Cyclosporin A increases basal intracellular calcium and calcium responses to endothelin and vasopressin in human coronary myocytes

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Abstract Cyclosporin A (CsA) is a widely used immunosuppressive agent with severe side effects including hypertension. Here, we investigated the effects of CsA on intracellular free calcium ($[Ca^{2+}]_i$) and the mechanisms involved in vasoconstriction in cultured human coronary myocytes. We used the Fura-2 technique for Ca^{2+} imaging. Acute application of CsA at therapeutic concentrations (0.1–10 μmol/l) had no effect. Chronic exposure to CsA (1 μmol/l) for 24 h induced a small (20 nmol/l) but highly significant increase of basal $[Ca^{2+}]_i$ and enhanced the occurrence of spontaneous Ca^{2+} oscillations. Endothelin- and vasopressin-induced rises of $[Ca^{2+}]_i$ were also enhanced. The demonstration that CsA increases basal $[Ca^{2+}]_i$ in addition to its impact on agonist receptor stimulation is of major importance for new therapeutic approaches. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cyclosporin; Coronary; Hypertension; Ca^{2+} imaging; Endothelin; Vasopressin

1. Introduction

Cyclosporin A (CsA) is the most commonly used immunosuppressive drug used after organ transplantation and in the treatment of a variety of autoimmune diseases [1]. Vasoconstriction induced by CsA results in major side effects including arterial hypertension [2] and renal failure [3]. Direct intracoronary injection of CsA induces a vasoconstrictive response through endothelium-dependent and myogenic mechanisms in the dog coronary artery [4,5]. Possible mechanisms include a direct increase of blood vessel tonus [6], stimulation of the renin–angiotensin system [7], increased production of vasoconstrictor agents such as thromboxane A_2 [8] and endothelin-1 (ET) [9,10], up-regulation of ET [11], vasopressin (AVP) [12] or angiotensin II receptors [13], increases in the phosphorylation of specific contractile regulatory proteins [14] or, yet, reduced production of vasodilating agents such as prostacyclins [15] and nitric oxide [16]. Thus, an alteration of both endothelium-dependent and -independent vasodilating responses may be involved [4,17]. Since contraction of vascular smooth muscle cells (VSMCs) depends on intracellular calcium ($[Ca^{2+}]_i$), the measurements of the effect of CsA on $[Ca^{2+}]_i$ have been performed in rat [12,18–21] and human [13] aortic cells but diverging results were shown. However, there is a lack of information concerning the effect of CsA on $[Ca^{2+}]_i$ in coronary myocytes. We investigated the effects of CsA on $[Ca^{2+}]_i$ in cultured human coronary myocytes.

2. Material and methods

2.1. Myocyte isolation and culture

Tissue samples were obtained from coronary arteries of six Caucasian male patients (18–60 years) undergoing heart transplantation over a period of 18 months. Patients had end-stage heart failure (New York Heart Association, classes III and IV) caused by idiopathic cardiomyopathy. Arteries were devoid of pathology (in particular atheroma). The procedures were approved by the Ethical Committee of the Hospital. Cells were isolated and grown in culture as described before [22–24]. Briefly, arteries were collected after cardiectomy. Media were incubated for 50 min at 37°C in Hanks' solution containing 1 mg/ml collagenase (Worthington, Newark, NJ, USA) and 0.6 mg/ml elastase (Boehringer, Mannheim, Germany) and then dispersed. Next, the cells were centrifuged ($100\times g$, 7 min) and resuspended in a medium for VSMCs (Clonetics Bio-Whittaker, Emerainville, France) containing: gentamicin sulfate 25 μg/ml, amphotericin 25 ng/ml, human fibroblast growth factor 2 ng/ml, bovine insulin 5 μg/ml, human epidermal growth factor 0.5 ng/ml and fetal bovine serum 20%. Cells were then plated (10 000 cells/ml) in 25 cm^3 disposable flasks (Falcon) and placed at 37°C in an air/CO_2 incubator. The medium was changed every day during the first week. Thereafter, the cells were transferred to maintenance medium (Dulbecco's minimum essential medium and Ham's F-10, 1:1 v/v; Eurobio, Les Ulis, France) containing 5% human serum and 5% Myoclone Super Plus (fetal bovine; Life Technologies, Cergy Pontoise, France) which was changed every 2–3 days. Confluence was reached 15–20 days after plating. Then, cells were trypsinized and grown in LAB-TEK dishes (Polylabo, Strasbourg, France). A total of 15 different cultures were used. We tested one set of cells originating from a healthy 24 year old Caucasian male (cells commercially available: Bio-Whittaker Europe, Verviers, Belgium).

