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Expression of Urocortin 2 and its Inhibitory Effects on Intracellular Ca^{2+} Via L-Type Voltage-Gated Calcium Channels in Rat Pheochromocytoma (PC12) Cells

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Urocortin 2, a new member of the corticotrophin-releasing factor (CRF) neuropeptide family, was reported to be widely expressed in the central nervous system and peripheral tissues. Here, we detected urocortin 2 mRNA in PC12 cells using reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, we observed its effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using confocal microscopy and flow cytometry and on voltage-gated calcium channel (VGCC) currents using whole-cell patch clamp. Our results showed that urocortin 2 mRNA was coexpressed with CRF, and CRF receptor (CRFR) 2β in undifferentiated PC12 cells, but not CRFR1 or CRFR2α. KCl (40 mM) or Bay K8644 (1 μM), an L-type VGCC activator, increased $[Ca^{2+}]_i$. Pretreatment of the cells with urocortin 2 significantly diminished the effect of Bay K8644 or KCl. Urocortin 2 showed no influence on $[Ca^{2+}]_i$ in tyrode's solution containing EGTA or Ca^{2+}-free tyrode's solution. It reversibly inhibited the VGCC currents in a concentration-dependent manner, but had no apparent effects on the cells treated with nifedipine (1 μM), an L-type VGCC blocker. Urocortin 2 up-shifted the current–voltage curves. No frequency-dependence of urocortin 2 effects on I_{Ba} was observed. The inhibitory effects of urocortin 2 on VGCC currents or $[Ca^{2+}]_i$ were not affected by astressin 2B, an antagonist of CRFR2. As calcium overload play a key role in some neuronal degenerative diseases such as Alzheimer's and Parkinson's diseases, our results suggest that urocortin 2 may be a potentially interesting agent for the treatment of these diseases.

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Keywords: urocortin 2; PC12 cells; voltage-gated calcium channel (VGCC); whole-cell patch clamp; intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

INTRODUCTION

Corticotropin-releasing factor (CRF), a 41-amino-acid peptide, belongs to a family of structurally related peptides that includes fish urotensin 1, amphibian sauvagine, and the recently identified urotensin homolog discovered in mammals, urocortins, which includes urocortin 1, urocortin 2 (a homolog of human stresscopin-related peptide), and urocortin 3 (a homolog of human stresscopin) (Reul and Holsboer, 2002; Suda *et al*, 2004). Urotensin 1, sauvagine, and urocortin 1–3 bind with up to 1000-fold higher affinities to the CRFR2 than species homologues of CRF (Lewis *et al*, 2001; Risbrough *et al*, 2004). Urocortin 2 and urocortin 3 are generally considered to represent endogenous ligands for mammalian CRFR2 variants, whereas urocortin 1 is thought to be an endogenous ligand for both CRFR1 and CRFR2 (Valdez *et al*, 2002). Urocortin 2 was reported to be expressed extensively in the brain, paraventricular hypothalamic nucleus, locus coeruleus, spinal cord, heart, adrenal gland, and peripheral blood cells (Reul and Holsboer, 2002; Takahashi *et al*, 2004; Brar *et al*, 2004; Venihaki *et al*, 2004; Chen *et al*, 2004; Nemoto *et al*, 2005). Previous reports showed that urocortin 2 had a number of physiological properties, such as suppression of appetite (Hsu and Hsueh, 2001), inhibition of inflammatory processes (Tsatsanis *et al*, 2005), etc., by interacting with its CRFR2. However, urocortin 2 could also cause neural activation in cell groups that are involved in autonomic activation, but do not express either type of CRFR (Parkes *et al*, 2001). It has also been demonstrated that urocortin 2 protected neonatal cardiac myocytes *in vitro* when administered before hypoxia/ischemia (Ikeda *et al*, 2005), which

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suggests that calcium channels may play some roles since ischemia and hypoxia damage is highly associated with Ca^{2+}-overload. Furthermore, it was reported that urocortin 2 played the cardiovascular protective role via protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathway, which might lead to changes in the intracellular Ca^{2+} (Kageyama *et al*, 2003).

Intracellular calcium plays a key role in gene expression, neuronal migration, neurotransmitter release (Catterall, 1998), and is involved in the developing of some neuronal degenerative diseases such as Alzheimer's and Parkinson's diseases, which are highly associated with calcium overload (Verkhratsky and Toescu, 2003). Voltage-gated calcium channels (VGCC), including L-, N-, P/Q-, T- and R-type channels have been reported to exist in the central nervous system (CNS) (Catterall, 1995, 1998; Perez-Reyes, 2003). Although L-type VGCC are not the primary channel type in CNS, it was reported that L-type VGCC also play a role in hormone release in endocrine cells (Milani *et al*, 1990) and in gene expression in neurons (Bean, 1989). Most recently, it has been found that a dysregulation of intraspine Cav1.3 L-type VGCC in stratopallidal medium spiny neurons (MSN) leads to a loss of spines and glutamatergic synapses in these cells and is likely a key step in the pathogenesis of Parkinson's diseases (Day *et al*, 2006). Also, previous studies showed that neurodegenerative pathogenesis was inhibited by calcium channel blockade (Stefani *et al*, 1998), which indicates that inhibition of calcium channels and then reduction in intracellular Ca^{2+} could be responsible for neuroprotection.

Taken together, there is a strong hint that urocortin 2 might have an influence on intracellular calcium and hence exert its neuroprotective effects. Data on urocortin 2's effects on intracellular calcium in neurons would be a convincing way to clarify the mechanism of urocortin 2's neuroprotective effects. This study was designed to observe the effects of urocortin 2 on intracellular Ca^{2+} levels and VGCC currents in rat undifferentiated PC12 cells. Effects of urocortin 2 on VGCC currents in acutely dissociated rat cortex neurons were also examined.

MATERIALS AND METHODS

These studies were carried out in agreement with the official recommendations of the Chinese Community Guidelines.

Reagents and Solutions

Urocortin 2, astressin 2B, Bay K8644, CsCl, ATP–Mg, omega-agatoxin IVA, omega-conotoxin GVIA, antalarmin, and HEPES were purchased from SIGMA (USA). Undifferentiated rat pheochromocytoma (PC12) cells were obtained from CLONTECH (USA). Foetus bovine blood serum was from HYCLONE. DMEM was purchased from GIBCO. Incubation solution (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose, aerated with 5% CO_2–95% O_2 to a final pH of 7.4. The external solution was composed of (in mM): 20 $BaCl_2$, 125 Tetraethylammonium (TEA), 10 HEPES, 10 glucose, (pH 7.3, adjusted with CsOH). TTX (0.5 μM) was used in the bath solution. The pipette solution contained (in mM): 120 CsCl, 2 $MgCl_2$, 10 CsF, 11 EGTA, 15 HEPES, 4 Mg–ATP, 10 glucose (pH 7.3, adjusted with CsOH).

