Inotropic Effect, Binding Properties, and Calcium Flux Effects of the Calcium Channel Agonist CGP 28392 in Intact Cultured Embryonic Chick Ventricular Cells

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SUMMARY. CGP 28392 is a recently described dihydropyridine derivative with positive inotropic properties. To study the mechanism of action of this putative calcium channel agonist, we have related the effects of CGP 28392 on contraction (measured with an optical video system) and radioactive calcium uptake to ligand-binding studies in cultured, spontaneously beating chick embryo ventricular cells. CGP 28392 produced a concentration-dependent increase in amplitude and velocity of contraction \( \text{EC}_{50} = 2 \times 10^{-6} \) m; maximum contractile effect = 85% of the calcium 3.6 mm response). Nifedipine produced a shift to the right of the concentration-effect curve for CGP 28392 without decreasing the maximum contractile response, suggesting competitive antagonism \( \text{pA}_{2} = 8.3 \). Computer analysis of displacement of \(^{[3]H}\)nitrendipine binding to intact heart cells by unlabeled CGP 28392 indicated a \( K_D = 2.2 \pm 0.95 \times 10^{-7} \) m, in good agreement with the \( \text{EC}_{50} \) for the inotropic effect. CGP 28392 increased the rate of radioactive calcium influx (+39% at 10 seconds) without altering beating rate, while nifedipine decreased radioactive calcium influx and antagonized the CGP 28392-induced increase in calcium influx. Our results indicate that, in intact cultured myocytes, CGP 28392 acts as a calcium channel agonist and competes for the dihydropyridine-binding site of the slow calcium channel. In contrast to calcium channel blockers, CGP 28392 increases calcium influx and enhances the contractile state. (Circ Res 56: 676–682, 1985)

TRANSSARCOLEMMAL influx of calcium via the slow calcium channel appears to have an essential role in excitation-contraction coupling in cardiac and smooth muscle (Fabiato et al., 1979; Henry, 1980; Blinks et al., 1982; Reuter et al., 1982). Calcium channel-blocking agents, including verapamil, nifedipine, and diltiazem, have been developed, and possess negative inotropic and smooth muscle relaxant properties. Recently, CGP 28392 (4-[2-(difluoromethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxofuro[3,4-b]pyridine-3-carboxylic acid ethylester), a novel dihydropyridine derivative (Fig. 1) structurally similar to nifedipine, has been shown to have effects opposite to those of the calcium channel blockers (Preuss et al., 1984). Another dihydropyridine derivative, Bay k 8644, has been reported to have positive inotropic and vasoconstricting properties in the pentobarbital-anesthetized dog (Schramm et al., 1983). Schramm and colleagues have shown indirectly an increase in calcium influx in isolated rabbit aortic strips. CGP 28392 and Bay k 8644 have been referred to as calcium channel agonists.

We report here the effects of the calcium channel agonist CGP 28392 on cultured cardiac myocytes. To gain insights into the mechanism of the effects of CGP 28392 on the calcium channel in contracting myocardial tissue, we examined the pharmacological action of CGP 28392 in intact, spontaneously contracting cultured heart cells. This preparation obviates diffusion limitations inherent in isolated organ preparations and permits direct comparison of contractile function, binding properties, and calcium fluxes under similar conditions. Moreover, as reviewed by Reuter (1983), calcium channels, unlike sodium channels, are not functional in isolated membrane vesicles, and binding experiments should preferably be done in intact cells.

The preparation utilizes embryonic tissue, not intact heart, but basic cellular mechanisms observed in this preparation generally have been observed in mature hearts and in intact, loaded muscle, as well (Marsh, 1983).

Previous work done in this laboratory (Marsh et al., 1983) has shown two binding sites of differing affinities in intact myocardial cells for the dihydropyridine nitrendipine. Binding to the low-affinity site correlates well with the pharmacological negative inotropic effect of this drug. The relationships among inotropic effect, binding properties, and augmented calcium influx induced by CGP 28392 were examined in this study.