2.2. Dye loading and measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was measured in single cells as described before [25]. Briefly, the dual excitation radiometric Ca^{2+}-sensitive dye Fura-2 was used for $[Ca^{2+}]_i$ imaging studies with an Olympus-LSR system (Merlin; Life Science Resources Ltd, Cambridge, UK). The variations in $[Ca^{2+}]_i$ were detected with the sensing fluorescent probe Fura-2 by means of a digital CCD camera. Cells grown in LAB-TEK dishes were loaded by incubation with 2.5 μM of the acetoxymethyl ester

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Abbreviations: CsA, cyclosporin A; ET, endothelin; AVP, [Arg^8]vasopressin; VSMCs, vascular smooth muscle cells; $[Ca^{2+}]_i$, intracellular free calcium concentration; $FR_{340/380}$, fluorescence ratio 340 nm/380 nm

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of Fura-2 and 0.02% Pluronic F-127 (Fura-2/AM and Pluronic, Molecular Probes Inc., Eugene, OR, USA) for 35 min at 37°C in Locke buffer containing (mmol/l): NaCl, 140; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.8; glucose, 10; HEPES, 10; pH 7.2 adjusted with NaOH. The osmolarity of all the solutions ranged between 298 and 303 mosmol/l. Cells were then rinsed thoroughly in Locke buffer and mounted on the stage of an inverted microscope (Olympus IX70; Olympus France, Rungis, France) equipped for epifluorescence. Illumination was via a SpectraMASTER monochromator coupled to the microscope fitted with a UV-transparent oil objective (Uapo/340 40×/1.35). The image was detected with an Astrocam 12/14 bit frame transfer digital camera. The MERLIN system controlled the illuminator and camera, and performed image ratioing and analysis. The image acquisition rate was 1.13 frames/s. The intensity of fluorescent light emission at $\lambda = 510$ nm, using excitation at 340 and 380 nm, was monitored for each single Fura-2-loaded cell. The ratio of fluorescence emission when excited at 340 nm (the absorbance peak of Fura bound to Ca^{2+}) to 380 nm (the absorbance peak of free Fura) was used as an index of $[Ca^{2+}]_i$, with an increase in the fluorescence ratio ($FR_{340/380}$) signifying an increase in $[Ca^{2+}]_i$. In order to estimate absolute variations, the $[Ca^{2+}]_i$ was calculated from the ratio, in accordance with the equation given by Grynkiewicz et al. [26]. Fura-2 calibration was performed in ionomycin-treated (10 μmol/l) cells incubated in the presence and absence of external Ca^{2+}. The estimated $[Ca^{2+}]_i$ was deduced from the equation: $[Ca^{2+}] = K_d B(R-R_{min})/(R_{max}-R)$, where K_d is the dissociation constant (225 nmol/l). The other variables were readily accessible from the measurements performed in control cells and in cells incubated with CsA. R_{min} and R_{max} are the 340/380 ratios of the Ca^{2+}-free (i.e. when the dye is free of Ca^{2+} with 10 mmol/l EGTA added) and Ca-bound (5 mM Ca^{2+}) forms, respectively. The variability in R_{min} (range 0.4–0.6) and R_{max} (range 1.4–1.6) ratios was independent of the presence or absence of CsA; this is common, even in supposedly homogeneous cell populations. B is the ratio of fluorescence excitation intensities of the two latter forms at 380 nm (F_{380min}/F_{380max}).