Cell Culture

Rat undifferentiated pheochromocytoma (PC12) cells were grown in DMEM 13.4 g/l, $NaHCO_3$ 3.7 g/l, 10% foetus bovine blood serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. To measure the membrane currents, the cells from the stock culture were plated onto glass coverslips, and used for experiments 2–3 days after plating.

Acute Isolation of Cerebral Cortex Neurons

Single cerebral cortex neurons were acutely dissociated according to procedures reported previously (Munakata and Akaike, 1993). In brief, pentobarbital-anesthetized Sprague–Dawley rats of both sexes (8- to 20-day-old) were decapitated, the brains were removed, and the area 2–4 mm from the anterior tip of the frontal lobe was cut into coronal slices (400 μm thick) in a cutting solution using a WPI oscillating tissue slicer (Campden Instruments, London, UK). The brain slices were kept in an incubation solution saturated with 5% CO_2–95% O_2 at room temperature for 60 min. The enzyme treatment consisting of incubation in 0.5% pronase (Calbiochem, San Diego, CA, USA) for 20–25 min at 31°C. The brain slices were stored in an enzyme-free incubation solution. The frontal lobe cortex was micropunched and mechanically dissociated with fire-polished Pasteur micropipettes in a small plastic culture dish (diameter = 35 mm, Falcon, Lincoln Park, NJ, USA) filled with normal external solution. The dissociated neurons adhered to the bottom of the dish within 30 min. The experiments were performed within 12 h after animals were killed.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from PC12 cells with RNeasy mini kit (QIAGEN, USA) using the method provided by the manufacturer. Total RNA was denatured at 65°C for 5 min and then reverse transcribed at 37°C for 60 min in a total volume of 20 μl reaction buffer (Life Technologies Inc., Grand Island, NY) containing 0.5 μg oligo-deoxythymidine (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 nmol deoxy-NTPs, and 200 U SuperScript™ II reverse transcriptase. The reaction mixture was then heated to 95°C for 5 min and immediately chilled on ice. Subsequently, the synthesized complementary DNA (cDNA) products (4 μl) were subjected to polymerase chain reaction (PCR) in a reaction mixture (20 μl) containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 4 nmol deoxy-NTPs, 5 pmol of each primer, respectively, and 0.5 U Taq DNA polymerase (Promega, WI, USA). The sequences of the primers employed in this study are summarized in Table 1. Brain and left cardiac atrium were used as positive controls for the expression of CRFR1 and CRFR2β, respectively. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined as an internal control. After preheating at 96°C for 2 min, denaturation, annealing, and elongation were carried out at 96°C for 15 s, at 64°C

Table 1 Primers Used for RT-PCR Analysis of Urocortin 2, CRF, CRFR1, CRFR2α, and CRFR2β

Gene	Primers for RT-PCR	Predicted size (bp)	Accession number (GenBank)
Urocortin 2	5′-CCGGATCCATGACCAGGTGG-3′	401	AY044835
	5′-CCGAATTCCTCCAGAACTTC-3′		
CRF	5′-TGATCCGCATGGGTGAAGAATAC-3′	394	NM031019
	5′-CCCGATAATCTCCATCAGTTTC-3′		
CRFR1	5′-TCCACTACATCTGAGACCATTC-3′	248	NM030999
	5′-TCCTGCCACCGGCGCCACCTCT-3′		
CRFR2α	5′-AACTGCAGCCTGGCACTG-3′	104	U16253
	5′-ATCTGGTCCAAGGTCGTG-3′		
CRFR2β	5′-CTCTCTTCCCAGTGCACA-3′	186	NM022714
	5′-ATCTGGTCCAAGGTCGTG-3′		
GAPDH	5′-AAGGTCGGAGTCAACGGA-3′	346	AF106860
	5′-AAGGTGGAGGAGTGGGTG-3′		

(CRFR1, R2α, and R2β) or 60°C (GAPDH) for 30 s, and at 72°C for 1 min, respectively. PCR was carried out for 35 cycles (CRFR1, R2α, R2β), or 22 cycles (GAPDH), respectively. Amplification products were subjected to electrophoresis in a 1% polyacrylamide gel, stained with ethidium bromide, and viewed on a UV box. Negative controls contained all reagents, except that 4 μl H_2O was substituted for reverse transcriptase in the RT reaction product. PCR analysis was repeated at least twice with the same samples to confirm reproducibility of the results.

Measurements of $[Ca^{2+}]_i$ by Confocal Laser Scanning Microscope

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with the ratiometric fluorescent dye Fluo-3/AM utilizing an LSM 510 invert confocal laser scanning microscope (Carl Zeiss Jena, Germany) with an argonkrypton laser (488 nm) excitation source. PC12 cells were seeded on a sterile glass coverslip at an appropriate density to allow imaging of 10–20 single cells. These attached cells were loaded with Fluo-3/AM (2 mM) for 40 min in a dark place at room temperature. The dye-loaded cells were gently washed three times with a medium containing (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 1 $CaCl_2$, 0.5 NaH_2PO_4, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). The cells were kept in medium for a further 20 min to allow the hydrolysis of Fluo-3/AM into Ca^{2+}-sensitive free acid form (Fluo-3) by cell esterases. The coverslip with attached cells was then transferred to a 1-ml thermoregulated chamber (22°C) on the stage of a ZEISS LSM 510 inverted microscope. The excitation wavelength was selected by a dichroitic mirror (FT510). The fluorescence intensities were detected at wavelengths greater than 515 nm using an additional cutoff filter (LP515) in front of the detector. Fluorescence images were scanned and stored as a time series. Cells were pre-exposed to urocortin 2 for 48 h. Bay K8644 (1 μM) or KCl (40 mM) was applied to the cells 1 min after scanning began. Regions of interest (ROI's) were subsequently selected for determination of the fluorescence intensities in the cytosol and the nucleus. These data were stored as ASCII files and computed off-line. Different solutions were used as the extracellular medium (in mM): 140 NaCl, 4.6 KCl, 2 $CaCl_2$, 10 glucose: These media and active compounds offered various possibilities of investigating changes in the intracellular Ca^{2+} signal.