Methods

Tissue Culture

Primary monolayer cultures of beating chick embryo ventricular cells were prepared as previously described.
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95% air-5% CO₂ gas mixture. A constant temperature of 37°C was maintained by enclosing the microscope in a thermostated Lucite box. After a 15-minute equilibration period, cells were superfused with solution containing glucose. Final concentrations (HIM) in the culture medium were Na⁺, 144; K⁺, 4.0; Ca ++, 0.97; HCO₃⁻, 18; Mg++, 0.8; and Cl-, 131. Cultures were incubated in a humidified 5% CO₂-95% air atmosphere at 37°C. Spontaneously and synchronously contracting monolayer at 3 days in culture were made with a phase contrast microscope-video motion detector system (Barry and Smith, 1982). The medium bathing cells during contractility measurements was culture medium containing 0.6 mM calcium. The pH of the buffer solution was main-
tained in 100-mm culture dishes and grown in culture medium consisting of a bicarbonate-buffered physiological salt solution containing 40% medium 199 (Gibco), 6% fetal calf serum (FCS), and 54% balanced salt solution containing glucose. Final concentrations (max) in the culture medium were Na⁺, 144; K⁺, 4.0; Ca ++, 0.97; HCO₃⁻, 18; Mg++, 0.8; and Cl-, 131. Cultures were incubated in a humidified 5% CO₂-95% air atmosphere at 37°C. Spontaneously and synchronously contracting confluent monolayers were present by 3 days in culture. For contractility and ion flux experiments, cells were grown on 25-mm circular glass coverslips.

**Contractility Measurements**

Measurements of the amplitude and velocity of contraction of individual cells in a spontaneously contracting monolayer at 3 days in culture were made with a phase contrast microscope-video motion detector system (Barry and Smith, 1982). The medium bathing cells during contractility measurements was culture medium containing 0.6 mM calcium. The pH of the buffer solution was maintained at 7.4 by continuously gassing the chamber with a 95% air-5% CO₂ gas mixture. A constant temperature of 37°C was maintained by enclosing the microscope in a thermostated Lucite box. After a 15-minute equilibration period, cells were superfused with solution containing either CGP 28392 (10⁻⁸-10⁻⁵ M) or 3.6 mM Ca ++, and changes in the amplitude and velocity of motion of an individual cell were monitored. The first derivative with respect to time of the amplitude signal was obtained electronically and calibrated to indicate velocity of motion in μm/sec. For assessment of relaxation, the peak velocity of cell wall motion during relaxation was measured and compared to peak velocity of contraction. Only one cell from each coverslip was used. Observations were repeated at least five different preparations. At the end of each experiment, after complete washout of CGP 28392, cells were exposed to 3.6 mM Ca ++. The change in the amplitude and velocity of contraction induced by CGP 28392 was expressed as a percent of the change produced by 3.6 mM Ca ++, a concentration that produces maximal contrac-
tile response (Miura et al., 1981). All solutions containing nifedipine were assiduously protected from light.

**Calcium Fluxes**

The procedures for estimating calcium fluxes have been described in detail by Biedert et al. (1979) and Barry and Smith (1982). For determination of calcium uptake, cells were exposed to medium containing 45Ca (5 μCi/ml; [Ca ++] = 0.6 mM) for 10, 20, 30, or 60 seconds. Barry and Smith (1982) have shown that this period of exposure to 45Ca labels the rapidly exchangeable calcium pool. After appro-
priate periods of labeling with 45Ca in the presence or absence of CGP 28392, nifedipine, or both, cells were washed twice for 8 seconds each by gently agitating the glass coverslips sequentially in two 60-ml volumes of ice cold balanced salt solution containing no radiolabeled ions. Cells then were dissolved in medium containing 1% sodium dodecyl sulfate (SDS) and 10 mM sodium borate. Aliquots of the medium containing dissolved cells were assayed for radioactivity in a liquid scintillation spectrom-
eter (Packard, model 3330).

**Binding Studies**

Marsh et al. (1983) confirmed observations from other laboratories that binding sites for [³H]nitrendipine can be identified in heart homogenates and extended binding studies to intact cultured cells. In the present experiments, we studied the binding properties of CGP 28392 by ex-
amining the displacement of 0.5 nM [³H]nitrendipine from specific binding sites by graded concentrations of unla-
beled CGP 28392. Cells were harvested by gentle scraping of the culture plates to suspend sheets of cells. Nine hundred microliters of cell suspension were added to 50 μl of [³H]nitrendipine and 50 μl of buffered medium containing the desired amount of CGP 28392.