2.3. Drugs

Crystallized pure CsA (Novartis Pharma, Basel, Switzerland), was prepared daily with a first dilution of 1.5 mg in 250 μl pure sterile dimethyl sulfoxide (DMSO) leading to 5 mmol/l final concentration. Further dilutions to 10 μmol/l and 1 μmol/l were performed with the standard Locke solution. Then, the final DMSO concentration in the cell medium was less than 0.1%. ET (Sigma, Saint Quentin Fallavier, France) was aliquoted at 100 μmol/l in 10 μl of acetic acid (5%). [Arg^8]vasopressin (AVP; Calbiochem-Novabiochem, Läufelfingen, Switzerland) was aliquoted at 1 mmol/l in 10 μl deionized H_2O, and then frozen at −80°C. The high K^+ solution contained the following (in mmol/l): NaCl, 90; KCl, 50; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.8; glucose, 10; HEPES, 10; pH 7.2 adjusted with NaOH. In most experiments, sequences of image acquisition lasted for 4–5 min in order to avoid the decrease in fluorescence (fading) induced by repetitive irradiation during longer experiments. Acute (20–30 s), extracellular application of the test agonists (KCl, CsA, ET, AVP) was achieved using a multiple capillary gravity-driven perfusion system (200 μm inner diameter; 100 μl/min) placed in close proximity (< 0.5 mm) to each group of cells studied (3–10 cells in each experiment). Following each application, cells were washed with Locke buffer. For chronic exposure to CsA, cells grown in the LAB-TEKs were incubated with 1 μmol/l CsA for 24 h prior to experiments.

2.4. Analyses

Since some cells exhibited spontaneous oscillatory activity, the effects of agonists were analyzed only in cells devoid of spontaneous oscillations of $[Ca^{2+}]_i$ after a 2 min period of observation. In addition, in order to detect any spontaneous, drug-unrelated increase of $[Ca^{2+}]_i$ occurring during application of agonists, we estimated the delay observed between the time of application and the onset of the various responses (on average: 3.2 ± 0.2 s, $n = 12$, for KCl; 7.3 ± 0.2 s, $n = 19$, for ET; and 18.2 ± 2.5 s, $n = 12$, for AVP). The duration of the responses were also examined. Spontaneous oscillations were analyzed in cells recorded for at least 5 min in the absence of prior application of any drug. For each cohort of cells used, changes in $FR_{340/380}$ following exposure to the test agent(s) were compared to the matched baseline values by either paired Student's t-test or unpaired t-test (with Welch's correction when variances were not equal). All values are reported as mean ± S.E.M. Results were considered not significant (ns) with $P > 0.05$, significant with $0.01 < P < 0.05$, very significant with $0.001 < P < 0.01$ and extremely significant with $P < 0.001$. Statistical analysis was performed with SAS statistical software (SAS Institute, Inc., Cary, NC, USA).

3. Results

First, $[Ca^{2+}]_i$ was measured at rest and in response to the depolarizing cation K^+ (KCl) and the two vasoconstrictive agents AVP and ET on cells devoid of spontaneous oscillatory activity of $[Ca^{2+}]_i$. Addition of K^+ (KCl 50 mmol/l) induced a transient increase of $[Ca^{2+}]_i$ (Fig. 1A) in all cells tested ($n = 12$). On average, K^+ increased the fluorescence ratio $FR_{340/380}$ from 0.76 ± 0.01 to 1.23 ± 0.01. ET, at a concentration of 100 nmol/l, induced an all-or-none response (i.e. inducing no measurable increase or alternatively, only a large increase). There was a rise of the $FR_{340/380}$ in 76% of the cells ($n = 54$) which is illustrated in Fig. 1B. On average, the $FR_{340/380}$ was increased from 0.74 ± 0.01 to 1.03 ± 0.02. Similarly, AVP (100 nmol/l) induced a rise in $[Ca^{2+}]_i$ in 53% of the cells ($n = 17$). This effect is illustrated in Fig. 1C. On average, the $FR_{340/380}$ was increased from 0.73 ± 0.01 to 0.98 ± 0.03.

Next, we evaluated the effect of acute application of CsA on $[Ca^{2+}]_i$. CsA was applied for 20–30 s at 0.1, 1, or 10 μmol/l, and then, after wash-out, cells were exposed to ET, AVP, or K^+. The results showed that CsA had neither significant effect in 65 out of 69 cells analyzed at any of the concentrations tested nor when the drug was applied for a longer time (10 min). Four myocytes responded to CsA (6%) with only a moderate increase ($FR_{340/380}$ less than 0.88). The majority of cells that were not responsive to acutely applied CsA responded to AVP (Fig. 2A), ET (100 nmol/l) or to depolarization by 50 mM K^+ (data not shown).