Whole-Cell Patch Clamp Recording

PC12 cells were placed in a 0.5 ml chamber mounted on an inverted microscope (IX70, Olympus). Cells were superfused at room temperature (19–23°C). Complete replacement of external solution 2 ml/min in the chamber was achieved within 2–3 min. The currents were recorded by a patch clamp amplifier (Axon 200B Inc.), Digidata 1322A, and p-Clamp software 8.2 (Axon Instrument, Foster City, CA, USA). Borosilicate glass electrodes were pulled using a level puller (Sutter Instruments, Model P-97) and had a resistance of 2–3 MΩ when filled with the electrode internal solution. Cell capacitance was measured by integrating the area of the capacitive transient. Data acquisition, storage, and analysis running on a personal computer were accomplished with p-Clamp 8.2 software (Axon Instrument). Urocortin 2 (0.01–5 μM), nifedipine (1 μM), omega-conotoxin GVIA (1 μM), and omega-agatoxin IVA (1 μM) were used in the external solution, respectively. Voltage-gated barium currents were evoked by a 200 ms-long depolarizing step pulse from the holding potential of −90 to 10 mV. Current–voltage (*I–V*) relationship was obtained by applying pulses for 200 ms from −60 to 60 mV in 10 mV increments. The current density of was calculated by normalizing the peak amplitude of current with cell capacitance (pA/pF). To study the frequency-dependent effect of urocortin 2, Currents were elicited by a train of 20 depolarizing voltage step (200 ms in duration) from a holding potential of −90 mV to a test potential of 10 mV delivered at frequency of either 1 or 3 Hz.

Determination of $[Ca^{2+}]_i$ by Flow Cytometry

All experiments were performed on a Coulter Elite flow cytometer equipped with an air-cooled 488 argon laser and a helium-cadmium UV laser. At least 10 000 cells were collected for each sample, and data were analyzed using the

Coulter software package and MultiTime (Phoenix Flow). Cells, detached with trypsin/EDTA and suspended in DMEM, were loaded for 30 min at room temperature with urocortin 2 (0.1 μM), astression 2B (1 μM), EGTA (3 mM) in normal Tyrode's solution, or ryanodine (1 nM) in Ca^{2+}-free Tyrode's solution. Ca^{2+}-free Tyrode's solution contained (mM): 135 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 0.33 NaH_2PO_4, 5 glucose, and 10 HEPES, pH value was adjusted to 7.4 with NaOH. Cells were then washed three times and resuspended in PBS solution. Experiments started 30 min later to ensure adequate loading of the dye, Fluo-3. Cells were analyzed on a flow cytometer with excitation at 325 nm and emission at 405 and 525 nm corresponding to the peak emission of the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. The 405/525 ratio was used as an index of $[Ca^{2+}]_i$. Cells with fluorescence <50 were excluded. No auto-fluorescence of unloaded cells was detected in any of the experimental conditions. Neither of the drugs (urocortin 2 and Bay K 8644) used affected the 405/525 ratio measured in the cells loaded with Fluo-3 during control conditions.

Data Analysis

Data were expressed as means $\pm$ standard error of the mean (SEM). The changes of $[Ca^{2+}]_i$ and barium currents induced by different concentrations of urocortin 2 *vs* control were compared statistically by an analysis of variance (ANOVA) followed by Student–Newman–Keuls (S–N–K) *post hoc* test. To estimate the frequency-dependent effect of urocortin 2, the analysis of covariance and the group *t*-test were used. The threshold of significance retained was $P<0.05$ or <0.01.

RESULTS

Expression of Urocortin 2 and CRF Receptors in PC12 Cells

RT-PCR analysis demonstrated that urocortin 2, CRF, and CRFR2β mRNA (specific predicted size of transcripts 401 and 186 bp, respectively) were coexpressed in PC12 cells (Figure 1a). However, neither CRFR1 nor CRFR2α mRNA could be detected in the cells (Figure 1b). The mRNA for CRFR1 and CRFR2α were detected in the positive controls. PCR products were further analyzed by direct sequencing and confirmed to be identical to the registered cDNA sequences for urocortin 2, CRF, CRFR1, CRFR2α, and CRFR2β (data not shown). GAPDH mRNA was similarly expressed in all samples. The negative control showed no band.

Reduction of $[Ca^{2+}]_i$ by Urocortin 2 in PC12 Cells

Bay K8644 (1 μM), an L-type VGCC activator, can increase the intracellular Ca^{2+} levels. When the cells were pre-exposed to nifedipine (1 μM), an L-type VGCC blocker, this effect of Bay K8644 was remarkably diminished, which confirmed that the Ca^{2+} influx took place through voltage-activated calcium channels (Figure 2a, upper panel). Pre-exposure of the cells to urocortin 2 at the concentration of 0.1, 1, and 10 μM, respectively, reduced the intracellular Ca^{2+} levels induced by Bay K8644 (Figure 2a, lower panel). Urocortin 2 could also attenuate the increase in Ca^{2+} concentration induced by KCl (40 mM) (Figure 2b). The inhibitory effects of urocortin 2 on intracellular Ca^{2+} increase induced by Bay K8644 or KCl were clearly shown to be statistically significant.

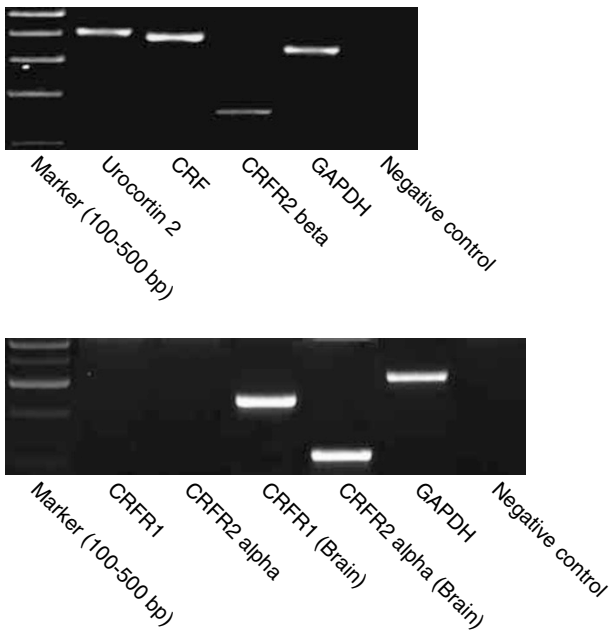

Figure 1 Expression of urocortin 2 and CRF receptors in PC12 cells. (a) Expression of urocortin 2, CRF, and CRFR2β in PC12 cells. (b) The positive control indicates mRNA from rat brain following RT. Negative control indicates mRNA without prior RT.

