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Inhibition of cyclic GMP hydrolysis with zaprinast reduces basal and cyclic AMP-elevated L-type calcium current in guinea-pig ventricular myocytes

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1 Cyclic GMP (cGMP) has been shown to be an important modulator of cardiac contractile function. A major component of cGMP regulation of contractility is cGMP-mediated inhibition of the cardiac calcium current (I_{Ca}). An under-appreciated aspect of cyclic nucleotide signalling is hydrolysis of the cyclic nucleotide (i.e., breakdown by phosphodiesterases (PDEs)). The role of cGMP hydrolysis in regulating I_{Ca} has not been studied. Thus the purpose of this study was to investigate if inhibition of cGMP hydrolysis can modulate I_{Ca} in isolated guinea-pig ventricular myocytes.

2 Zaprinast, a selective inhibitor of cGMP-specific PDE (PDE5), caused a significant increase in cGMP levels in myocytes, but was without affect on basal or β-adrenergic stimulated cAMP levels (consistent with its actions as a specific inhibitor of PDE5).

3 Zaprinast inhibited I_{Ca} that was pre-stimulated with cAMP elevating agents (isoproterenol, a β-adrenergic agonist; or forskolin, a direct activator of adenylate cyclase). The effect of zaprinast was greatly reduced by KT5823, an inhibitor of cGMP-dependent protein kinase (PKG).

4 Zaprinast also significantly inhibited basal I_{Ca} when perforated-patch or whole-cell recording with physiological pipette calcium concentration (10^{-7} M) was used. However, this effect was not observed when using standard calcium-free whole-cell recording conditions.

5 These results indicate that inhibition of cGMP hydrolysis can decrease both basal and cAMP-stimulated I_{Ca}. Thus, cGMP hydrolysis may likely be an important step for physiological modulation of I_{Ca}. This regulation may also be important in disease states in which cGMP production is increased and PDE5 expression is altered, such as heart failure.
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Abbreviations: cAMP, cyclic AMP (adenosine 3′,5′-cyclic monophosphate, sodium salt); cGMP, cyclic GMP (guanosine 3′,5′-cyclic monophosphate, sodium salt); DMSO, dimethylsulphoxide; DNAase, deoxyribonuclease; E-C, excitation-contraction; EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; forskolin, 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; IBMX, 3-isobutyl-1-methylxanthine; I_{Ca}, L-type Ca^{2+} current; NO, nitric oxide; PDE: phosphodiesterase; PDE5, phosphodiesterase type 5 (cGMP-selective); PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; SR, sarcoplasmic reticulum; zaprinast, (M&B 22948), 1,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one

Introduction

The L-type cardiac calcium current (I_{Ca}) plays a central role in excitation-contraction (E-C) coupling, and therefore is an important determinant of cardiac contractility (for review see Bers, 2001). An important physiological modulator of E-C coupling is the β-adrenergic pathway. Upon β-adrenergic stimulation, activation of adenylate cyclase leads to increased cAMP levels, which results in cAMP-dependent protein kinase (PKA)-mediated phosphorylation of L-type Ca^{2+} channels, phospholamban, Ca^{2+} release channel of the sacroplasmic reticulum (SR), and the myofilaments (Bers & Ziolo, 2001). Phosphorylation of the L-type Ca^{2+} channel by PKA leads to an increase in Ca^{2+} influx, which increases SR Ca^{2+} release, SR Ca^{2+} loading, and to a lesser extent the direct activation of myofilaments (Bers, 2001). Through these mechanisms, I_{Ca} contributes to the positive inotropic effect of β-adrenergic stimulation.

The hydrolysis of cyclic nucleotides occurs *via* enzymes termed phosphodiesterases (PDEs). There are multiple isoforms of PDEs in cardiac myocytes that can hydrolyze cAMP and/or cGMP (for review see Francis *et al*., 2001). Previous studies have found not only that PDE2-4 inhibitors can increase basal I_{Ca}, they can also potentiate the effect of β-adrenergic stimulation on I_{Ca} (e.g., Kawamura & Wahler, 1994; Kajimoto *et al*., 1997; Verde *et al*., 1999). These data suggest that both the synthesis and the rate of hydrolysis of cAMP are important in regulating I_{Ca}.

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cGMP also regulates cardiac contractility, frequently acting on the same proteins in the E-C coupling pathway as cAMP. Activation of guanylate cyclase leads to increased cGMP synthesis that results in activation of the cGMP-dependent protein kinase (PKG). As with the cAMP/PKA pathway, the cGMP/PKG pathway also modulates I_{Ca} (e.g., Wahler & Sperelakis, 1985; Fischmeister & Hartzell, 1987; Levi *et al*., 1989; 1994; Mery *et al*., 1991; Wahler & Dollinger, 1995; Sumii & Sperelakis, 1995; Ziolo *et al*., 2001a). Both stimulation and inhibition of I_{Ca} have been reported with cGMP, depending on cardiac tissue used and experimental conditions (e.g., prior β-adrenergic stimulation). It appears that in mammalian ventricular myocytes, the inhibitory effect of cGMP on I_{Ca}, predominates (Mery *et al*., 1991; Wahler & Dollinger, 1995). The role of cGMP hydrolysis by PDEs in regulating I_{Ca} has not been previously investigated. We hypothesize that hydrolysis of cGMP may be an overlooked important regulatory step in the modulation of I_{Ca} by the cGMP pathway. In order to test this hypothesis, zaprinast was used to inhibit hydrolysis of cGMP. Zaprinast is a specific inhibitor of PDE5, which is a cGMP-selective PDE (Prigent *et al*., 1988; Kotera *et al*., 1998).

Methods

Adult male guinea-pigs (200–250 g, Hartley strain, Charles River Labs, Wilmington, MA, U.S.A.) were anaesthetized with pentobarbitone sodium (45–60 mg kg^{-1}, i.p.) and the heart was rapidly removed for myocytes isolation. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication # 85-23, revised 1985).