The consequences of 24 h exposure to CsA (0.1 and 1 μmol/l) were also assessed in a cohort of myocytes ($n = 442$; from seven different cultures). We compared the basal $[Ca^{2+}]_i$ after 24 h incubation to the baseline value of $[Ca^{2+}]_i$ in control cells ($n = 338$). To avoid undesirable non-specific culture effects, time-matched control experiments were conducted in which test and control cells were grown in parallel from the same tissue samples. Thus test and control experiments were performed randomly. The results showed that the resting $[Ca^{2+}]_i$ was increased significantly after pre-incubation with 1 μM CsA (no effect at 0.1 μmol/l). On average, the basal $FR_{340/380}$ was increased very significantly from 0.758 ± 0.003 to 0.791 ± 0.003 ($P < 0.01$) as shown in Fig. 2B. After calibration in an attempt to estimate the variation in terms of absolute value (see Section 2), this corresponded to an extremely significant increase in basal $[Ca^{2+}]_i$ from 100 ± 2 nmol/l to 120 ± 2 nmol ($P < 0.001$). In addition, many cells chronically exposed to CsA exhibited oscillatory activity with spontaneous increases of $[Ca^{2+}]_i$ (Fig. 2C). On average, the $FR_{340/380}$ increased to 0.92 ± 0.03, corresponding to an estimated maximal increase of $[Ca^{2+}]_i$ to 210 ± 22 nmol/l, which was similar to the average maximal increase observed during oscillations in control cells ($FR_{340/380}$ 0.94 ± 0.01). There was also no significant difference in the time course of these oscillations which lasted, on average, 91 ± 7 s ($n = 6$) in control cells and 109 ± 7 s ($n = 16$) in CsA-treated cells. However, spontaneous increases of $[Ca^{2+}]_i$ were detected in more cells from the pop-

ulation of CsA-treated cells (35%, $n=119$) than in the population of control cells (7%, $n=83$; $P<0.001$ as determined with Fisher's exact test). Since there was no significant difference in oscillation frequency between the CsA-treated (0.007 ± 0.0003 Hz, $n=16$) and the control cells (0.008 ± 0.0004 Hz, $n=6$), CsA is likely to increase the number of cells with oscillatory activity rather than the frequency of oscillations of a given cell. Similar results were observed in cells originating from one normal heart. For example, the basal $FR_{340/380}$ increased from 0.793 ± 0.04 ($n=96$) to 0.821 ± 0.04 ($n=42$, $P<0.001$) in those cells after pre-incubation with 1 μM CsA.

Next, we assessed the consequences of 24 h exposure to CsA on the responses of $[Ca^{2+}]_i$ to ET and AVP (100 nmol/l). Here also, time-matched control and test experiments were conducted in parallel from the same tissue samples. The effects of ET and AVP were analyzed only in cells devoid of spontaneous oscillations (a majority of cells) to minimize interference. Furthermore, analysis of their delay, duration helped us to distinguish drug-related and spontaneous increases of $[Ca^{2+}]_i$ for the interpretation of the results. Fig. 3A,B illustrates the distinct effects of ET on the $FR_{340/380}$ in control cells and in cells pre-incubated with 1 μmol/l CsA. In control cells, the mean basal fluorescence ratio rose from 0.76 ± 0.01 to a peak of 1.06 ± 0.02 ($n=71$). In CsA-treated cells, the ratio increased from 0.79 ± 0.01 to an absolute value of 1.12 ± 0.02 ($n=35$). The amplitude of the difference (mean peak ratio minus mean basal ratio) was very significantly higher (0.35 ± 0.02) in the CsA-treated cells than in the control group (0.27 ± 0.02; $P<0.01$). We estimated that the augmentation of $[Ca^{2+}]_i$ induced by ET was increased from 393 ± 43