Concentration-Dependent Inhibitory Effects of Urocortin 2 on I_{Ba}

A series of evoked current traces were recorded when a PC12 cell was given designed depolarizing test pulses (Figure 3a). The current could be completely blocked by 200 nM $CdCl_2$ ($n=5$, Figure 3b), a nonselective calcium channel blocker.

After exposure of the cells to urocortin 2 at higher than 0.01 μM, the current was significantly reduced with a maximum inhibitory effect at 5 μM. Urocortin 2 at 0.01, 0.1, 0.5, 1, 5 μM markedly inhibited I_{Ba} by $6.7\pm1.7\%$ ($n=6$, $P>0.05$), $21.6\pm2.9\%$ ($n=6$, $P<0.05$), $23.4\pm3.8\%$ ($n=6$, $P<0.05$), $32.7\pm6.1\%$ ($n=6$, $P<0.01$), $45.6\pm3.9\%$ ($n=6$, $P<0.01$), respectively (Figure 3c). After washout, the peak of I_{Ba} partially returned (Figure 3d). In the presence of the CRFR2 antagonist, astressin 2B (1 μM), urocortin 2 (0.1 μM) inhibited I_{Ba} at 10th minute by about 22%, from 10.2 ± 3.7 to 8.6 ± 1.9 pA/pF ($n=6$, $P<0.05$) (Figure 3e), which showed no difference from the inhibition in the absence of astressin 2B. After pretreatment of PC12 cells with nonpeptide CRFR1 antagonist, antalarmin (1 μM), urocortin 2 (0.1 μM) inhibited I_{Ba} by 22.6% at the 10th minnute, from 9.8 ± 1.3 to 7.4 ± 1.7 pA/pF ($n=6$, $P<0.05$) (Figure 3f), which also showed no difference from the inhibition in the absence of antalarmin.

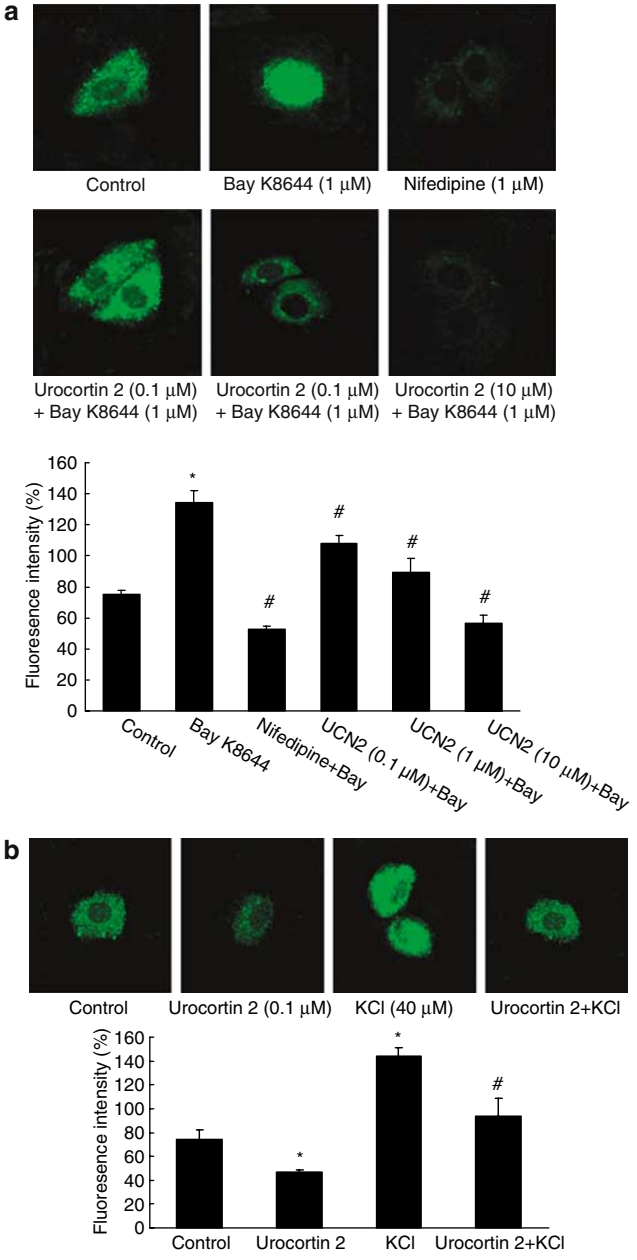

Figure 2 Effects of urocortin 2 on $[Ca^{2+}]_i$ in PC12 cells. (a) In the presence of Bay K8644 (1 μM), nifedipine (1 μM), and urocortin 2 (0.1–10 μM). $^*P<0.05$ *vs* control, $^{\#}P<0.05$ *vs* Bay K8644, $n=6$. (b) In the presence of KCl (40 mM) and urocortin 2 (0.1 μM). $^*P<0.05$ *vs* control, $^{\#}P<0.05$ *vs* KCl, $n=6$.

Effects of Nifedipine, Omega-Conotoxin GVIA, and Omega-Agatoxin IVA on Urocortin 2's Inhibition

It is well known that undifferentiated PC12 cells coexpress multiple VGCC including L-, N-, and P/Q-type calcium channels. To further distinguish which type of calcium channel was inhibited by urocortin 2, the effects of 0.1 μM urocortin 2 on whole-cell calcium currents in undifferentiated PC12 cells were observed in the presence of 1 μM nifedipine, a dihydropyridine calcium channel blocker. If urocortin 2 could specifically block L-type calcium channels, there should be no significant inhibition in the presence of nifedipine. Figure 4 summarized the results of this experiment. Urocortin 2 (0.1 μM) had no apparent effects on cells treated with 1 μM nifedipine (Figure 4b). This result suggests that urocortin 2 may be specific for L-type calcium channels. After pretreatment of the cells with omega-conotoxin GVIA (1 μM) (Figure 4c), an N-type calcium channel blocker, or omega-agatoxin IVA (1 μM) (Figure 4d), a P/Q-type calcium channel blocker, urocortin 2 inhibited the calcium currents by about 36.8 and 28.8% respectively.