Isolation of ventricular myocytes

Ventricular myocytes were isolated as previously described (e.g., Kawamura & Wahler, 1994). Briefly, the hearts were retrogradely perfused with a collagenase solution (Type II, 40–65 units ml^{-1}; Worthington Biochemical Corp., Freehold, NJ, U.S.A.). At the end of the perfusion period, the ventricles were minced and dissociated by trituration at 37°C. Subsequently, the myocytes were filtered, centrifuged and 'cleaned' with a protease and DNAase solution and suspended in a high K^+ storage solution for at least 30 min prior to being used. This latter cleaning step was omitted when myocytes were isolated for cyclic nucleotide assays.

Cyclic nucleotide assays

The cyclic nucleotide assay protocol used in the present study was performed as previously described (Ziolo *et al*., 1998). Briefly, sample aliquots of myocyte preparations were resuspended in 1.5 ml of bath solution (control, zaprinast, isoproterenol or isoproterenol + zaprinast) at room temperature. After 10 min, the myocyte samples were centrifuged at $14{,}000 \times g$ for 45 s, the supernatant was aspirated, and the cells were resuspended in 600 μl of cold 65% ethanol. The ethanol-myocyte mixture was sonicated and then centrifuged at $14{,}000 \times g$ for 20 min at 4°C. The supernatant was aspirated and placed in a separate microcentrifuge tube, the ethanol was evaporated, and the remaining residue was stored at −20°C. The pellet was resuspended in 1% SDS for measurement of total protein content using a bicinchonic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). For measurement of cAMP and cGMP, the frozen residue was reconstituted with 50 mM acetate buffer and cAMP or cGMP content was determined by radioimmunoassay using a commercially available kit (Biomedical Technologies Inc., Stoughton, MA, U.S.A.).

Whole-cell recording of calcium currents

Calcium currents were recorded at room temperature (22–25°C) as we have described previously (e.g., Kawamura & Wahler, 1994), using (in most instances) standard whole-cell voltage clamp methods. Patch pipettes were pulled from borosilicate glass (1B150F, World Precision Instruments, Sarasota, FL, U.S.A.) and fire-polished. The bath solution contained (in mM): NaCl 140, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, glucose 10, HEPES 5; pH = 7.4. The nominally 'Ca-free' pipette solution for standard whole-cell recording contained: CsCl 120, $MgCl_2$ 6, EGTA 10, Na_2ATP 5, HEPES 10; pH = 7.2. This was the pipette solution used for all whole-cell recordings, except where indicated. Under these conditions, we have previously determined that the pipette free Ca^{2+} concentration is less than 10^{-9} M (Kawamura & Wahler, 1994).

After making a seal and rupturing the patch, cell membrane capacitance was estimated by integrating the capacitive current response to small voltage pulses and dividing by the voltage (5 mV). Subsequently, much of the series resistance was compensated. Several minutes were permitted to pass to allow for cell dialysis. Thereafter, currents were elicited by 200 ms pulses to 0 mV from a holding potential of −80 mV (following a pre-pulse to −40 mV) at a frequency of 0.2 Hz. The prepulse inactivated the Na^+ current, and the replacement of K^+ with Cs^+ eliminated the K^+ currents. This procedure isolated the L-type calcium current (I_{Ca}). I_{Ca} was measured as the peak inward current minus the current at the end of the 200 ms pulse. Periodically, current-voltage (I-V) curves were also generated by 10 mV steps over the range from −40 to +50 mV (or +60 mV). Commercial software (pCLAMP, Axon Instruments, Foster City, CA, U.S.A.) was used for generating voltage pulses, and also for data acquisition and current analysis.

In some additional experiments, sufficient calcium was added to the pipette solution to result in a pipette Ca^{2+} concentration of 10^{-7} M (Kawamura & Wahler, 1994). All other aspects of the recording methodology for these experiments were identical to the standard whole-cell recording with the calcium-free pipette solution.

Perforated-patch recording of calcium currents

In addition to the whole-cell recording methods described above, in some experiments perforated-patch recording methods were used in order to maintain a more physiological intracellular environment. The bath solution used for perforated-patch recording was identical to the bath solution used for whole-cell recording (above). The pipette solution used for perforated-patch recording contained (in mM) Cs-glutamate 110, CsCl 20, $CaCl_2$ 1.8, $MgCl_2$ 1.1, HEPES 5, pH = 7.0. The tip of the perforated-patch pipette was dipped

in this pipette solution for a few seconds, just prior to use. The rest of the pipette was backfilled with pipette solution in which 240 μg ml^{-1} amphotericin B and 2.5 μg ml^{-1} Pluronic F-127 (Calbiochem, La Jolla, CA, U.S.A.) had been added.

For perforated-patch recording, after obtaining a gigaohm seal between the pipette and cell in the cell-attached mode, no further suction was applied. Once electrical access had been spontaneously achieved, we recorded I-V curves every few minutes until we were satisfied that voltage control was adequate before continuing (for a discussion of determining adequacy of voltage control see Kawamura & Wahler, 1994). With the use of relatively large-tipped pipettes, the perforated-patch access resistance in these experiments was 3.2 ± 0.5 MΩ. Since the control currents under perforated-patch conditions were typically much less than 1 nA, this normally resulted in a voltage error of only a few mV. The protocols for data acquisition and analysis were identical as for whole-cell recording.

Drugs and solutions

Zaprinast (Rhône-Poulenc, Dagenham, U.K.; Calbiochem, San Diego, CA, U.S.A.) was dissolved in 1 N NaOH. Forskolin was dissolved in dimethylsulphoxide (DMSO, final concentration = 0.0083%). Appropriate solvent concentrations were also added to the control bath solutions. All compounds were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise indicated.

Statistical analysis

Results from multiple experiments are expressed as the mean ± s.e.mean. Statistical analyses were performed with Student's paired or unpaired *t*-test, or repeated measures analysis-of-variance followed by a Newman-Keuls' post-test (with $P < 0.05$ being considered significant).