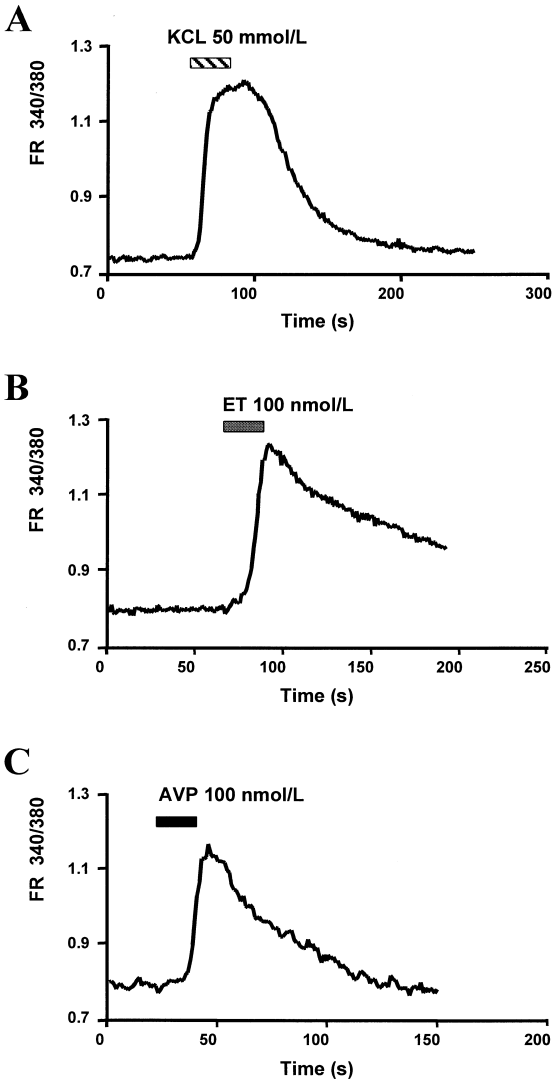

Fig. 1. Representative $FR_{340/380}$ traces of the increasing effects of (A) K^+ (50 mmol/l), (B) ET (100 nmol/l), and (C) AVP (100 nmol/l) on $[Ca^{2+}]_i$ in fura-2-loaded human coronary myocytes. Cells were loaded with the Ca^{2+} indicator and studied as described in Section 2. The time and duration of applications are represented by the horizontal bars at the top of each panel. Experiments were conducted on three different cells.

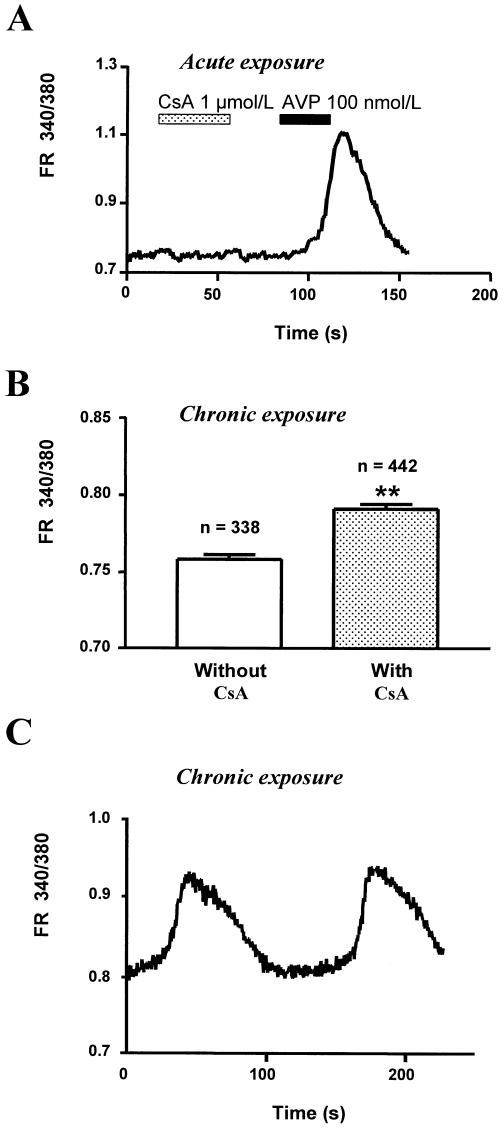

Fig. 2. Effect of CsA. A: Lack of effect following acute exposure to CsA (1 μmol/l) on $[Ca^{2+}]_i$ in a Fura-2-loaded human coronary myocyte. After wash-out of CsA, AVP (100 nmol/l) induced a rise in $[Ca^{2+}]_i$ which was similar to that shown in Fig. 1. B: Bar graphs show the mean increase in basal $[Ca^{2+}]_i$ induced by incubating human coronary myocytes with CsA (1 μmol/l) for 24 h prior to the experiments ($^{**}P<0.01$). C: Typical spontaneous increases in $[Ca^{2+}]_i$ observed in a human coronary myocyte incubated with CsA (1 μmol/l) for 24 h prior to the experiment.