Effects of Urocortin 2 on Electrophysiological Properties of I_{Ba}

As demonstrated in Figure 5a, urocortin 2 significantly up-shifted the I-V curve concentration-dependently, but did not alter eletcrophysiological properties of I_{Ba}. The current density at 20 mV was declined from 10.7 ± 2.2 to 7.6 ± 1.4 pA/pF ($n=6$, $P<0.05$) and 5.1 ± 0.8 pA/pF ($n=6$, $P<0.05$) in the presence of urocortin 2 at 0.1 and 1 μM, respectively. Figure 5b shows the time–course effects of urocortin 2 on I_{Ba} in PC12 cells. When the cells were held at −90 mV, and given a 200 ms, 1 Hz depolarizing pulse, I_{Ba} reached its maximum. After exposure of the cells to urocortin 2 at 0.01, 0.1, and 1 μM for 10 min, the currents were reduced by about 4, 21, and 32%, respectively, at the same time point ($n=6$). Frequency-dependency was tested by change of the stimulating frequency. I_{Ba} was elicited by a train of 20 depolarizing voltage steps from −90 mV (holding) to 10 mV with 200 ms duration at a frequency of either 1 or 3 Hz. After exposure of the cells to the urocortin 2 (0.1 μM) within 10 min, I_{Ba} was reduced to a similar extent at 1 or 3 Hz, which appeared to be hardly frequency-dependent (Figure 5c).

Determination of $[Ca^{2+}]_i$ by Flow Cytometry

To calibrate the system, blank (buffer without the Fluo-3) and control (buffer with Fluo-3) were measured. As shown in Figure 6, Urocortin 2 (0.1 μM) could reduce $[Ca^{2+}]_i$ in normal Tyrode's solution (Figure 6a, upper panel), but had no influence on $[Ca^{2+}]_i$ in normal Tyrode's solution containing EGTA (3 mM) or Ca^{2+}-free Tyrode's solution (Figure 6a, lower panel), which showed that urocortin 2 might inhibit $[Ca^{2+}]_i$ not via internal calcium store. After pretreatment of the cells with 1.0 nM ryanodine, the fluorescence intensity of PC12 cells was remarkably increased. Urocortin 2 (0.1 μM) had no significant influence on this increase induced by ryanodine (Figure 6b).

Effects of Urocortin 2 on I_{Ba} in Single Cerebral Cortex Neurons in Rat

Although undifferentiated PC12 cells are considered as a useful model for neuronal-like L-type VGCC investigation (Pummer *et al*, 1989), we still examined the effect of urocortin 2 on I_{Ba} in acutely dissociated rat cerebral cortex neurons for more precisely evaluating its neuroprotective potential. As shown in Figure 7a, I_{Ba} were evoked by a 200 ms-long depolarizing step pulse from the holding potential of −90 to 10 mV. I_{Ba} were obtained in the absence or presence of urocortin 2. Urocortin 2 at 0.1 μM markedly