Results

Effects of zaprinast on cyclic nucleotide levels

The cAMP and cGMP levels were measured in isolated myocyte preparations to determine the effects of zaprinast on cyclic nucleotide levels. Figure 1 shows the overall results from isolated myocytes exposed to 10 μM zaprinast. Figure 1A demonstrates that zaprinast exposure greatly increased cGMP levels (from 14.0 ± 7.0 fmol mg^{-1} protein without zaprinast to 40.0 ± 6.0 fmol mg^{-1} protein with zaprinast; $P < 0.05$, paired *t*-test, $n = 3$), but had no effect on basal cAMP levels (i.e., in the absence of other exogenous agents), 4.7 ± 1.1 pmol mg^{-1} protein without zaprinast to 4.4 ± 0.9 pmol mg^{-1} protein with zaprinast); $P > 0.05$, paired *t*-test, $n = 4$). Furthermore, Figure 1B indicates that following a substantial increase in cAMP levels due to β-adrenergic stimulation with isoproterenol, zaprinast again had no effect on the cAMP levels (control: 4.6 ± 0.4 pmol mg^{-1} protein; isoproterenol: 9.2 ± 1.3 pmol mg^{-1} protein, $P < 0.05$ compared to control; isoproterenol + zaprinast: 9.9 ± 1.0 pmol mg^{-1} protein, $P < 0.05$ compared to control, NS compared to isoproterenol; ANOVA, $n = 6$). These results suggest that zaprinast did act as a specific inhibitor of the cGMP-selective PDE in ventricular myocytes, while having little or no effect on other PDE isozymes.

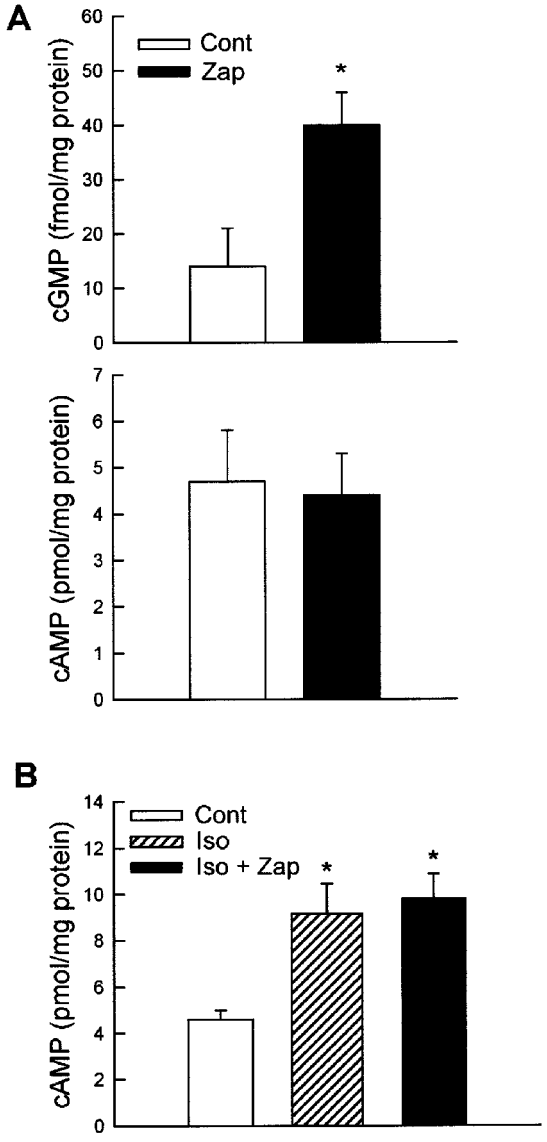

Figure 1 Effects of zaprinast on cGMP and cAMP levels under basal conditions (A) and in the presence of isoproterenol (B). Shown are the mean ± s.e.mean of duplicate measurements from three hearts (cGMP) and four hearts (cAMP) (A) or six hearts (B). Zaprinast (ZAP, 10 μM) greatly enhanced cGMP levels whereas zaprinast did not alter cAMP levels under either basal conditions or in the presence of isoproterenol (Iso, 1 μM). *$P < 0.05$ from myocytes in control bath solution; paired *t*-test (A) or ANOVA (B).

Effects of zaprinast on basal I_{Ca} *(using whole-cell (Ca-free) recording)*

Superfusion of the cells with either 10 μM or 100 μM zaprinast resulted in no change in basal I_{Ca}. Figure 2 shows a time plot indicating the lack of an effect of zaprinast superfusion on basal current. Thus, following superfusion with 100 μM zaprinast, I_{Ca} was $95 \pm 6\%$ of the control, pre-zaprinast value ($P > 0.05$; paired *t*-test; $n = 5$). Zaprinast also had no effect on the calcium current inactivation kinetics during whole-cell recording (data not shown).

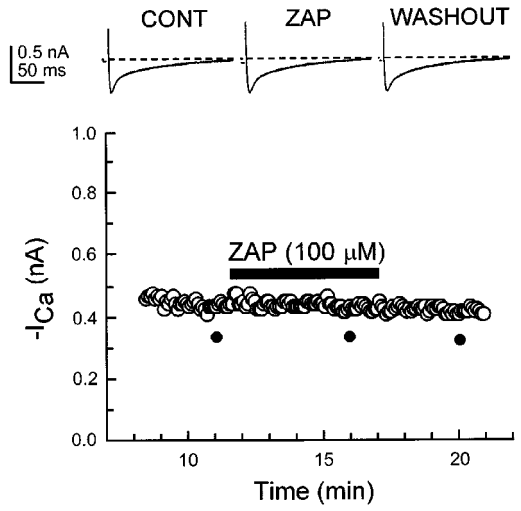

Figure 2 Zaprinast had no effect on basal I_{Ca} using standard whole-cell recording methods with a nominally calcium-free pipette solution. Shown are the original currents (upper panel) and plot of the amplitude of I_{Ca} at 0 mV over time (lower panel) during superfusion of the cells with control (CONT) or 100 μM zaprinast-containing (ZAP) solutions. The original current traces shown in the upper panel are the average of three to five individual traces and were obtained at the approximate times indicated in the lower panel. The dotted lines in the upper panel indicate the zero current level. The currents plotted in the lower panel were measured as the peak minus end-of-pulse currents.

