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The function of calcineurin and ERK1/2 signal in the antihypertrophic effects of κ-opioid receptor stimulation on myocardial hypertrophy induced by isoprenaline

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Received June 29, 2011, accepted July 29, 2011

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Pharmazie 66: 182–186 (2011) *doi: 10.1691/ph.2012.1622*

The aim of the present study, performed in an *in vitro* model of cardiac hypertrophy, was to examine the possible function of calcineurin and ERK1/2 in the inhibitory effects of κ-opioid receptor stimulation on Ca^{2+} transients and myocardial hypertrophy induced by β1-adrenoceptor stimulation. We determined the effects of trans-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamid methanesulfonate salt (U50,488H), a selective κ-opioid receptor agonist, on the enhancement of spontaneous Ca^{2+} transients and the induction of hypertrophy by isoprenaline, a β-adrenoceptor agonist, in cultured neonatal ventricular myocytes. Total protein content, [^{3}H]leucine incorporation and cell size were used as indices of hypertrophy; calcineurin activity and phospho-ERK1/2 level were determined by immunoblotting. Isoprenaline (10 μmol·L^{-1}) increased all the three indices of hypertrophy, Ca^{2+} transients, calcineurin activity and the level of phospho-ERK1/2. The effects of isoprenaline were abolished by 1 μmol·L^{-1} U50,488H in the absence but not in the presence of nor-binaltorphimine, a κ-opioid receptor antagonist. The inhibitory effects of U50,488H were reproduced by cyclosporine-A, an inhibitor of calcineurin, U0126, the inhibitor of ERK1/2 and verapamil, a L-type Ca^{2+} channel antagonist. In addition, suppression of calcineurin activity by cyclosporine-A was associated with modest suppression of ERK1/2 phosphorylation. Meanwhile, suppression of ERK1/2 phosphorylation by U0126 was associated with modest suppression of calcineurin activity. In conclusion, the inhibitory effects of κ-opioid receptor stimulation involved calcineurin and ERK1/2, and the two signaling pathways showed interaction in the mechanism of antihypertrophic effects afforded by κ-opioid receptor stimulation.

1. Introduction

In the heart, δ- and κ-opioid receptors are found. Stimulation of the δ-opioid receptor enhances proliferation (Zhao et al. 2008) and reduces apoptosis (Wang et al. 2009) in neonatal cardiomyocytes. Activation of the κ-opioid receptor with its selective agonist trans-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamid methanesulfonate salt (U50,488H), inhibits the Ca^{2+} transients and cardiac hypertrophy in neonatal rats (Wu et al. 2008) as well as hypertrophy (Jaiswal et al. 2010), fibrosis (Yin et al. 2009) and arrhythmias (Jin et al. 2008) in the whole hearts induced by isoprenaline. The inhibitory effects of U50,488H are abolished by blockade of the κ-opioid receptor with its antagonist, nor-binaltorphimine (Nor-BNI) (Wang et al. 2004). These observations suggested that the κ-opioid receptor inhibits the effects of β-adrenoceptor stimulation — "cross-talk" between the κ-opioid receptor and the β-adrenoceptor. Our previous study has provided the first evidence that the cross-talk is occurred between the κ-opioid receptor and the β1-adrenoceptor subtype in cardiac hypertrophy (Shan et al. 2007). Further studies also indicated that cross-talk between the κ-opioid receptor and the β1-adrenoceptor was due mainly to an interaction at the G-protein level, including Gs and Gi proteins (Sheng et al. 1997; Yu et al. 2001). Zou et al. (1999) have reported that isoprenaline activated ERK1/2 through both Gs- and Gi-dependent pathways and induced cardiomyocyte hypertrophy. This is in agreement with other reports that isoprenaline significantly activated ERK1/2 through cAMP and PKA in cardiac myocytes (Bogoyevitch et al. 1996; Tutor et al. 2007). Calcineurin is a calcium regulated protein phosphatase that functions as a positive regulator of cardiac hypertrophic growth through a direct mechanism involving activation of nuclear factor of activate T-cell (NFAT) transcription factors. Myocardial hypertrophy induced by isoprenaline was also accompanied by enhancement of intracellular calcineurin activity, and inhibition of calcineurin activity was associated with inhibition of cardiac hypertrophy induced by isoprenaline (Zou et al. 2001). These results suggested that ERK1/2 and calcineurin were involved in isoprenaline induced myocardial hypertrophy. Particularly, the cross-talk between calcineurin and ERK1/2 signaling pathways as regulatory mechanisms of cardiac gene regulation and growth has been demonstrated (Sanna et al. 2005), and it has been shown that suppression of calcineurin and activation of ERK1/2 are interacting mechanisms involved in cardioprotection by δ-opioid receptor activation (Ikeda et al. 2006). However,