nmol/l to 677 ± 161 nmol/l in CsA-treated cells ($P < 0.01$). The time course of the response was markedly different from that of spontaneous oscillations. For example, the increase in $[Ca^{2+}]_i$ induced by ET lasted 200 ± 5 s ($n = 29$). Similar effects were observed with AVP (Fig. 4A,B). AVP increased the mean basal fluorescence ratio from 0.73 ± 0.01 to a peak of 0.98 ± 0.03 in the control group of cells ($n = 12$) and from 0.81 ± 0.01 to 1.19 ± 0.02 in the CsA-incubated group ($n = 39$). Again, the amplitude of difference was extremely significantly higher (0.39 ± 0.02) in the incubated group than in the control group (0.25 ± 0.02; $P < 0.001$). These results are illustrated in Fig. 4A,B. We estimated that the augmentation of $[Ca^{2+}]_i$ induced by AVP was increased from 277 ± 36 nmol/l to 761 ± 62 nmol ($P < 0.001$) in CsA-treated cells. In contrast with ET, the duration of the AVP response was not significantly different from that of spontaneous oscillations, due to some variability in the duration among cells. However, the amplitude of the rise in $[Ca^{2+}]_i$ was significantly higher than the amplitude of spontaneous oscillations which excluded a misinterpretation of the results. We found no difference in the number of cells that responded to ET and AVP after incubation with CsA. Similar results were found for the cells originating from the single donor devoid of pathology (data not shown).

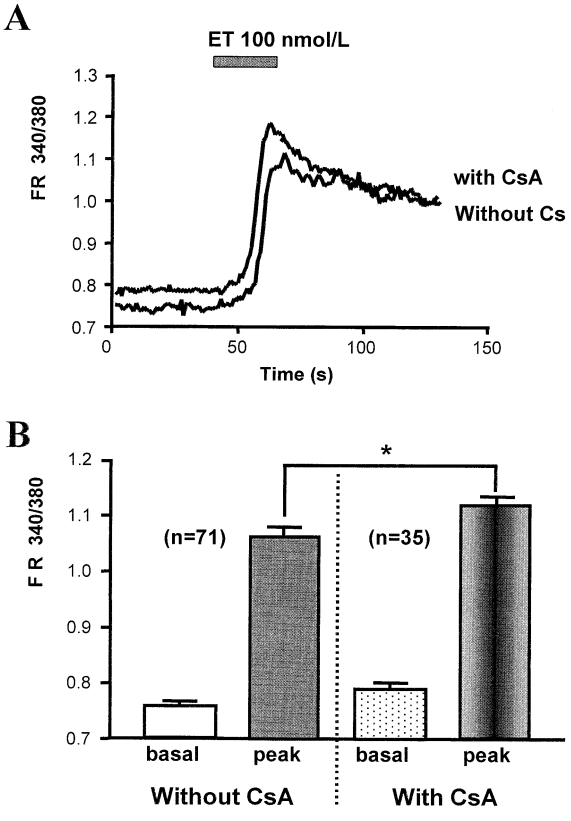

Fig. 3. Effect of chronic exposure to CsA (1 μmol/l) for 24 h on the ET-induced rise of $[Ca^{2+}]_i$ in cultured human coronary myocytes. A: Illustration of the maximal effect of ET as observed in two different cells incubated with and without CsA (1 μmol/l) for 24 h prior to the experiments. B: Bar graphs show the mean effects of the two conditions. The maximal increase in $[Ca^{2+}]_i$ induced by ET was higher in cells incubated with CsA (*$P < 0.05$).