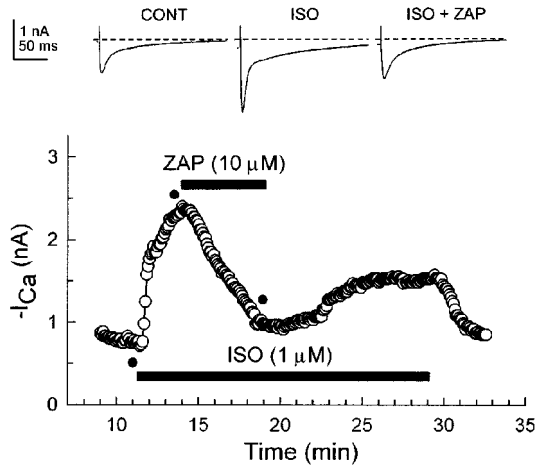

Figure 3 Zaprinast inhibited the isoproterenol-stimulated I_{Ca}. Shown are the original currents (upper panel) and the amplitude of I_{Ca} at 0 mV over time (lower panel) during superfusion of the cells with control (CONT), 1 μM isoproterenol-containing (ISO), or isoproterenol plus 10 μM zaprinast-containing (ISO + ZAP) solutions. Superfusion of the cell with 1 μM isoproterenol caused a large stimulation of I_{Ca}. Subsequent superfusion of the cell with 10 μM zaprinast caused a substantial inhibition of the isoproterenol-induced stimulation of I_{Ca}. Upon washout of zaprinast there was a partial recovery of the isoproterenol-stimulated I_{Ca}. Standard whole-cell recording (calcium-free pipette solution) was used.

Effects of zaprinast on I_{Ca} *pre-stimulated with cAMP elevating agents*

The effects of zaprinast were also examined on I_{Ca} that was first stimulated with isoproterenol, a β-adrenergic agonist. As expected, superfusion of myocytes with isoproterenol (1 μM) caused a substantial stimulation of I_{Ca}. Thus, in the presence of 1 μM isoproterenol, I_{Ca} was $296 \pm 37\%$ of control ($n = 18$). In contrast to the lack of an effect of zaprinast on the basal I_{Ca} (above), superfusion with zaprinast (10 μM) in the presence of isoproterenol caused a substantial inhibition of I_{Ca} (e.g., Figure 3). On average, 10 μM zaprinast inhibited $69 \pm 15\%$ of the isoproterenol-induced increase in I_{Ca} ($P < 0.05$ from I_{Ca} prior to zaprinast; paired t-test; $n = 18$).

In a number of additional experiments, forskolin (a direct activator of adenylate cyclase that enhances I_{Ca} (e.g., Wahler & Sperelakis, 1986) while bypassing the β-adrenoceptor) was used to enhance I_{Ca} instead of isoproterenol. Following superfusion with 0.3 μM forskolin, I_{Ca} was $351 \pm 26\%$ of control ($n = 35$). As was the case with isoproterenol-stimulated I_{Ca}, subsequent superfusion of the cells with zaprinast also caused an inhibition of the forskolin-stimulated I_{Ca} (Figures 4 and 5). This inhibition of forskolin-stimulated I_{Ca} was dose-dependent (Figure 4), with zaprinast causing a statistically significant decrease in forskolin-stimulated I_{Ca} over the range of 1–100 μM. Zaprinast inhibition was maximal at 10 μM. Therefore, most experiments were done at that concentration. On average, the degree of inhibition attained with 10 μM zaprinast was similar whether isoproterenol or forskolin were used to enhance I_{Ca} ($-63 \pm 9\%$ of the forskolin stimulation versus $-69 \pm 15\%$ of the isoproterenol stimulation).

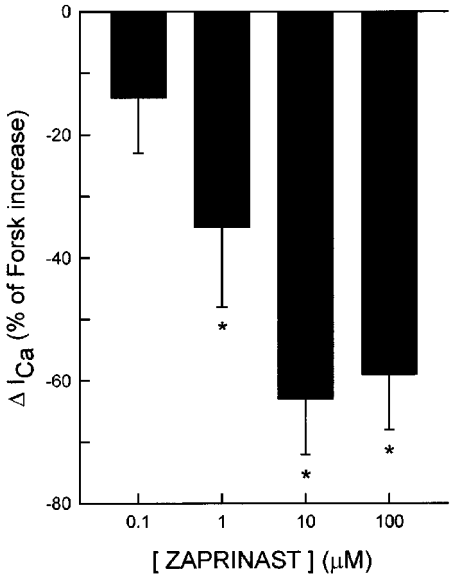

Figure 4 Dose-dependent inhibition of forskolin-stimulated I_{Ca} by zaprinast. The data shown is the maximum decrease of forskolin-stimulated I_{Ca} (as a percentage of the increase in I_{Ca} caused by 0.3 μM forskolin) caused by superfusion with various concentrations of zaprinast. Responses shown are the mean $\pm$ s.e.mean of five to 15 experiments per concentration of zaprinast. A single concentration of zaprinast was applied per cell. $^*P < 0.05$ from forskolin-stimulated I_{Ca} value prior to addition of zaprinast; paired t-test. Standard whole-cell recording was used.

Effects of PKG inhibitor KT5823 on zaprinast-mediated inhibition of I_{Ca}

The effects of KT5823, a specific inhibitor of PKG, were examined on the I_{Ca} response to zaprinast (after stimulation