whether calcineurin and ERK1/2 as well as interaction between protein phosphatases and protein kinases are involved in the mechanism of antihypertrophic effects afforded by κ-opioid receptor stimulation on myocardial hypertrophy induced by β-adrenoceptor stimulation has not been examined.

The purpose of the present study was twofold. Firstly, we tested the hypothesis that calcineurin and ERK1/2 were involved in the inhibition afforded by κ-opioid receptor stimulation to isoprenaline. Secondly, we tested the hypothesis that interaction between calcineurin and ERK1/2 pathways was operative in the mechanism of antihypertrophic effects afforded by κ-opioid receptor stimulation on myocardial hypertrophy. Both the two series of experiments were performed in ventricular myocytes from neonatal rats. In the first, we compared the effects of U50,488H with cyclosporine-A (CsA), the inhibitor of calcineurin, U0126, the inhibitor of ERK1/2 and verapamil, a L-type Ca^{2+} channel antagonist on the hypertrophy and $[Ca^{2+}]_i$ transients induced by isoprenaline. In the second, calcineurin activity and phospho-ERK1/2 level were compared and analyzed using immunoblotting. Hypertrophy was characterized by increases in protein content, [^{3}H]leucine incorporation and cell size, and $[Ca^{2+}]_i$ transients was measured by Till image system by cell-loading Fura-2/AM. Results showed that calcineurin and ERK1/2 and their interaction were involved in the inhibitory effects afforded by κ-opioid receptor stimulation.

2. Investigations and results

2.1. Effects of different agents on isoprenaline-induced enhancement of total protein content, cell size and [^{3}H]leucine incorporation

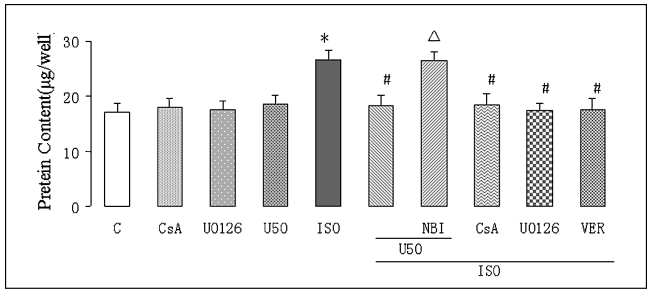
Cyclosporine A (1 μmol·L^{-1}) CsA, 10 μmol·L^{-1} U0126 and 1 μmol·L^{-1} U50,488H alone had no effects on the basal total protein content (Fig. 1A), cell size (Fig. 1B) and [^{3}H]leucine incorporation(Fig. 1C). Isoprenaline (10 μmol·L^{-1}) significantly increased all the three indices in myocytes, and these effects were abolished by 1 μmol·L^{-1} U50,488H, 1 μmol·L^{-1} CsA, 10 μmol·L^{-1} U0126 and 1 μmol·L^{-1} verapamil. The inhibitory effects of U50,488H were abolished by 1 μmol·L^{-1} nor-binaltorphimine.