The medium in which experiments were conducted was identical to the physiological salt solution in which con-
tractility experiments were conducted, except that medium for binding studies was buffered by 4 mM HEPES and contained 1% bovine serum albumin (BSA) rather than FCS. Incubation was for 15 minutes at 37°C in the dark. Binding was terminated by adding 10 ml of wash buffer (50 mM potassium phosphate, pH 7.5) at 37°C to the assay mixture, incubating 5 seconds, then rapidly filtering through a Whatman GF/C filter, followed by two rapid 5-ml washes with buffer at 37°C. Filters were dried and counted at 24% efficiency in a Packard liquid scintillation counter. Displacement curves were analyzed with the iterative, nonlinear least squares method of Munson and Rodbard (1980) on an IBM 370 computer to determine ligand affinity and number of binding sites.

**Cell Density Correction**

To normalize for cell density on each culture dish or coverslip, the monolayers were incubated in l-[4,5-³H(N)] leucine (0.1 μCi/ml) for 24 hours before the experiment. [³H]Leucine was incorporated into cell protein, and the ³H counts permitted estimation of cell density on each glass coverslip. The relationship between radioactive counts and protein concentration (Lowry et al., 1951) allowed esti-
mation of protein content of cells on each glass coverslip. Thus, simultaneous counting of $^3$H and $^{45}$Ca permitted normalization of calcium flux per milligram cell protein.

Materials

CGP 28392 was kindly provided by Dr. K. Scheibli of Ciba Geigy A.G., Basel, Switzerland, and nifedipine was obtained from Pfizer. Stock solutions of $10^{-3}$ M CGP 28392 and nifedipine were made up in 75% ethanol. For contractility and $^{45}$Ca uptake studies, control medium contained the same percentage of ethanol present in final CGP 28392 or nifedipine solutions. $[^3]$H Nitrendipine (specific activity 88 Ci/mmol) and $^{45}$Ca were obtained from New England Nuclear, and unlabeled nitrendipine was from Miles Laboratories. Other reagents were obtained from Fisher Scientific and were the highest grade commercially available. Tissue culture media were from sources previously described (Marsh et al., 1982).

Results

Effects on Contractility

Both amplitude and velocity of contraction of spontaneously beating myocytes were increased by CGP 28392, as illustrated in Figure 2. This inotropic effect was concentration dependent, and was not accompanied by a significant change in beating rate over the range from $10^{-8}$ to $10^{-5}$ M. The increase in contractile state had a rapid onset, reached plateau values within 3–5 minutes, and was fully reversible on washing CGP 28392 from the myocyte chamber with return to control levels within 5–10 minutes. The positive inotropic effect had its threshold at $10^{-8}$ M CGP 28392. The maximum effect achieved at $10^{-5}$ M represented 85 ± 4% of the contractile response to $[Ca^{++}]_o = 3.6$ mM. Log-logit transformation of the sigmoid concentration effect curve yielded an EC$_{50}$ of $2 \times 10^{-7}$ M for the increase in contractility produced by CGP 28392. The peak velocity of contraction and of relaxation were increased by CGP 28392 in a concentration-dependent fashion, but their ratio was unchanged, suggesting that relaxation was not directly altered over this range of concentrations (Miura et al., 1981).

To determine whether the calcium channel blocker nifedipine was able to antagonize the CGP 28392 response, we made additional contractility measurements. After 10 minutes of superfusion of myocytes with CGP 28392, the dihydropyridine calcium channel antagonist nifedipine was added to the superfusion medium. The decrease in contractility had a rapid onset and reached a plateau within 5–10 minutes. In Figure 3, the contractile state observed at equilibrium in the presence of nifedipine and CGP 28392 is expressed as a percentage of the $Ca^{++} = 3.6$ mM response. Under these conditions, nifedipine produced a shift to the right of the concentration-effect curve for CGP 28392. Contractile response to CGP 28392 in the presence of $3 \times 10^{-4}$ M nifedipine was indistinguishable from that to $1 \times 10^{-5}$ M CGP 28392 in the absence of nifedipine. Drug solubility limitations prohibited testing higher CGP 28392 concentrations in the presence of nifedipine to ensure a plateau in response, but we consider it unlikely that nifedipine would enhance CGP 28392 efficacy. The curve shift is roughly parallel, suggesting competitive pharmacological antagonism. The pA$_2$ was 8.31. These results indicate

![Figure 2](image-url)

**Figure 2.** Amplitude of contraction of an individual cultured myocardial cell. Tracing of a record from a typical experiment. In panel A, the amplitude of contraction is shown as a function of time during superfusion of the monolayer with medium containing 0.6 mM Ca$^{++}$.