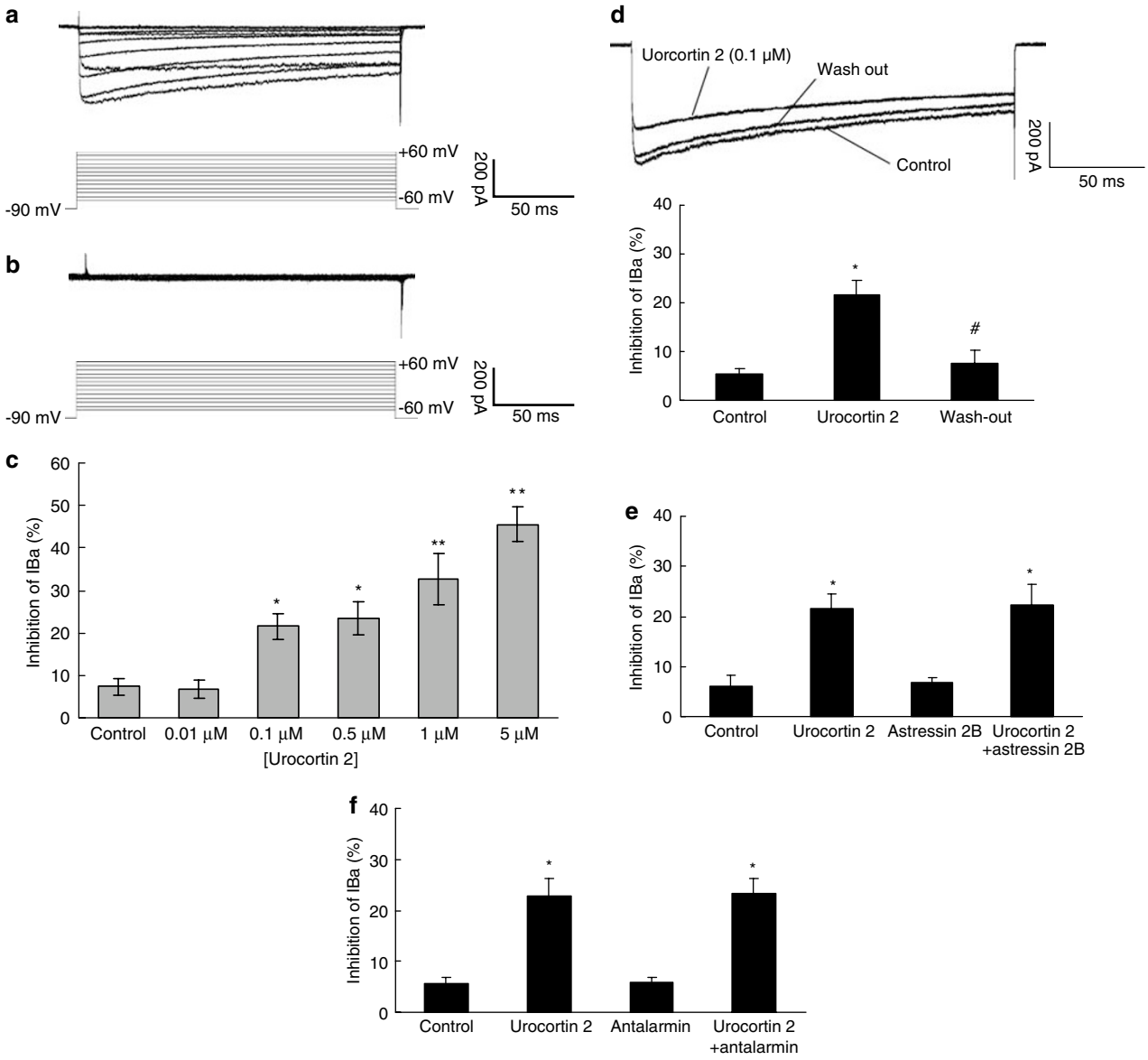

Figure 3 Effects of urocortin 2 on I_{Ba} in PC12 cells. (a) Original records of the currents evoked by a series of depolarizing pulses from a holding potential of −90 mV to test potentials between −60 and + 60 mV, in 10-mV increments. (b) Effects of $CdCl_2$ (200 nM) on the current. (c) Concentration-dependent effects of urocortin 2 on I_{Ba} (*$P<0.05$ *vs* control, **$P<0.01$ *vs* control, $n=6$). (d) Current traces of I_{Ba} obtained in the absence, at presence of urocortin 2 at 0.1 μM, and washout ($n=6$). (e) Effect of urocortin 2 (0.1 μM) and urocortin 2 (0.1 μM) + astressin 2B (1 μM) on I_{Ba} in PC12 cells (*$P<0.05$ *vs* control, $n=6$). (f) Effect of urocortin 2 (0.1 μM) and urocortin 2 (0.1 μM) + antalarmin (1 μM) on I_{Ba} in PC12 cells (*$P<0.05$ *vs* control, $n=6$).

inhibited I_{Ba} at 10th minute by 28.3%, from 8.6 ± 0.7 to 6.2 ± 1.5 pA/pF ($P<0.05$, $n=6$) (Figure 7b).

DISCUSSION

As mentioned above, urocortin 2 possesses a wide spectrum of physiological and pharmacological actions (Brar *et al*, 2004; Takahashi *et al*, 2004; Venihaki *et al*, 2004; Nemoto *et al*, 2005) including its extensively neuroprotective effects (Valdez *et al*, 2002; Bayatti *et al*, 2003). However, the mechanisms of this protection remain to be unknown. Here, we firstly report that urocortin 2 reduces the intracellular Ca^{2+} levels via inhibiting L-type VGCC in undifferentiated PC12 cells, where both urocortin 2 and CRFR2β, but not CRFR1 or CRFR2α, are expressed.

Intracellular calcium played very important roles in the developing of brain ischemia and neuronal degenerative diseases such as Alzheimer's and Parkinson's disease, which are highly associated with intracellular Ca^{2+} overload (Kostyuk and Verkhratsky, 1994). Several notes have been proposed to explain the drop in $[Ca^{2+}]_i$, such as the inhibition of the voltage-gated and receptor-operated Ca^{2+} channels, or the reduction of Ca^{2+} release from the endoplasmic reticulum (ER) (Verkhratsky and Toescu, 2003). In the present study, urocortin 2 inhibited the intracellular Ca^{2+} increase induced by Bay K8644 or KCl, which was not affected by asstressin 2B, the CRFR2 blocker.

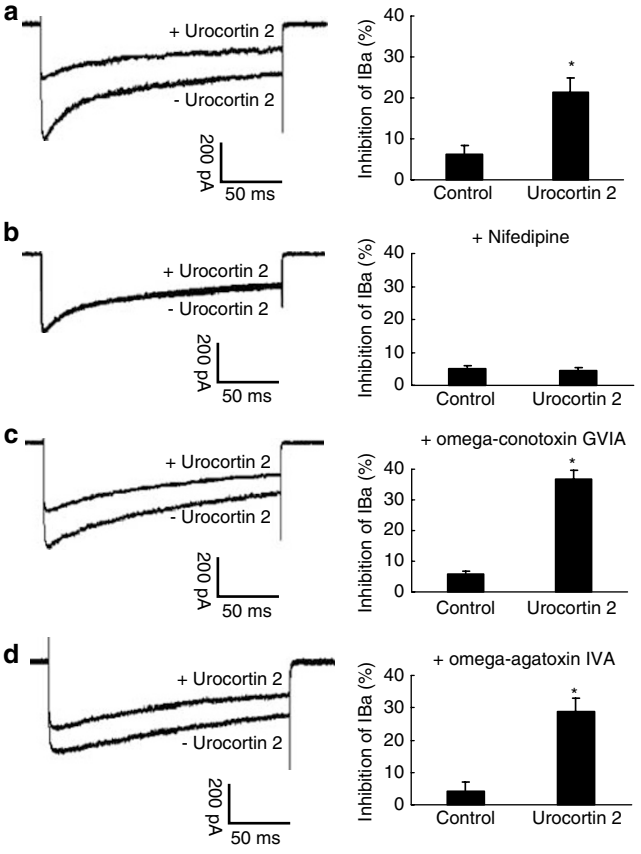

Figure 4 Effects of nifedipine, omega-conotoxin GVIA, and omega-agatoxin IVA on the whole-cell calcium currents in undifferentiated PC12 cells. I_{Ba} were evoked by a 200 ms-long depolarizing step pulse from the holding potential of −90 to 10 mV. (a) Data traces showing the effects of 0.1 μM urocortin 2 on I_{Ba} (*$P<0.05$ *vs* control, $n=6$). (b) Data showing the effects of 0.1 μM urocortin 2 on I_{Ba} with 1 μM nifedipine in the bath solution. (c) Data showing the effects of 0.1 μM urocortin 2 on I_{Ba} with omega-conotoxin GVIA (1 μM) in the bath solution (*$P<0.05$ *vs* control, $n=6$). (d) Data showing the effects of 0.1 μM urocortin 2 on I_{Ba} with omega-agatoxin IVA (1 μM) in the bath solution (*$P<0.05$ *vs* control, $n=6$).

