that cGMP and cGMP-elevating agents inhibit mesangial-cell proliferation and that the rate of cell proliferation is decreased at higher cell densities [12]. Thus it is possible that enhanced sensitivity to cGMP may play a role in the decrease in cell proliferation in high-density cultures. However, the failure of growth arrest to abrogate the effect of cell density on the decrease in cytosolic free Ca^{2+} indicates that the cells' proliferative status is unlikely to play a major role in the

cell-density-dependent differential responsiveness of the cells to cGMP-elevating agents or 8-bromo cGMP.

In summary, our results indicate that cell density is an important determinant of the responsiveness of rat mesangial cells to ANP (99–126) and to a NO-generating vasodilator, SNAP, in relation to the vasodilators' ability to decrease cytosolic free Ca^{2+} and induce the accumulation of cGMP. The greater responsiveness of high-density mesangial cells to the aforementioned agents appears to be mediated, at least in part, by enhanced effectiveness of cGMP in eliciting decreases in cytosolic free Ca^{2+} . However, differential fold stimulation of cGMP accumulation elicited by ANP (99–126) or SNAP may also contribute to the observed differences. The ability to modulate, at will, the response of mesangial cells to cGMP may be of use in future studies dealing with the mechanisms of action of cGMP-mediated events.

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