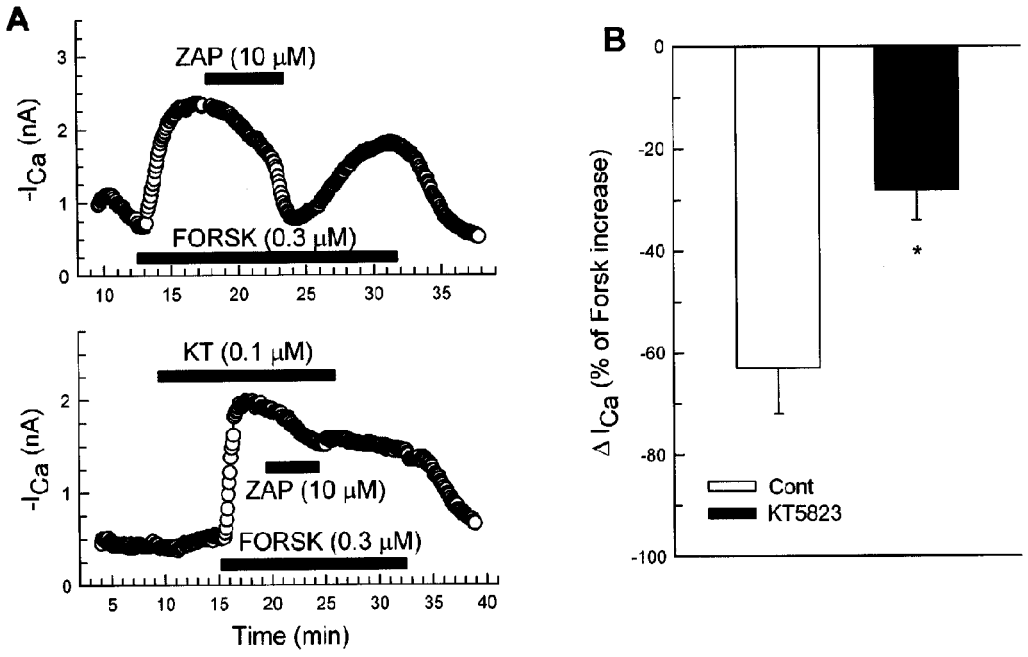

Figure 5 Zaprinast inhibition of the forskolin-stimulated I_{Ca} in the absence and presence of an inhibitor of cGMP-dependent protein kinase (KT5823). (A) Superfusion of a cell with 0.3 μM forskolin (FORSK) caused a large stimulation of I_{Ca}. Under control conditions (i.e., in the absence of KT5823) (upper panel), subsequent superfusion of the cell with 10 μM zaprinast (ZAP) caused a substantial inhibition of the forskolin-induced stimulation of I_{Ca}. In contrast, in the presence of 0.1 μM KT5823 (KT) (lower panel), the inhibitory effect of zaprinast was greatly attenuated. Standard whole-cell recording was used. (B) Shown are the mean$\pm$s.e.mean of the reduction in I_{Ca} (as a percentage of the increase caused by 0.3 μm forskolin) for 15 experiments under control conditions (Cont) and 15 experiments in the presence of 0.1 μM KT5823. *$P<0.05$ from control reduction; unpaired t-test.

with forskolin). An example of the effect of 0.1 μM KT5823 on the inhibitory response to 10 μM zaprinast is shown in Figure 5A. The overall effect of KT5823 (0.1 μM) is shown in Figure 5B. Superfusion of the cells with 0.1 μM KT5823 before application of 0.3 μm forskolin had no significant effect on either the basal I_{Ca} or the response to forskolin. Thus, the basal I_{Ca} following superfusion with KT5823 was 98$\pm$4% of the pre-KT5823 value ($P>0.05$, paired t-test, $n=15$). Additionally, 0.3 μM forskolin enhanced I_{Ca} by 351$\pm$26% in the absence of KT5823 ($n=35$), and enhanced I_{Ca} by 351$\pm$41% in the presence of 0.1 μM KT5823 ($n=15$). While KT5823 had no effect on basal or forskolin-stimulated I_{Ca}, it did significantly attenuate the inhibitory effect of 10 μM zaprinast on forskolin-enhanced I_{Ca} (Figure 5) (from a 63$\pm$9% reduction in the absence of KT5823 to a 28$\pm$5% reduction in the presence of KT5823; $P<0.05$).

Effects of zaprinast on basal I_{Ca} *using perforated-patch recording technique*

Conventional whole-cell voltage clamp results in dialysis of the cytoplasmic constituents. Thus, in order to examine whether the lack of an effect of zaprinast on basal I_{Ca} was due to the altered intracellular environment, we undertook some additional experiments using the perforated-patch technique. Using this technique, one has electrical access to the cell interior and most intracellular constituents remain largely undisturbed (Horn & Marty, 1988). In contrast to the lack of an effect of zaprinast on basal I_{Ca} using conventional whole-cell recording (e.g., Figure 2), when perforated-patch recording was used, zaprinast now had a significant inhibitory effect (Figure 6). That is, zaprinast now reduced basal I_{Ca} by 30$\pm$5% (from 2.9$\pm$1.0 to 2.0$\pm$0.7 pA pF^{-1}, $P<0.05$, paired t-test, $n=7$). Zaprinast again had no effect on calcium current inactivation kinetics under perforated-patch recording conditions (data not shown).

Effects of zaprinast on basal I_{Ca} *using whole-cell recording* ($Ca^{2+}=10^{-7}$M)

In a previous study (Kawamura & Wahler, 1994), we had shown that the unphysiologically low intracellular Ca^{2+} ($<10^{-9}$ M) generated by the typical nominally Ca^{2+}-free pipette solution used in conventional whole-cell recording altered the response to the non-specific PDE inhibitor isobutylmethylxanthine (IBMX). Therefore, in the present study, we undertook additional zaprinast experiments with a pipette solution containing more physiological Ca^{2+} concentration (10^{-7} M). In contrast to the lack of an effect of zaprinast on basal I_{Ca} using conventional whole-cell recording with Ca^{2+}-free pipette solution (e.g., Figure 2), when using the 10^{-7} M Ca^{2+} pipette solution, zaprinast now had a significant inhibitory effect on basal I_{Ca} (Figure 6). That is, zaprinast inhibited basal I_{Ca} by 24$\pm$8% (from 3.5$\pm$0.3 to 2.6$\pm$0.3 pA pF^{-1}; $P<0.05$, paired t-test, $n=7$) when a more physiological intracellular calcium concentration was used. The effect of zaprinast on basal I_{Ca} utilizing different recording conditions (nominally calcium-free standard whole-cell, perforated-patch, and whole-cell with more physiological $[Ca^{2+}]_i$) is summarized in Figure 6B. As was the case for standard whole-cell recording and perforated-patch, zaprinast again had no effect on calcium current decay