2.2. Effects of different agents on isoprenaline-induced enhancement of spontaneous $[Ca^{2+}]_i$ transients

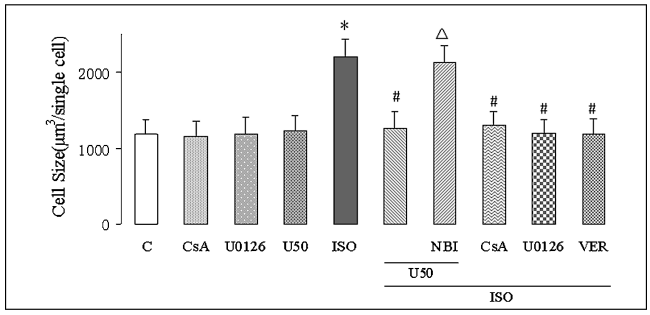
Cyclosporine A (1 μmol·L^{-1}), 10 μmol·L^{-1} U0126 and 1 μmol·L^{-1} U50,488H alone had no effects on the amplitude and frequency of spontaneous $[Ca^{2+}]_i$ transients. 10 μmol·L^{-1} isoprenaline significantly increased both the amplitude (Fig. 2A and B) and frequency (Fig. 2A and C) of spontaneous $[Ca^{2+}]_i$ transients. Both were abolished by 1 μmol·L^{-1} U50,488H, 1 μmol·L^{-1} CsA, 10 μmol·L^{-1} U0126 and 1 μmol·L^{-1} verapamil. None of the treatments had any effects on the resting $[Ca^{2+}]_i$. The effects of U50,488H were abolished by 1 μmol·L^{-1} nor-binaltorphimine.

2.3. Effects of different agents on calcineurin activity and level of phospho-ERK1/2

Isoprenaline increased the calcineurin activity and level of phospho-ERK1/2 by 120.5% (Fig. 3A and C) and 229.4% (Fig. 3B and D) respectively. U50,488H which itself had no effects on basial calcineurin activity and level of phospho-ERK1/2 level abolished the effects of isoprenaline. The inhibitory effects of U50,488H was abolished by nor-binaltorphimine.



(A)



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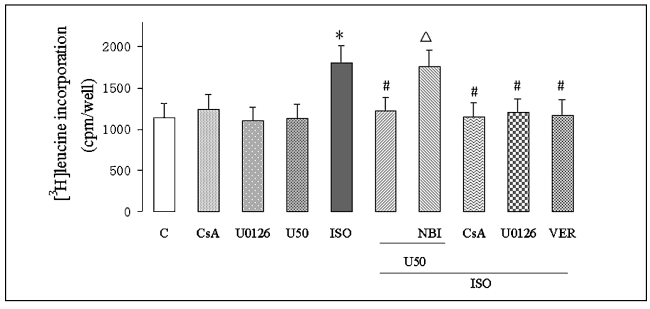

(C)

Fig. 1: Effects of different agents on protein content (A), cell size (B) and [^{3}H]leucine incorporation (C) of the cultured ventricular myocytes from the neonatal rat. Methods and times of cell culture are as described in the methods section. Values are presented as mean ± S.E.M.; n = 6 for protein content and [^{3}H] leucine uptake and n = 80 for cell size. $^*P < 0.05$, compared with control group; $^{\#}P < 0.05$ compared with ISO group; $^{\triangle}P < 0.05$, compared with ISO + U50488H group. ISO: isoprenaline; U50: U50,488H; NBI: nor-binaltorphimine; CsA: cyclosporine-A; VER: verapamil

To examine the interaction between calcineurin and ERK1/2, we also determined the effects of CsA on ERK1/2 phosphorylation and U0126 on calcineurin expression. Though CsA and U0126 alone had no effects on both basal calcineurin expression and basal ERK1/2 phosphorylation, suppression of calcineurin activity by CsA was associated with modest suppression of ERK1/2 phosphorylation. Meanwhile, suppression of ERK1/2 phosphorylation by U0126 was associated with modest suppression of calcineurin activity enhanced by isoprenaline. These date indicated that an interaction exists between calcineurin and ERK1/2.