Panel B shows the response to 0.6 mM Ca$^{++}$ + 0.3 $\mu$M CGP 28392. Control amplitude was then reestablished in 0.6 mM Ca$^{++}$ solution. Panel C shows the amplitude of contraction when the medium was changed to 3.6 $\mu$M Ca$^{++}$. In 0.6 $\mu$M Ca$^{++}$ medium, the increase in amplitude induced by CGP 28392 0.3 $\mu$M was 46% of the increase elicited by exposure to 3.6 $\mu$M Ca$^{++}$ medium.

![Figure 3](image-url)

**Figure 3.** Concentration-effect curve for increase in amplitude of contraction of cultured heart cells by CGP 28392. Percent maximal amplitude of contraction (100% response = response to 3.6 $\mu$M Ca$^{++}$) is plotted against CGP 28392 concentration. Each point is the mean ± SEM of the response of five different preparations to CGP 28392 alone (■) or CGP 28392 + nifedipine $10^{-6}$ M (●).
that the novel calcium agonist CGP 28392 is able to increase contractility in cultured heart cells, and that this effect is antagonized by nifedipine.

Radioligand-Binding Experiments

Previous work from this laboratory demonstrated that [3H]nitrendipine bound rapidly to intact cultured heart cells, reaching equilibrium at 5 minutes at 37°C; the binding was saturable and reversible with a Kd of 2.6 × 10^{-10} M for the high-affinity site and 1.9 × 10^{-8} M for the low-affinity site. Binding properties of CGP 28392 were studied by examining the displacement of 0.5 nM [3H]nitrendipine from the dihydropyridine-binding site by graded concentrations of unlabeled CGP 28392. Binding was analyzed by fixing dissociation constants for nitrendipine at values previously determined (Marsh et al., 1983) and solving for dissociation constants for CGP 28392, Bmax, and nonspecific binding. Analysis of four 16 to 18-point nitrendipine displacement curves indicated that CGP 28392 bound to a single class of sites with Kd = 2.2 ± 0.95 × 10^{-7} M (mean ± SEM) and Bmax = 145 ± 17 fmol/mg protein. In each case, a single-site model for CGP 28392 binding was preferred over a two-site model (P < 0.01) (Fig. 4).

45Ca Uptake

To determine whether the positive inotropic effect of CGP 28392 could be correlated with altered calcium influx via the slow calcium channel, we studied 45Ca uptake in cultured heart cells in the presence and absence of a calcium channel antagonist. Cells were preincubated in HEPES-buffered medium (pH 7.4) containing 0.9 mM Ca++ for 5 minutes, then were incubated further in medium containing 45Ca, with or without 10^{-6} M CGP 28392. In control cells, 45Ca content increased with time. CGP 28392 at 10^{-6} M did not produce a measurable increase in 45Ca influx under these conditions. Therefore, cells were preincubated in calcium-free medium for 5 minutes, then were exposed to medium containing 0.9 mM Ca++, 45Ca tracer, and (1) 0.05% ethanol, (2) 10^{-6} M CGP 28392, (3) 10^{-5} M nifedipine, or (4) 10^{-6} M CGP 28392 + 10^{-5} M nifedipine. As shown in Figure 5, under these conditions CGP 28392 markedly increased the rate of calcium uptake such that 45Ca influx observed at 10 seconds was 39% greater than that observed under control conditions. When 10^{-5} M nifedipine was added during the 5-minute preincubation in calcium-free medium, it lowered significantly the rate of 45Ca uptake and 45Ca content throughout the 30-second period. When 10^{-5} M nifedipine was present during the preincubation and calcium uptake period, the CGP 28392-induced augmentation of 45Ca uptake was abolished. These results support the thesis that CGP 28392 augments Ca++ entry via the slow calcium channel, and that this contributes to the positive inotropic effect observed in cultured chick heart cells.