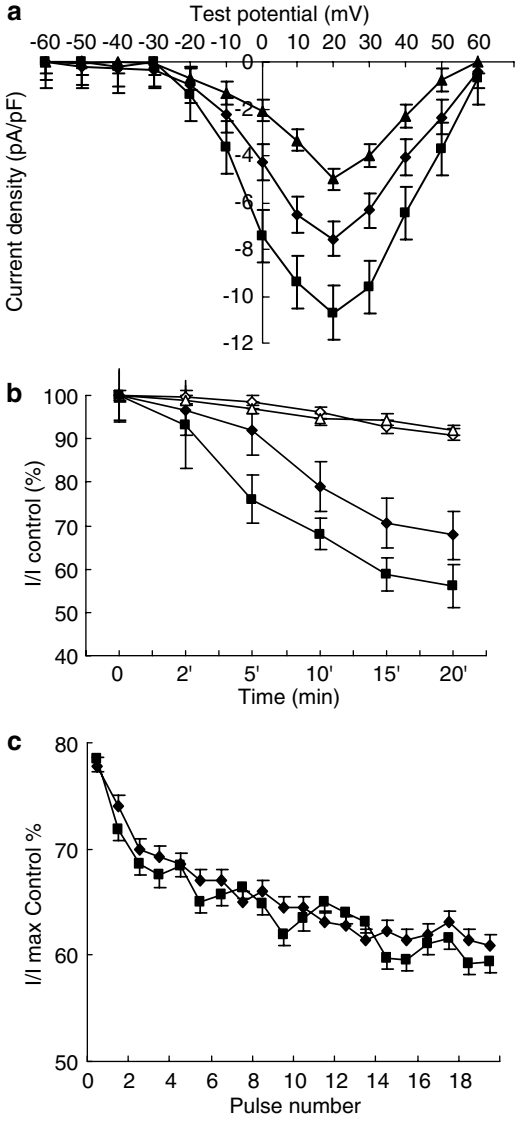

Figure 5 Effects of urocortin 2 on electrophysiological properties I_{Ba}. (a) Effects of urocortin 2 at different concentrations on current–voltage (*I–V*) relation. Control is marked with (■), urocortin 2 at 0.1 μM with (◆), and urocortin 2 at 1 μM with (▲). 200 ms steps from a holding potential of −90 mV were applied between −60 and +60 mV (increments of 10 mV) ($n=6$). (b) Frequency-independent effects of urocortin 2 on I_{Ba}. Urocortin 2 (0.1 μM) inhibited I_{Ba} at 1 Hz (■) and 3 Hz (◆) to a similar extent, which appeared to be frequency-independent ($n=6$). (c) Time course of urocortin 2 effects on I_{Ba} ($n=6$). The control current was marked with (◇), and 0.01 μM with (□), 0.1 μM with (◆), 1 μM with (■), the currents show time-dependence.

This result demonstrated that urocortin 2 might decrease intracellular Ca^{2+} via inhibiting L-type calcium channels, instead of binding to its receptors firstly. Urocortin 2 could also reduce $[Ca^{2+}]_i$ in normal tyrode's solution, but no inhibitory effects were observed in Ca^{2+}-free or EGTA contained tyrode's solution, which indicated that urocortin 2 inhibited $[Ca^{2+}]_i$ not via internal Ca^{2+} store. This result was confirmed by the fact that urocortin 2 did not affect ryanodine-induced intracellular calcium increase.

To further investigate the effects of urocortin 2 on calcium channels, we used whole-cell patch clamp technique to detect I_{Ba} in undifferentiated PC12 cells, which express L-, N-, T-, and P/Q-type calcium channels (Avidor *et al*, 1994; Gage *et al*, 2002; Del Toro *et al*, 2003; Liu *et al*, 2003; Day *et al*, 2006). Under the present experimental conditions, Ba^{2+} was the only charge carrier for inward current (Ma *et al*, 2005), which was confirmed by the result that VGCC currents were completely blocked by 200 nM $CdCl_2$, a nonselective calcium channel blocker, indicating that it was not contaminated by K^+ and Na^+ currents (Tao *et al*, 2005a). Barium was used as the charge carrier because Ba^{2+} is more permeable than Ca^{2+} through calcium channels (Hagiwara and Byerly, 1981) and Ca^{2+}-dependent inactivation of calcium channels is much less in barium media (Eckert and Chad, 1984). Rundown of ionic currents is always a concern in whole-cell voltage clamp recording. We minimized time-dependent changes in barium currents by using high resistance pipettes filled with MgATP 4 μM and beginning the experiments within 10 min after membrane rupture (Tao *et al*, 2005a). After wash-out, the currents partially recovered, indicating the effect of urocortin 2 on