3. Discussion

Investigations in the past have indicated the involvement of multiple signaling pathways mediated by protein kinases, including AC/cAMP/PKA and CaMKII signaling pathway, in the mechanism of κ-opioid receptor stimulation on β-adrenoceptor stimulation (Yu et al. 1999; Wu et al. 2008). The present study confirmed previous investigations that κ-opioid receptor stimulation with 1 μmol·L^{-1} U50,488H inhibited the enhanced Ca^{2+} transients and hypertrophy (Wu et al. 2008) of the rat heart in response to β-adrenoceptor stimulation. The novel finding is

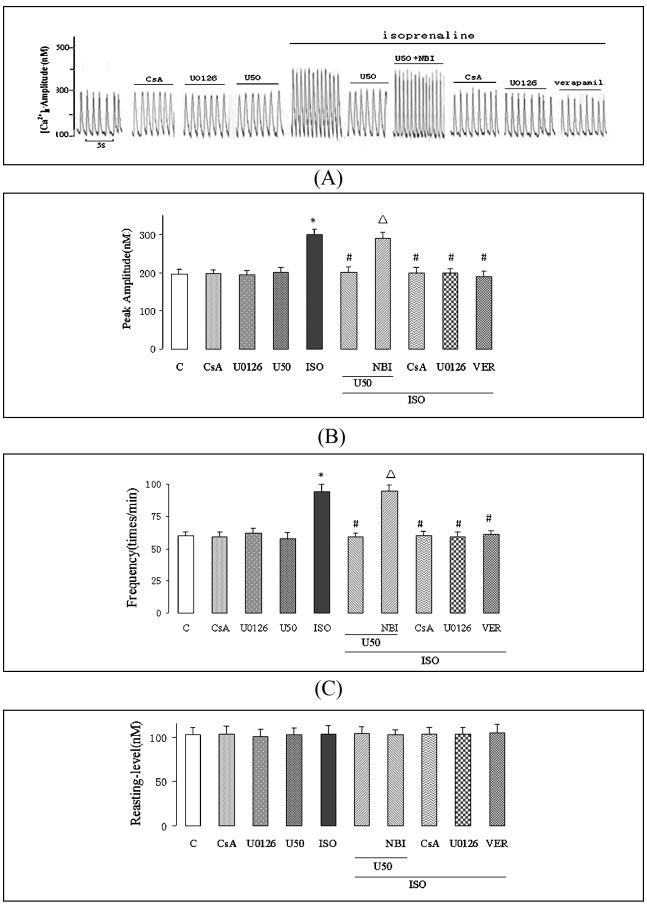

Fig. 2: Effects of different agents on the peak amplitude (B) and frequency (C) of the spontaneous $[Ca^{2+}]_i$ transients and resting Ca^{2+} (D) in the cultured ventricular myocyte from the neonatal rat. (A) shows the representative tracings. Methods for Measurement of cytosolic calcium transient are as described in the Methods section. Values are presented as mean ± S.E.M.; n = 6 in each group. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$ compared with ISO group; $^{\triangle}P < 0.05$, compared with ISO + U50,488H group