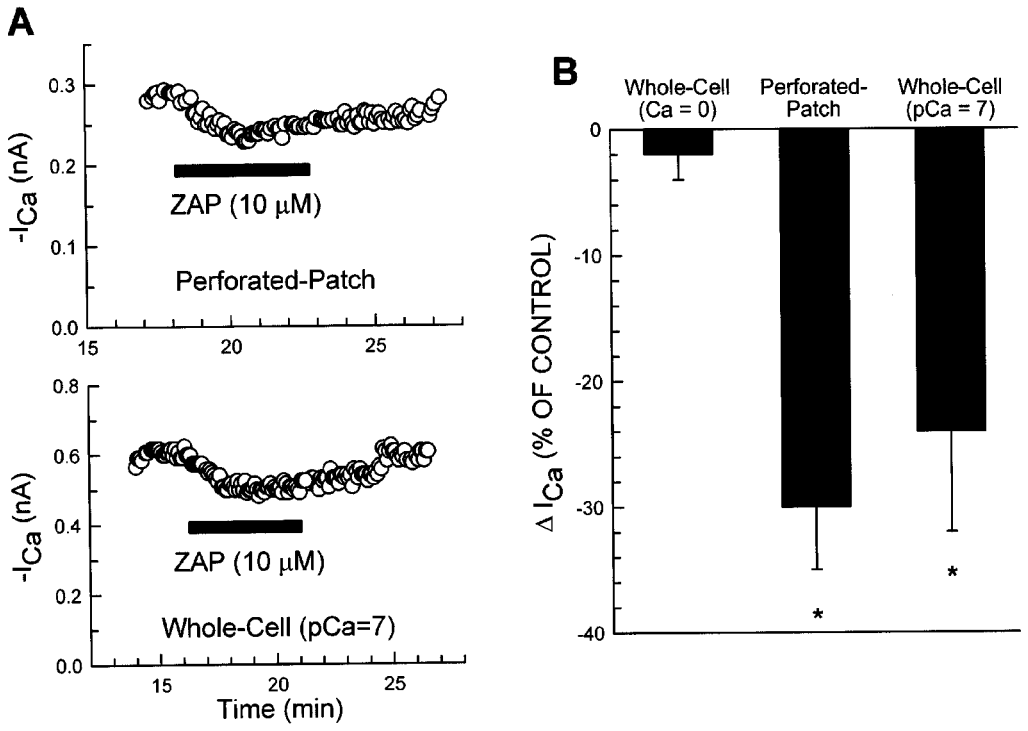

Figure 6 The effect of zaprinast on basal I_{Ca}, using different recording methods. (A) Shown is an example of the effect of 10 μM zaprinast on basal I_{Ca} using perforated-patch recording (upper trace). Zaprinast inhibited basal I_{Ca} over a period of a few minutes. Upon washout of the zaprinast, I_{Ca} gradually recovered. A virtually identical effect of zaprinast was observed using whole-cell recording with 10^{-7} M Ca^{2+} pipette solution (lower trace). (B) Shown are the maximal responses (mean $\pm$ s.e.mean of five to seven cells each) to zaprinast using whole-cell voltage clamp recording with either zero calcium pipette (Whole-Cell Ca = 0) or 10^{-7} M Ca^{2+} pipette (Whole-Cell (pCa = 7)) solutions, or perforated-patch recording. Zaprinast (10 μM) inhibited basal I_{Ca} by about 25–30% when using perforated-patch or whole-cell recording with 10^{-7} M Ca^{2+} pipette solution (*$P<0.05$ from basal I_{Ca} prior to zaprinast), whereas zaprinast had no significant effect on basal I_{Ca} when whole-cell recording was undertaken using a calcium-free pipette solution.

under whole-cell recording with added pipette calcium (data not shown). In addition to not affecting the decay of the current, zaprinast also did not substantially shift the calcium current I-V relationship under any of the recording conditions (Figure 7).

Discussion

Calcium channels play an important role in E-C coupling in cardiac myocytes. Upon depolarization, Ca^{2+} entering through the calcium channels leads to Ca^{2+} release from the SR by Ca^{2+}-induced-Ca^{2+}-release. This influx of Ca^{2+} also loads the SR and also directly causes activation of the myofilaments. Thus, controlling the amount of Ca^{2+} influx through I_{Ca} is a key determinant of contractility. Many pathways that alter contractility act through regulation of I_{Ca}. One such pathway is stimulation of the β-adrenergic pathway. Activation of adenylate cyclase and increased cAMP synthesis activates PKA, which will lead to phosphorylation of many proteins including the L-type Ca^{2+} channel. It has also been shown that the rate of cAMP hydrolysis by PDEs is also an important determinant of cAMP regulation of I_{Ca}, (e.g., Kawamura & Wahler, 1994; Kajimoto *et al*., 1997; Verde *et al*., 1999).

Another pathway that regulates I_{Ca} activity is the cGMP pathway (e.g., Wahler & Sperelakis, 1985; Fischmeister & Hartzell, 1987; Levi *et al*., 1989; 1994; Mery *et al*., 1991; Wahler & Dollinger, 1995; Sumii & Sperelakis, 1995; Ziolo *et al*., 2001a). Thus, like the cAMP pathway, increases in cGMP synthesis (e.g., with nitric oxide (NO)) leads to activation of PKG and phosphorylation of the L-type Ca^{2+} channel (or an associated regulatory protein). In contrast to PKA, activation of PKG in mammalian ventricular myocytes generally leads to a decrease in I_{Ca}. Additionally, cGMP can activate or inhibit different PDE isoforms, thereby either increasing or decreasing cAMP levels and indirectly modulating I_{Ca}.