Discussion

The monolayer preparation of spontaneously beating cultured chick embryo heart cells allows direct comparison among contractile function, bind-
ing properties, and Ca\(^{++}\) fluxes, studied under similar conditions. Moreover, the lack of major diffusion limitation inherent in isolated organ preparations and the lack of endogenous neuroeffectors simplify interpretation of the results.

CGP 28392 induced a rapid, sustained, and concentration-dependent increase in contractility. The relationship between CGP 28392 concentration and contractile response is sigmoidal, as shown in Figure 3, with a threshold at \(10^{-8}\) M. The efficacy of this new compound in our cultured cell system was high, since the maximum response represents 85% of the 3.6 mM Ca\(^{++}\) response, which, in our experience, is the maximum response obtainable in this preparation (Miura et al., 1981). The efficacy of CGP 28392 was greater than that of isoproterenol, which at a saturating concentration of \(10^{-6}\) M induced only 50 ± 5% of the 3.6 mM Ca\(^{++}\) response. However, the potency of CGP 28392 is lower than that of isoproterenol, since the EC\(_{50}\) values are respectively \(2 \times 10^{-7}\) and \(1.3 \times 10^{-8}\) M. The dihydropyridine calcium channel antagonist nifedipine antagonized the CGP 28392 contractile effect, suggesting a direct effect of CGP 28392 on the calcium channel or a closely related structure. However, the EC\(_{50}\) of CGP 28392 for the contractile effect was high, compared to the IC\(_{50}\) of 23 nM for nifedipine (Marsh et al., 1983). The lower affinity of CGP 28392 for the calcium channel site in our system suggested by this result was confirmed by binding experiments. The density and affinity of \(\beta\)-receptors and dihydropyridine-binding sites are age and species dependent (Renaud, et al., 1984), so extrapolation of relative efficacy to other systems must be considered cautiously.

The shift to the right in the concentration-contractile response curve for CGP 28392 produced by nifedipine is roughly parallel. This shift is consistent with competitive antagonism, although the present data do not exclude the possibility of spare calcium antagonist-binding sites and a noncompetitive interaction. The observation that the CGP 28392-[\(^{3}H\)nitrendipine displacement curve is steep (\(N_i = 1.26 \pm 0.09\)) is also consistent with the hypothesis that these structurally similar dihydropyridine derivatives interact with the same binding site according to the law of mass action.

There was a close relationship between the pharmacological effects and binding properties of CGP 28392, since the \(K_d\) obtained from the [\(^{3}H\)nitrendipine displacement curve (2.2 \(\times 10^{-7}\) M) and the EC\(_{50}\) from contractility experiments (2.0 \(\times 10^{-7}\) M) done under comparable conditions were quite similar. Computer analysis of the [\(^{3}H\)nitrendipine displacement curve indicated a single class of binding sites for CGP 28392 competing with nifedipine. High (\(K_d = 0.3-3\) nM) and low (\(K_d = 19-67\) nM) affinity sites have been described for the calcium channel antagonist nifedipine in intact cells (Marsh et al., 1983) and guinea pig cardiac membrane fractions (Bellemann et al., 1981) and for the calcium channel agonist BAY k 8644 in membranes from rabbit ventricles (Sarmiento et al., 1984). These high- and low-affinity sites have been suggested to mediate positive and negative inotropic effects induced by dihydropyridines (Grupp et al., 1984). Whether or not the calcium channel agonist-positive inotropic effect is mediated by a common dihydropyridine-binding site remains to be determined. This might be examined by comparing in the same preparation the binding properties and pharmacological effects of the recently described Ca\(^{++}\) channel agonists CGP 28392 and Bay k 8644.