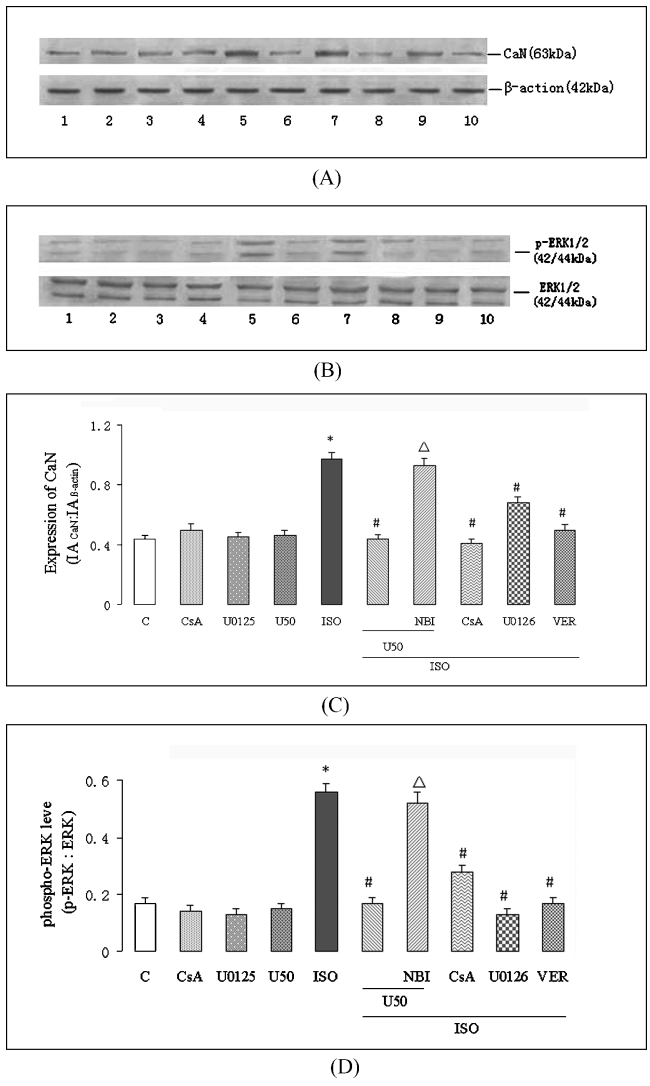

Fig. 3: Effects of different agents on calcineurin expression and level of phospho-ERK1/2 in the cultured myocardial cell from neonatal rat. (A) and (C) expression of calcineurin. (B) and (D) level of phoso-ERK1/2. phospho-ERK level = pERK/ERK. Lane 1: control; lane 2: CsA; lane 3: U0126; lane 4: U50,488H (1 μmol·L^{-1}); lane 5: isoprenaline (10 μmol·L^{-1}); lane 6: isoprenaline (10 μmol·L^{-1}) + U50,488H (1 μmol·L^{-1}); lane 7: ISO (10 μmol·L^{-1}) + NBI (1 μmol·L^{-1}) + U50,488H(1 μmol L^{-1}); lane 8: isoprenaline (10 μmol·L^{-1}) + CsA (1 μmol·L^{-1}); lane 9: isoprenaline (10 μmol·L^{-1}) + U0126 (10 μmol L^{-1}); lane 10: isoprenaline (10 μmol·L^{-1}) + verapamil (1 μmol·L^{-1}); mean ± S.E.M., n = 4 in each group. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$ compared with ISO group; $^{\triangle}P < 0.05$, compared with ISO + U50488H group

that the inhibitory action of U50,488H on Ca^{2+} transients and hypertrophy induced by isoprenaline was reproduced by inhibition of calcineurin and ERK1/2. Therefore, κ-opioid receptor stimulation inhibited the cardiac responses to β1-adrenoceptor stimulation by firstly suppressing the Gs-protein, which in turn suppressed not only the AC/cAMP/PKA and CaMKII pathways, but also the calcineurin and ERK1/2 pathways.

In the present study, significant elevation of calcineurin expression and phospho-ERK1/2 level were detected accompanied with hypertrophy induced by isoprenaline. U50,488H showed the function of suppressing the enhancement of calcineurin activity and level of phospho-ERK1/2 induced by isoprenaline in the absence but not in the presence of nor-binaltorphimine. Furthermore, suppression of calcineurin with CsA and suppression of phospho-ERK1/2 level with U0126 were associated with inhibition of hypertrophy induced by isoprenaline. These results suggest that suppression of enhanced calcineurin activity and phospho-ERK1/2 level induced by isoprenaline is crucial for the inhibiton of hypertrophy afforded by κ-opioid receptor stimulation, and this is the first evidence that calcineurin and ERK1/2 are involved in the antihypertrophic effects of κ-opioid receptor stimulation.