Cyclic nucleotide PDE exists as several isozymes. While some PDE isozymes preferentially hydrolyze cAMP, and others hydrolyze both cAMP and cGMP, one family of PDE isozymes (PDE5) preferentially hydrolyzes cGMP (Francis *et al*., 2001). A commonly used inhibitor of the cGMP-selective PDE is zaprinast. Zaprinast has been shown to be a specific inhibitor of this PDE isoform in the heart (IC_{50} for PDE5 of approximately 0.2–1 μM, whereas the IC_{50}'s for the other PDE isozymes are orders of magnitude higher (Prigent *et al*., 1988; Kotera *et al*., 1998). The previous results of other investigators are consistent with the effects of zaprinast being mediated by inhibition of cGMP hydrolysis. Thus, cardiac myocytes express PDE5 (Senzaki *et al*., 2001) and zaprinast inhibits cardiac PDE5 at micromolar concentrations (Prigent *et al*., 1988; Kotera *et al*., 1998). Zaprinast enhances intracellular cGMP levels in ventricular myocytes (present study, Figure 1; Clemo *et al*., 1992; Straznicka *et al*., 1999;

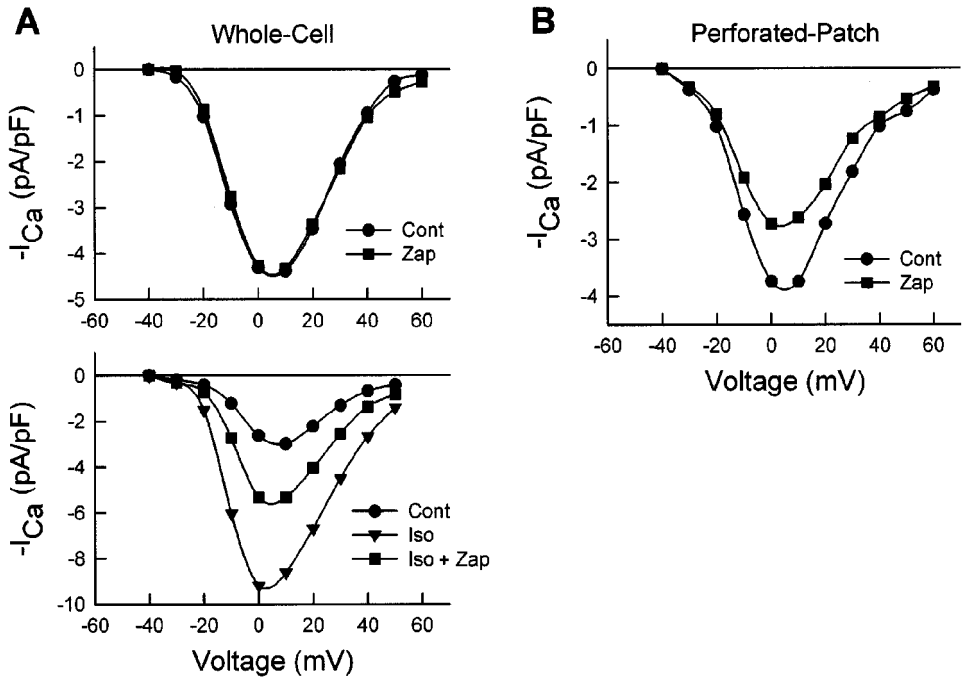

Figure 7 Effect of zaprinast on current-voltage (I-V) relationships. (A) Zaprinast (Zap, 10 μM) had no effect on the basal current I-V curve (upper panel) when standard whole-cell recording (i.e., nominally calcium free) was used. Isoproterenol (Iso, 1 μM) caused a large increase in I_{Ca} and a leftward voltage shift of the I-V curve, which remained following inhibition of the current by zaprinast. (B) When perforated-patch recording was used, zaprinast (Zap, 10 μM) caused a reduction in basal I_{Ca}, but did not voltage shift the I-V relationship.

Yan *et al*., 2000), consistent with inhibition of PDE5. Since basal cAMP levels were not altered by zaprinast (Figure 1), significant non-specific inhibition of other PDE isoforms does not appear to occur with zaprinast at 10 μM, the concentration primarily used in this study. In addition, there was no effect of zaprinast on cAMP levels pre-stimulated with isoproterenol. Thus, in the present and previous studies, zaprinast increased cGMP levels, consistent with its action as a selective inhibitor of PDE5, while having no apparent direct effect on other PDE isoforms. These results also suggest that under the present conditions the effects of zaprinast were unlikely to be due to indirect effects of cGMP-induced alterations in cAMP levels (see below).

Previous studies have examined the functional effects of PDE5 inhibition by zaprinast. Most relevant to the present study, zaprinast has been shown to enhance cGMP levels and decrease myocytes shortening in mammalian cardiac myocytes (basal and pre-stimulated with cAMP elevating agents) (Straznicka *et al*., 1999; Yan *et al*., 2000; Senzaki *et al*., 2001). These studies suggest that the activity of PDE5 may be an important determinant of cardiac contractile function. Thus, the present study was undertaken to try to discover which mechanism is responsible for the depressed myocyte function with PDE5 inhibition. More specifically, we examined if the L-type calcium channel is involved in this PDE5-mediated depression in myocyte function.

The present study found that zaprinast had an 'anti-adrenergic effect' on I_{Ca}. That is, zaprinast had a substantial inhibitory effect on I_{Ca} that had been stimulated by isoproterenol or forskolin (Figures 3–5), in contrast to the lack of zaprinast on basal I_{Ca} when using standard whole-cell recording methodology (nominally calcium-free pipette solution) (Figure 2). This anti-adrenergic action of zaprinast is similar to the anti-adrenergic effects of exogenous cGMP or agents that stimulate guanylate cyclase or PKG (e.g., Wahler & Sperelakis, 1985; Fischmeister & Hartzell, 1987; Levi *et al*., 1989; 1994; Mery *et al*., 1991; Wahler & Dollinger, 1995; Sumii & Sperelakis, 1995; Ziolo *et al*., 2001a). We conclude that the inhibition of PDE5 by zaprinast, and the resulting increase in intracellular cGMP levels, is responsible for the inhibition of I_{Ca}.

In mammalian ventricular myocytes, cGMP-mediated inhibition of I_{Ca} may be largely due to the activation of PKG (Mery *et al*., 1991; Wahler & Dollinger, 1995; Sumii & Sperelakis, 1995). Thus, we examined if KT5823, a specific inhibitor of PKG (Kase, 1988), blocks the effects of zaprinast on I_{Ca}. KT5823 has been shown to reduce the negative contractile effects of 8-Br-cGMP and zaprinast in isolated myocytes (Shah *et al*., 1994; Straznicka *et al*., 1999) and the effects of NO/cGMP on I_{Ca} (Wahler & Dollinger, 1995; Ziolo *et al*., 2001a). In the present study, the anti-adrenergic effect of zaprinast on I_{Ca} was also reduced by KT5823 (Figure 5). This data further supports the hypothesis that zaprinast inhibition of I_{Ca} is mediated through increased cGMP and activation of PKG.