To determine the effects of CGP 28392 on calcium influx via the slow calcium channel, we studied \(^{45}\)Ca uptake by myocytes in the presence and absence of a dihydropyridine calcium channel antagonist. No measurable change in \(^{45}\)Ca uptake was found in response to CGP 28392 (10\(^{-8}\) M) or nifedipine (10\(^{-5}\) M) in the presence of 0.9 mM Ca. Why CGP 28392 at the same concentration in 0.9 mM Ca\(^{++}\) medium was able to increase contractility, whereas no detectable change in \(^{45}\)Ca uptake was found, is not clear. It is possible that, despite an increase in \(^{45}\)Ca influx, \(^{45}\)Ca content did not increase appreciably because of a compensating increase in \(^{45}\)Ca efflux (Barry and Smith, 1982). It must also be recognized that it is difficult to measure true unidirectional transmembrane fluxes of calcium in intact cardiac tissue because of back diffusion of tracer, as discussed by Atwell et al. (1979) and Horres et al. (1979). Therefore, it appears that the CGP 28392-induced increase in \(^{45}\)Ca influx is sufficient to induce a change in contractility, but small enough to escape detection by the method used. When the sensitivity of \(^{45}\)Ca uptake was enhanced by preincubation in zero calcium medium, CGP 28392 clearly increased the measured \(^{45}\)Ca uptake rate, whereas nifedipine decreased it. Nifedipine antagonized the CGP 28392-induced increment in \(^{45}\)Ca uptake.

The interpretation of \(^{45}\)Ca uptake experiments done after acute exposure to zero calcium medium must be viewed cautiously. Observations relevant to the interpretation of this experiment include the fact that prolonged slow channel activation was shown to occur when cardiac muscle was superfused with calcium-free solutions (Garnier et al., 1969; Linden and Brooker, 1981). It is of interest that, in frog ventricular strips, verapamil produced a dramatic reduction in the duration of long action potentials induced by low [Ca\(^{++}\)], isoproterenol, or both (Linden and Brooker, 1981). Recent experiments in our own laboratory with spontaneously beating chick embryo ventricular cells confirmed these findings in that superfusion of the cells with calcium-free medium enhanced dramatically the duration of the transmembrane action potential. The rate of isoproterenol-induced \(^{45}\)Ca uptake was also enhanced after preincubation in calcium-free medium as compared to the rate of isoproterenol-induced \(^{45}\)Ca uptake after 0.9 mM Ca\(^{++}\) preincubation (Kim et al., in
preparation). Additional observations include the fact that when cells are superfused with calcium-free medium, they cease contracting; when normal calcium is restored in the superfusate, contractions return to baseline in less than 2 minutes (data not shown). Under these conditions, intracellular calcium content returns to 90% of control in 60 seconds. Thus, withdrawal of extracellular calcium for 5 minutes has no sustained effect on contractile performance. Since binding experiments are conducted over 15 minutes (equilibrium conditions), transient depression of contractility and Ca++ content would not be expected to alter equilibrium binding.

Although prolonged slow channel activation presumably ceases following the return of cells to medium containing 0.9 mm Ca++ plus 45Ca, resumption of normal slow channel kinetics may be delayed by (1) the decreased intracellular calcium content and the increased intracellular sodium content (measured at the end of the calcium-free preincubation), and (2) the need for a 16- to 20-second period to equilibrate fully the extracellular space with the new calcium concentration (Barry and Smith, 1982). Thus, when cells are exposed to calcium-free medium, one may speculate that the slow channels open more frequently and/or stay open longer, that CGP 28392 interacts preferentially with this activated state, and that the slow channels maintain that property, at least in part, for the first 20 seconds of the subsequent exposure to 0.9 mm Ca++.

In summary, our results demonstrate that CGP 28392 acts as a calcium channel agonist in that it increases nifedipine-sensitive 45Ca uptake in intact cardiac cells. There is concomitant augmentation of contractility, and the drug appears to occupy the [3H]nitrendipine-binding site at concentrations that produce contractile and ion flux effects. By enhancing calcium influx into the myocardial cell, CGP 28392 appears to augment the contractile state of the heart cell. Although another dihydropyridine derivative (Bay k 8644) has been shown in a preliminary report to enhance calcium uptake in vascular tissue (Anderson et al., 1984), we are not aware of other data directly demonstrating an increase in calcium influx by a calcium channel agonist in cardiac tissue. Our results are consistent with electrophysiological findings that the calcium channel agonist Bay k 8644 enhances calcium channel current in myocardial cells (Cohen and Chung, 1984; Hess et al., 1984). This novel class of compounds should be useful probes for the study of calcium channel properties. They may also be prototypes of clinically useful drugs that act by a mechanism different from other conventional or investigational inotropic agents.

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