Regarding the molecular mechanisms linking isoprenaline to hypertrophy, strong evidence supports the idea that changes in cytoplasmic $[Ca^{2+}]_i$ plays an essential role in hypertrophic signaling in the heart (Bishopric et al 1992; Nakajima et al. 2009). The present study further demonstrated the previous conclusion and found that not only blockade of the κ-opioid receptor but also inhibition of calcineurin and ERK1/2 abolished the response of Ca^{2+} transients to isoprenaline, suggesting that Ca^{2+} homeostasis played an important role in the inhibitory effects of κ-opioid receptor stimulation to isoprenaline. These were supported by following observations that verapamil, the L-type Ca^{2+} channel blocker, also abolished the hypertrophic response to isoprenaline. Administration of verapamil reduced the enhancement of calcineurin expression and ERK1/2 phosphorylation induced by isoprenaline. The results suggest that κ-opioid receptor stimulation may abolish the hypertrophic response induced by isoprenaline, which is partially *via* attenuating the augment of intracellular Ca^{2+} and then suppressing the expression of calcineurin and ERK1/2 phosphorylation.

It is interesting to find that in the inhibition of κ-opioid receptor stimulation, blockade of the calcineurin with CsA was associated with modest suppression of ERK1/2 phosphorylation. Likewise, blockade of the ERK1/2 with U0126 was associated

with modest suppression of calcineurin activity enhanced by isoprenaline, though CsA and U0126 showed no effects on both the basal calcineurin activity and ERK1/2 phosphorylation. These results suggest that in addition to the involvement of calcineurin and the ERK1/2 pathway, the interaction between the two pathways is also involved in the mechanism of antihypertrophic effects afforded by κ-opioid receptor stimulaion to isoprenaline. It is not really strange that multiple intracellular signaling pathways are necessary for orchestrating hypertrophic response, and that these pathways are interdependent. Mice expressing the activated calcineurin transgene, which are characterized by a robust hypertrophic response, showed constitutive ERK1/2 activation in the heart (De et al. 2000). Isoprenaline-induced activation of ERK1/2 was significantly suppressed by calcineurin inhibitors both *in vitro* and *in vivo* in a model of cardiac hypertrophy (Zou et al. 2001). Antithetically, Ras activation was shown to promote NFATc4 activity in cardiac myocytes through a MEK1–ERK1/2-dependent pathway (Ichida et al. 2001). These studies suggest a reciprocal, yet reinforcing signaling relationship between calcineurin and MEK1–ERK1/2, such that calcineurin activation promotes ERK1/2 activation and Ras–MEK1–ERK1/2 activation enhances NFAT activation through an unknown mechanism. Of course there is an opposite report that activation of ERK1/2 was associated with suppression of calcineurin activity in the cardioprotection afforded by δ-opioid receptor stimulation (Ikeda et al. 2006). Anyway, these various reports provide consistent data suggesting coordination of the cardiac growth response through interconnected regulatory pathways. In our present study, we observed a reciprocal relationship between the calcineurin and ERK1/2, which contributed to our conclusion that inhibitory effects of κ-opioid receptor stimulation on β1-adrenoceptor stimulation may involve calcineurin and ERK1/2, and the two signaling pathways showed interaction at least at the level of proteins in the mechanism of antihypertrophic effects afforded by κ-opioid receptor stimulation.

Though several lines of evidence have suggested the existence of cross-talk between the calcineurin and ERK1/2 pathways, the mechanism of the interaction between calcineurin and ERK1/2 is still unknown at present. In our study we found that not only the administration of verapamil showed the function of reducing the enhanced calcineurin expression and ERK1/2 phosphorylation, but also the administration of CsA and U0126 showed the function of reducing the intracellular Ca^{2+}, suggesting that intracellular Ca^{2+} may be the key nodal point which links the phosphatase and protein kinases. Nevertheless, further investigations are still warranted to delineate the mechanisms of the interation between calcineurin and ERK1/2 pathways.