The zaprinast inhibition of forskolin-stimulated I_{Ca} that remains after KT5823 inhibition could reflect an additional PKG-independent action of zaprinast. Stimulation of cGMP-stimulated PDE2 is one such possible additional mechanism, since this would be expected to result in a decreased I_{Ca}. It has been previously demonstrated that cGMP-stimulated PDE2 plays a major role in regulating I_{Ca} in some preparations, such as frog ventricular myocytes and human atrial myocytes (e.g., Rivet-Bastide *et al*., 1997). However, the

same study that described an important role for PDE2 in regulating I_{Ca} of human atrial myocytes found no such role for PDE2 in mammalian (rat) ventricular myocytes (Rivet-Bastide *et al*., 1997). Similarly, while some studies suggest a major role for PDE2 in the inhibition of I_{Ca} and negative inotropic effects of cGMP in mammalian ventricular myocytes (for review, see Balligand, 1999), other studies do not support such a role for PDE2 (e.g., Wegener *et al*., 2002). The lack of an effect of zaprinast on cAMP levels in the present study, either under basal conditions or following elevation of cAMP, argues against a significant PDE2-mediated effect of zaprinast on I_{Ca} in our preparation. Of course, since cAMP levels might be changed in some compartment without affecting global cAMP levels, a PDE2-mediated mechanism cannot be completely excluded. A simpler explanation is that 0.1 μM of KT5823 does not completely inhibit PKG. We have previously shown that this concentration of KT5823 reduces the depression of cAMP-elevated I_{Ca} by SIN-1 (an NO donor) to a similar extent as the reduction of the zaprinast effect in the present study (Wahler & Dollinger, 1995). A higher concentration (1 μM) of KT5823 completely blocked the effects of SIN-1, but also had considerable non-specific effects and was, therefore, not used in the present study. In the absence of any evidence for a role for PDE2 in the observed effects of zaprinast, we conclude that the effects of zaprinast are largely, if not entirely, mediated by PKG.

In the present study, PDE5 inhibition increased basal cGMP levels, which suggests that there is considerable basal activity of both guanylate cyclase and PDE5. Furthermore, in addition to the anti-adrenergic actions of zaprinast observed when using standard whole-cell recording techniques, zaprinast also inhibited basal I_{Ca} during perforated-patch recording or when more physiological levels of pipette calcium were maintained (Figure 6). The NO donor SIN-1 has similarly been reported to inhibit basal I_{Ca} only in the presence of physiological pipette calcium concentrations (Matsumoto, 1997). Thus, agents which increase cGMP levels (zaprinast, NO donors) are capable of inhibiting basal I_{Ca} in mammalian ventricular myocytes; however, this effect may be not be observed under standard whole-cell recording conditions due to the unphysiologically low pipette calcium concentrations. These results suggest that cGMP inhibition of basal I_{Ca} is likely to occur physiologically. We speculate that only PKA-phosphorylated calcium channels may be inhibited by cGMP and the relatively modest inhibition of I_{Ca} under basal conditions may be due to a low level of basal PKA phosphorylation, which is further reduced under standard whole-cell conditions.

Several investigators have reported a relatively modest cGMP inhibition of basal shortening (e.g., Shah *et al*., 1994; Straznicka *et al*., 1999; Yan *et al*., 2000; Senzaki *et al*., 2001), which has been suggested to be independent of changes in basal I_{Ca}, in large part due to the reported lack of an effect of cGMP on basal I_{Ca} in mammalian myocytes. However, cGMP inhibition of basal shortening has been observed in intact cells, whereas the lack of cGMP inhibition of basal I_{Ca} has previously been observed using standard whole-cell voltage clamp techniques with unphysiologically low pipette Ca^{2+} concentrations, which 'masks' cGMP inhibition of basal I_{Ca}. The present study and that of Matsumoto (1997) suggest that cGMP may in fact inhibit basal I_{Ca} in intact myocytes, though to a lesser extent than the 'anti-adrenergic' inhibition of cAMP-stimulated I_{Ca}. Thus, we suggest that GMP inhibition of basal I_{Ca} may be responsible for the cGMP inhibition of basal shortening in intact ventricular myocytes after all. However, this remains to be directly determined.

In addition to the physiological significance of the rate of cGMP hydrolysis in modulating cardiac function, this pathway may also be important under pathological conditions. Thus, during heart failure there is a depressed I_{Ca} response to β-adrenergic stimulation. There have also been some reports that basal I_{Ca} is reduced in heart failure; however, there is significant disagreement on this point (for review see Mukherjee & Spinale, 1998), which may be due to the use of standard whole cell conditions with a nominally Ca^{2+} free pipette solution. NO production is also increased in heart failure, and part of the contractile dysfunction of heart failure is apparently due to overproduction of NO (Hare *et al*., 1998; Ziolo *et al*., 2001b). Additionally, it has been found that PDE5 expression and activity is reduced in heart failure (Senzaki *et al*., 2001). This also would be expected to lead to an increase in cGMP levels, which could further contribute to the decreased I_{Ca} seen in heart failure. Thus, the reduced I_{Ca} observed in heart failure may also be due, in part, to PKG-mediated inhibition of I_{Ca}. This remains to be directly demonstrated.

The major finding of this study is that zaprinast inhibits both the cAMP-stimulated I_{Ca} (an 'anti-adrenergic' action) and, to a lesser extent, basal I_{Ca} in guinea-pig ventricular myocytes. The present results provide evidence in support of the hypothesis that the cGMP-selective PDE (PDE5) may be an important pathway for regulating I_{Ca} under both basal conditions and in response to β-adrenergic stimulation. Thus, while several studies have focused on the role of guanylate cyclase and the synthesis of cGMP in regulating I_{Ca}, the present study demonstrates that the rate of cGMP hydrolysis by the cGMP-selective PDE (PDE5) is likely to be an additional important determinant of I_{Ca} and, therefore, cardiac function.

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