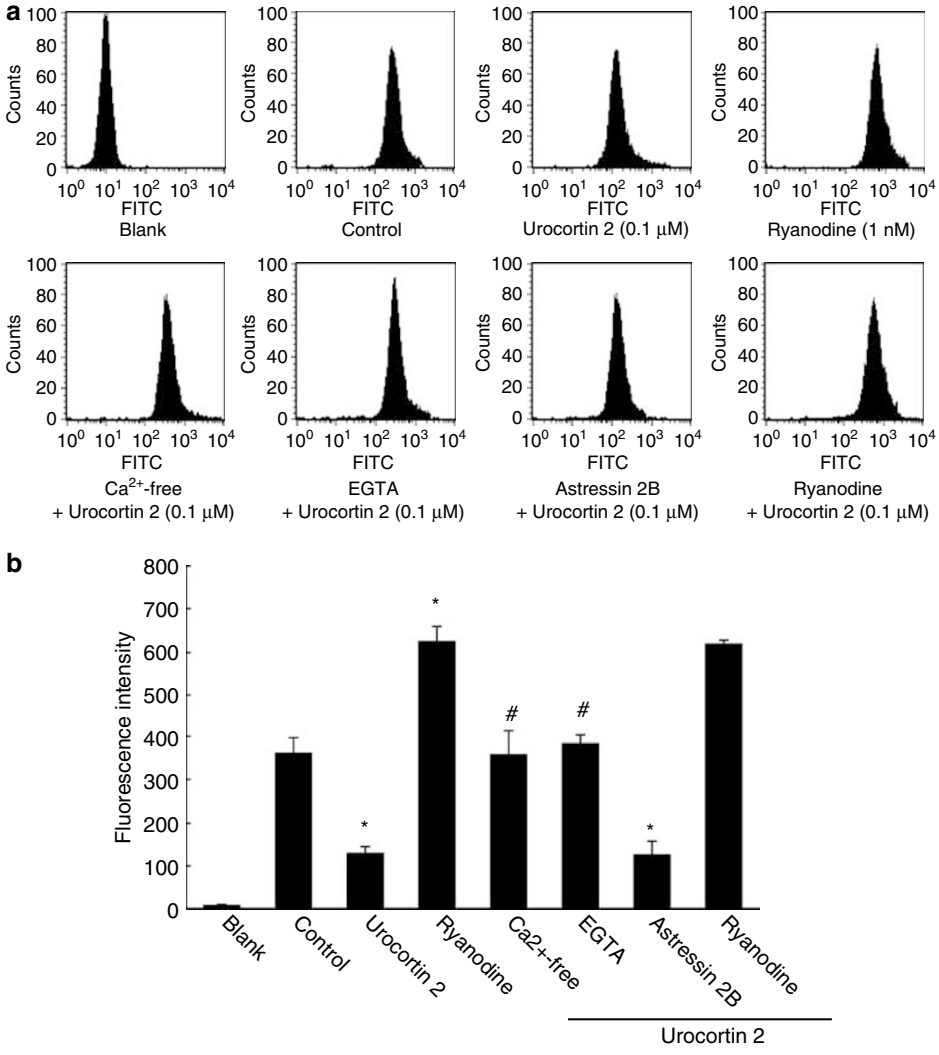

Figure 6 (a) Effects of urocortin 2 (0.1 μM), astressin 2B (1 μM), ryanodine (1 nM), Ca^{2+}-free tyrode's solution, or tyrode's solution containing EGTA (3 mM) on $[Ca^{2+}]_i$ by flow cytometry. No fluorescence was detected in the control without loading the Fluo-3 (blank). (b) Statistical data of urocortin 2 on the intracellular Ca^{2+} levels (*$P<0.05$ *vs* control, $^{\#}P<0.05$ *vs* urocortin 2, $n=6$).

I_{Ba} was not the consequence of the rundown. The effect of urocortin 2 on I_{Ba} is concentration-dependent. Urocortin 2 significantly shifted the I-V curve to the more positive level. Pretreatment of the cells with antalarmin, a nonpeptide CRFR1 blocker, or astressin 2B, a CRFR2 blocker, did not influence the inhibitory effect of urocortin 2 on the currents, suggesting that urocortin 2 may exert the inhibitory effect directly via VGCC channel instead of via binding firstly to its CRFR1 or CRFR2. When PC12 cells were treated with L-type calcium channel blocker, nifedipine, the cells became insensitive to urocortin 2. This result provides strong evidence that urocortin 2 targets L-type calcium channels. After pretreatment of the cells with omega-conotoxin GVIA, an N-type calcium channel blocker, and omega-agatoxin IVA, a P/Q-type calcium channel blocker, urocortin 2 inhibited I_{Ba} differently, which is consistent with the different contribution of L-, N-, and P/Q-type calcium currents to the total in these cells. Our previous reports also showed that urocortin, another member of CRF related peptides, inhibited T-type VGCC currents in mouse spermatogenic cells (Tao *et al*, 2005b). In the present study, nifedipine-treated PC12 cells were insensitive to urocortin 2, which indicated that T-type VGCC may play little role in the inhibitory effect of urocortin 2.

The inhibitory effects of urocortin 2 on $[Ca^{2+}]_i$ via L-type VGCC would be expected to exert a neuroprotective action by reducing calcium overload, consistent with the report that urocortin 2 protected the neuron against apoptosis (Bayatti *et al*, 2003), which is also thought to be highly associated with intracellular Ca^{2+} overload. Also, previous report showed that a dysregulation of intraspine Cav1.3 L-type VGCC leads to a loss of spines and glutamatergic synapses in stratopallidal MSNs, which is likely a key step in the emergence of pathological activity that is responsible for symptoms in Parkinson diseases (Day *et al*, 2006). In addition, we also found that urocortin 2 could inhibit I_{Ba} in acutely dissociated rat cerebral cortex neurons. Therefore, it is reasonable to infer that urocortin 2 exerts its protection on neuron at least partly due to the ability to inhibit the L-type VGCC. However, our results are inconsistent with some previous reports. It was found that urocortin, another

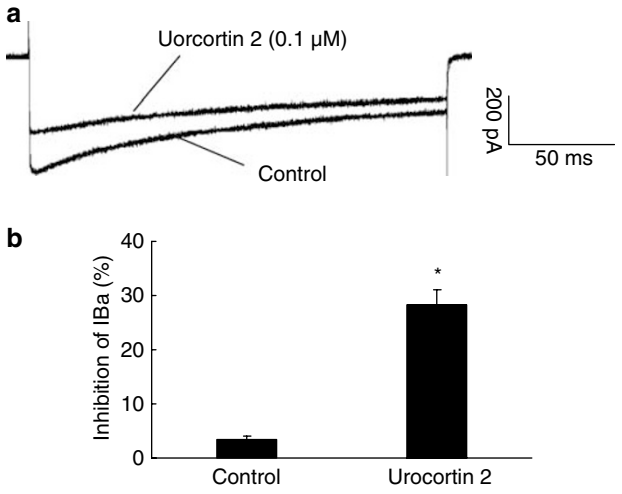

Figure 7 Effects of urocortin 2 on I_{Ba} in acutely dissociated cerebral cortex neurons. (a) Current traces of voltage-gated barium currents obtained in the absence or presence of urocortin 2 at 0.1 μM. I_{Ba} were evoked by a 200 ms-long depolarizing step pulse from the holding potential of −90 to 10 mV. (b) Inhibition of I_{Ba} by urocortin 2 (0.1 μM) (*$P<0.05$ *vs* control, $n=6$).

member of CRF family, stimulated Ca^{2+} uptake in skin cells (Wiesner *et al*, 2003). In addition, urocortin-induced increase of ANP and BNP secretion was completely abolished by a-helical CRF, a specific CRFR2 antagonist, and partially inhibited by diltiazem, another L-type VGCC blocker in cultured neonatal cardiomyocytes (Ikeda *et al*, 1998). In the same paper, the authors also stated that urocortin caused cAMP accumulation, which may lead to activation of L-type VGCC. In the present study, urocortin 2 was found to inhibit I_{Ba} in undifferentiated PC12 cells, and this effect was not influenced by antalarmin, a CRFR1 antagonist or astressin 2B, a CRFR2 antagonist. The interpretation for the differences remains to be explored, which might be attributed to the diversity of urocortins' action mechanisms and the different cell species/ages used for the experiments.

In conclusion, the present study provides evidence that urocortin 2, coexpressed with CRFR2β in undifferentiated PC12 cells, decreases the intracellular calcium levels via inhibiting the L-type VGCC. It may be a potential endogenous protective agent under some pathophysiological conditions, such as brain ischemia and some neuronal degenerative diseases like Alzheimer's and Parkinson's diseases (Lipscombe *et al*, 1988; Kostyuk and Verkhratsky, 1995), in which Ca^{2+}-overload plays very important roles. Further studies will be performed on calcium-channel-transfected HEK293 cells to distinguish urocortin 2's target channel type between $Ca_V1.2$ or $Ca_V1.3$, the two main subunits of L-type VGCC expressed in undifferentiated PC12 cells (Avidor *et al*, 1994; Liu *et al*, 2003; Tang *et al*, 2004).

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