4. Experimental

The experimental protocols were approved by Committee of Liaoning Medical College for the Use of Experimental Animals for Research and Teaching.

4.1. Culture of neonatal rat ventricular myocytes

Sprague–Dawley rats, 1–3 days old, were killed and their heart was removed. The ventricle was separated from the atrium, trisected, and digested with trypsin (0.6 mg/ml, Sigma) for 20 min at 37 °C. Ventricular myocytes were cultured as described previously (Sheng et al. 1996). The cell supernatant was removed after centrifugation, and the pellet was re-suspended in fetal bovine serum. The above steps were repeated 7–10 times until the cardiac ventricle was completely digested. The cell suspension was diluted to 1×10^6/ml and placed in 24-well tissue culture plates in humidified 5% CO_2/95% air at 37 °C for 48 h. The culture medium consisted of 15% heat-inactivated fetal bovine serum, 84% Dulbecco's Modified Eagle's Medium (DMEM) and 1% penicillin–streptomycin, conditions shown to enhance the growth of cultured ventricular myocytes (Wang et al. 1995). Bromodeoxyuridine (0.1 mmol/l) was added to prevent non-myocyte proliferation without being toxic to myocytes (Wang et al. 2004).

4.2. Treatment protocols

After 48 h incubation, the wells were divided into test groups and the medium was replaced with 0.4% calf serum medium or 0.4% calf serum medium supplemented with different agents. Myocardial cells became "quiescent" in low serum medium and grew without multiplication and/or proliferation (Berk 1989). In experiments involving treatment with U50,488H, isoprenaline, CsA, U0126, verapamil or Fura-2/AM were used. All drugs were initially dissolved in distilled water and subsequently diluted in culture medium, except for CsA, U0126 and Fura-2/AM, which were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO was less than 0.1%, which itself had no effect.

4.3. Cellular protein content determination

Cells were cultured for 72 h in the presence of various compounds (72 h was chosen because preliminary studies showed that the maximum effects were obtained at that time). Dishes were washed rapidly three times with Hanks solution, the cells were dissolved in 1% sodium dodecylsulphate (SDS), and the protein content was measured by Lowry's method (Wu et al. 2008).

4.4. Measurement of cell size

The size of ventricular myocytes was obtained by measuring cell diameter (Zheng et al. 1996). The medium was aspirated and cells were washed rapidly three times with D-Hanks solution. Cells were then treated with 0.3 ml of 0.1% trypsin per well at 37 °C for 10 min and the process was terminated with 10% fetal bovine serum (0.2 ml/well). Digested cells were collected and measured on an inverted microscope. For measurements, four or five fields were randomly chosen from 16 or 20 field in the direction of random table and photographed at high power (×400), and 80 individual cell areas were calculated by CIAS Daheng computer photograph analysis system.

4.5. Incorporation of [^{3}H]Leucine

[^{3}H]Leucine uptake, was used as an index of protein synthesis. The medium from myocardial cells grown in 24-well plates was aspirated and replaced with a medium contaning 1 Ci [^{3}H] leucine. Drugs were added and incubation continued for 48 h. The medium was then aspirated and cells were washed rapidly three times with cold Hanks solution. They were then lysed by addition of 1 ml per well 1% SDS. Lysates were collected and precipitated by addition of 1 ml 5% trichloroacetic acid and then applied to fiberglass GF/C filters. After washing three times with 5 ml Hanks solution, filters were dried and transferred to vials containing 4 ml scintillation fluid and the radioactivity was determined by liquid scintillation counting (Luo et al. 2001). The radioactivity, which represented the [^{3}H]leucine incorporated into newly synthesized protein, was expressed as cpm per 10^5 cells.