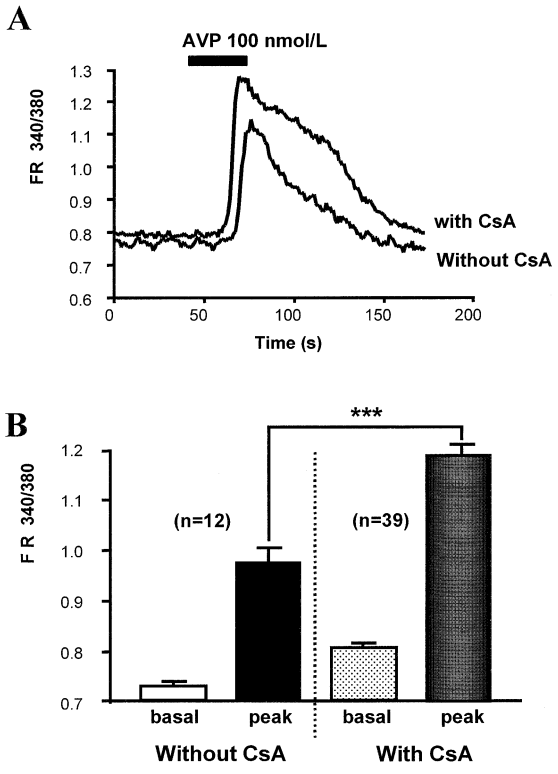

Fig. 4. Effect of chronic exposure to CsA (1 μmol/l) for 24 h on the AVP-induced rise of $[Ca^{2+}]_i$ in cultured human coronary myocytes. A: Illustration of the maximal effect of AVP as observed in two different cells incubated without and with CsA (1 μmol/l) for 24 h prior to the experiments. B: Bar graphs show the mean effects of the two conditions. The maximal increase in $[Ca^{2+}]_i$ induced by AVP was higher in cells incubated with CsA (***$P < 0.001$).

4. Discussion

Numerous studies have reported data on the many events associated with development of arterial hypertension and nephrotoxicity induced by CsA during therapeutic use. However, the exact mechanism(s) is (are) still unclear and there is no satisfying therapy available to prevent side effects. The major findings of the present study are that, in human coronary myocytes, CsA (i) has no acute effect on $[Ca^{2+}]_i$, (ii) increases basal $[Ca^{2+}]_i$ moderately but significantly after 24 h exposure, (iii) increases the frequency of occurrence of spontaneous oscillatory activity of $[Ca^{2+}]_i$, and (iv) potentiates the effects of ET and AVP, two potent vasoconstrictor hormones.

Coronary constriction has been shown in vivo after acute exposure to CsA [4,6,17]. However, the mechanisms by which this occurs are still not well understood. Some of the results obtained on arterial rings are of little clinical interest due to the high concentrations employed (40 μmol/l–5 mmol/l) [5,14]. Although CsA has been reported to induce contraction of isolated rat VSMCs, this effect was suggested to be mediated by ET [10]. We show here that acute application of clinically relevant concentrations of CsA had no direct effect on $[Ca^{2+}]_i$ in cultured human coronary myocytes. However, these cells responded to K^+ depolarization which confirmed the presence of functional Ca^{2+} channels required for Ca^{2+} entry [22] and of an effective coupling with intracellular stores involved in the rise in $[Ca^{2+}]_i$. In particular, a majority of cells responded to AVP (53%) and ET (76%) with an all-or-nothing response

which also demonstrated that the major Ca^{2+} signalling pathways were present at the population level despite some variability among cells and between the AVP and ET pathways. Therefore, we hypothesize that the lack of effect of acute application of CsA on $[Ca^{2+}]_i$ was genuine. An indirect effect, mediated via the release of endothelial-derived contracting factors [4,5], or also a possible direct effect on contractile protein activity of muscle cells [14], might explain the discrepancy with some other studies performed in vivo or on arterial rings in vitro.

Whether CsA promotes a significant rise in the resting $[Ca^{2+}]_i$ of human arterial cells is of great interest because this elevation may trigger a variety of signals. Our study shows for the first time that therapeutic concentrations of CsA applied for 24 h increase $[Ca^{2+}]_i$ in human arterial cells. However, this finding contrasts with a similar study in human aortic cells [13]. The discrepancy may relate to the number of cells used. The increase found in our study was indeed moderate but proved highly significant due to the use of cohorts of cells (for both control and test conditions).