4.6. Loading of cells with Fura-2/AM

Myocytes were cultured in wells, each with a coverslip. The coverslips with myocytes were incubated with Fura-2/AM (4 μmol/l) in the medium for 25 min. The unincorporated dye was removed by washing twice with fresh medium. To allow the Fura-2/AM in the cytosol to de-esterify, the loaded cells were maintained at room temperature (24–26 °C) for 60 min before measurement of $[Ca^{2+}]_i$.

4.7. Measurement of cytosolic calcium transient

A spectrofluorometric method was used to measure the cytosolic Ca^{2+} transient, using Fura-2/AM as the Ca^{2+} indicator. After loading with Fura-2/AM, the coverslips with myocytes were transferred to a superfusion chamber on the stage of an inverted microscope, which was coupled to a TILL imaging system (Germany), and superfused with a Hanks buffer. The emitted light was filtered at 510 nm. Fluorescence signals at 340 nm (F340) and 380 nm (F380) were recorded in a personal computer for data processing and analysis. Maximal fluorescence for each coverslip was obtained after the addition of the Ca^{2+} ionophore ionomycin (20 μmol/l). Ethylene glycol tetraacetic acid (EGTA) was added to a final concentration of 20 mmol/l for the Ca^{2+}-free condition. Cytosolic $[Ca^{2+}]$ was calculated by the following formula: $[Ca^{2+}]_i = Kd \cdot (Sf2/Sb2) \cdot (R340/380 - R_{min})/(R_{max} - R340/380)$ (Grymkiewicz et al. 1985). Kd is the dissociation constant of Fura-2/AM for Ca^{2+} and was assumed to be 225 nmol/l at 37 °C. R340/380 is the ratio of corrected fluorescence signals. R_{max} is the ratio obtained after ionomycin treatment. R_{min} is the ratio of the corrected signals obtained after EGTA treatment. Sf2 and Sb2 represent the emission intensities at 380 nm excitation at saturation and under Ca^{2+}-free conditions, respectively.

4.8. Western blotting

Cells were washed once with ice-cold PBS containing 100 μmol/l sodium orthovanadate and solubilized in the lysis buffer (50 mmol/l Tris-HCl, 137 mmol/l NaCl, 10% glycerol, 100 μmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Nonident P-40; pH 7.4). After centrifugation at 12,000 g for 20 min, the supernatant was collected. Cells were dissolved in buffer containing 65 mmol/l Tris-HCl (pH 6.8), 3% SDS, 10% glycerol, and 6 mol urea. After measurement of protein concentration (BCA kit, Pierce, Rockford, IL), β-mercaptoethanol and bromophenol blue were added to the buffer for electrophoresis. Sixty micrograms of protein thus obtained was separated on 10% SDS-PAGE and transblotted to polyvinylidene difluoride membranes (BioRad, Hercules, CA). The blots were incubated at 4 °C overnight with antibodies and the resulting bands detected using enhanced chemiluminescence. Antibodies to ERK1/2 phosphorylated at Tyr-204 (1:2000 dilution; Santa Cruz) were used to detect the activated form of the kinase. Antibodies to ERK1/2 (1:2000 dilution; Santa Cruz) were used to detect the corresponding total protein level. Antibodies to calcineurin (1:2000 dilution; Santa Cruz) were used to detect the activated form of the phosphatase. Intensities in the resulting bands were quantified using CAMIAS008 image analysis system.
phospho-ERK level = pERK/ERK

4.9. Statistical analysis

All data are expressed as mean ± SEM. For the effects of drugs at different concentrations, analysis of variance (one-way ANOVA) was used to compare control and treatment groups. The post LSD test was used to evaluate differences between two groups. $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank Prof IC Bruce for advice particularly on the use of English, ZM Qi, XL Xu and ZH Zong for their expert technical assistance. This work was supported by the National Natural Science Foundation (30973898/C190702).

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