Here, we show that chronic treatment with CsA has two other major effects. The first is to enhance the occurrence of spontaneous short $[Ca^{2+}]_i$ rises, which differ from the larger and more prolonged increases observed during agonist receptor stimulation. This occurrence involves an increase in the number of cells likely to generate oscillatory activity. This is probably directly related to the elevation in the resting cytosolic $[Ca^{2+}]_i$ and the resulting periodic release of internally stored Ca^{2+}. The second is to potentiate the increases in $[Ca^{2+}]_i$ that are induced by AVP and ET in human coronary myocytes. We suggest that these effects may participate in increased arterial blood pressure. This finding extends similar observations made before on hormone responses in rat aortic myocytes where, in addition to AVP and ET, serotonin and angiotensin II actions were shown to be also potentiated by CsA [12,21] independently of its immunosuppressive effect and calcineurin inhibition [12,13,20,27]. It has been suggested that CsA acts on a target upstream of G protein activation, possibly at the receptor level [12]. In addition, it was shown that the potentiation of angiotensin responses in human aortic cells induced by therapeutic concentrations of CsA reflects a twofold increase in the number of angiotensin-1 receptors due to an upregulation of these receptors [13,28–30]. The CsA-induced increase in the resting $[Ca^{2+}]_i$ which we report here is probably too weak to account per se for any change in the basal tone of the artery. However, the resulting promotion of spontaneous increases of $[Ca^{2+}]_i$ may contribute to the increase in vasoconstriction. This contribution may be direct as well as indirect since Ca^{2+} oscillations have been shown to increase both the efficacy and the information content of Ca^{2+} signals in relation with activation of Ca^{2+}-dependent genes [31,32]. Variations in the frequency and duration of global Ca^{2+} signals activate different genes [31,33]. Thus, repetitive increases in cytoplasmic Ca^{2+}, which are probably related to increased periodic release of internally stored Ca^{2+}, could up-regulate the synthesis (or activity) of receptor proteins involved in vasoconstriction as shown for AVP [18]. This step may be a key event in enhanced vasoconstrictor hormone action by CsA. To overcome the problem of the side effects of CsA, several therapeutic solutions have been proposed including use of antagonists of ET receptors [34], inhibitors of the renin–angiotensin system [35,36], Ca^{2+} channel blockers [35–37], or restoration of NO-mediated vasodilatation by L-arginine [38]. However, these strategies provide only partial benefits. Further study of the mechanisms underlying the CsA-induced increase in $[Ca^{2+}]_i$ are clearly needed to find the putative pharmacological key point involved in triggering the multiple effects of the drug.

One limitation of this study may reside in the fact that, though they were devoid of atheroma, the arteries from which the cells derived were taken from pathological hearts (dilated cardiomyopathy). However, it should be noted that the results obtained with cells derived from one donor with a normal heart were consistent. Another limitation may concern the possible compartmentalization of the fluorescent Ca^{2+} indicator in some organelles (e.g. mitochondria, endoplasmic reticulum, nucleus) rather than in the cytoplasm. Further work, using for example confocal microscopy, is mandatory to localize precisely the initial rise in $[Ca^{2+}]_i$ at the subcellular level and to describe the mechanisms involved. Whatever the origin of the spontaneous oscillations, however, their large amplitude seems to be accounted for by variations of the cytosolic $[Ca^{2+}]_i$.

In conclusion, our results demonstrate that therapeutic concentrations of CsA lead to an elevation of the resting $[Ca^{2+}]_i$ with promotion of spontaneous oscillatory activity and an increased hormone-induced rise of $[Ca^{2+}]_i$ in human coronary myocytes. Although the latter effect occurs independently of potential drug effects on endothelium-dependent release of hormones, it may contribute to the potentiation of those effects by increasing the sensitivity of myocytes to various hormones. Hence, strategies aimed at reducing the side effects of CsA should not target a single hormone receptor. The demonstration that CsA increases basal Ca^{2+} upstream of its multiple effects on different hormone receptors is worth considering for the development of new therapeutic approaches.

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