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State-dependent calcium mobilization by urotensin-II in cultured human endothelial cells

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Abstract

Human endothelial cells express urotensin II (U-II) as well as its receptor GPR14. Using microfluorimetric techniques, the effect of human U-II on cytosolic Ca^{2+} concentrations $[Ca^{2+}]_i$ in cultured human aortic endothelial cells (HAEC) loaded with Fura-2 was evaluated in static or flow conditions. Under the static state, U-II (100 nM) abolished spontaneous Ca^{2+} oscillations, which occurred in a population of cultured HAEC. Similarly, U-II reduced thrombin-, but not ATP-induced calcium responses, suggesting that the peptide does not alter the $G_{q/11}/IP_3$ pathway; rather, it modifies the coupling between protease activated receptors and $G_{q/11}/IP_3$. Under the flow condition, U-II (1, 10 and 100 nM) produced a dose-dependent increase in $[Ca^{2+}]_i$, which was subjected to desensitization. The result demonstrates a state-dependent effect of U-II in cultured HAEC, which may explain the variable responses to U-II under different experimental conditions.

Keywords

Calcium mobilization; human endothelial cells; G protein-coupled receptor

Introduction

Urotensin II (U-II), a cyclic peptide, was first isolated from the caudal neurosecretory cells of teleost fish, and subsequently in the frog, rodent and human [19,54]. The human U-II is composed of 11 amino acid residues; the fish and frog U-II consists of 12 and 13 amino acids [20]. The cyclic region, where the biological activity resides, is fully conserved from fish to human [20].

U-II mRNA, or peptide, is expressed in ventral horn neurons of the spinal cord and brainstem in all the species that have been examined including the human [17,18,21,28,29,49,50]. For example, U-II-immunoreactivity of varying intensities is present in a population of ventral horn neurons in the rat spinal cord, hypoglossal nucleus, dorsal motor nucleus of the vagus, facial motor nucleus, nucleus ambiguus, abducens nucleus and trigeminal motor nucleus [28]. Information relative to the physiological or pharmacological action of U-II in the central nervous system is limited. U-II by intracerebroventricular injection causes hypertension and

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bradycardia, stimulates prolactin and thyrotropin secretion, promotes rapid eye movement sleep episode, and induces a number of behavioral responses indicative of anxiogenic and depressant-like behaviors [24,31,36]. A wide distribution of U-II receptors in the brain and spinal cord may contribute to the broad range of central effects elicited by exogenous U-II [39].

Results from several laboratories suggest that U-II is the endogenous ligand for the orphan G-protein coupled receptor GPR14, which has structural similarity with members of the somatostatin/opioid receptor family [5,42,44,47]. In addition to neural tissues, GPR14 mRNA is present in peripheral tissues including the vasculature, heart, and skeletal muscle [43]. Initial studies support a vasoconstrictive action of U-II, which is eight- to 109-fold more potent than endothelin 1 in certain vessels [25]. Subsequent reports show that the vascular response to U-II varied, depending on the species, type of blood vessel, concentration of U-II and route of administration. For example, intravenous infusion of U-II (3 to 300 pmol/min) was found to cause no significant changes in heart rate, mean arterial pressure or cardiac index in healthy male volunteers as compared to saline infusion [4]. In another study where the peptide was infused into the brachial artery, the forearm blood flow was reduced by U-II (1 to 300 pmol/min) in a dose-dependent manner, indicating a vasoconstrictive effect [10]. In human blood vessels *in vitro*, U-II has been found to cause a vasoconstriction, dilatation or no significant changes [7,34,59].

Using calcium flux as an index, the present study was undertaken to investigate the Ca^{2+} response to human U-II in cultured human aorta endothelial cells (HAEC) under flow or static conditions, which may simulate different experimental states.

Methods

HAEC culture

Human aortic endothelial cells (HAEC) (Clonetics Corp., San Diego, CA) were grown in M199 medium (Invitrogen, Grand Island, NY) containing 20% fetal calf serum (HyClone Laboratories, Logan, UT), 50 μg/ml endothelial cell growth supplement (BD Bioscience, Bedford, MA), and 50 μg/ml heparin (Sigma, St. Louis, MO). The culture medium was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells from passages 8-9 were used in the experiments.

Flow vs static peptide administration

HAEC were exposed to laminar shear stress (τ) of 10 dyne/cm^2, as calculated by the following formula [9,38]:

$$\tau = 6\mu Q/wh^2$$

where under our experimental conditions μ is the media viscosity (0.0085 g/cm/s), w is the channel width (1.0 cm), h is the channel height (0.2 cm), and Q is the volumetric flow rate (0.07843 cm^3/s).

For static administration, peptides or chemicals were added directly to the organ bath.

Ca^{2+} measurement

Cytosolic Ca^{2+}concentrations $[Ca^{2+}]_i$ were measured by the microfluorimetric technique, as previously described [14]. Cultured HAEC were loaded with the fluorescent Ca^{2+} indicator Fura-2 AM (3 μM) by incubation of the cells in Hank's balanced salt solution (HBSS) plus Fura-2 AM for 45 min, and HBSS alone for an additional 15-60 min to allow de-esterification

of the dye. Coverslips were mounted in a diamond-shaped recording chamber (model RC-25, Warner Instrument Inc., Hamden, CT) that provides laminar solution flow. The recording chamber was mounted on the stage of a TE2000U Eclipse Nikon inverted microscope equipped with a Photometrics CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ). The volume of the chamber was 500 μl. For laminar flow experiments, the coverlips were perfused with HBSS at 2.5 ml/min using a Minipuls 3 peristaltic pump (Gilson Inc, Middleton, WI). Fura-2 fluorescence (emission = 520 nm), following alternate excitation at 340 nm and 380 nm, was acquired at a frequency of 0.2 Hz using a MetaFluor software.

Statistics

Statistical significance between groups was evaluated using one-way ANOVA followed by Bonferroni test, $p< 0.05$ being considered significantly different.

Chemicals

ATP and thrombin were from Sigma Aldrich (St. Louis, MO), and human urotensin II from Phoenix Pharmaceuticals, Inc. (Burlingame, CA).

Results

$[Ca^{2+}]$ in flow stimulated HAEC

The basal value of $[Ca^{2+}]_i$ in cultured HAEC was 68 ± 4.2 nM (n= 85). Saline perfusion at a flow rate of 0.07843 cm^3/s (equivalent to 10 dyne/cm^2 of shear stress) rapidly raised the $[Ca^{2+}]_i$ to 283 ± 5.7 nM (n= 50). Addition of U-II (1, 10, 100 nM) to perfusing saline produced a rapid rise in $[Ca^{2+}]_i$ by an additional 72 ± 4 nM (n=16), 168 + 5 nM (n=12) and 463 ± 8.4 nM (n=15), respectively (Fig. 1). In a Ca^{2+}-free saline, U-II (100 nM) induced a transitory elevation in $[Ca^{2+}]_i$ by 348 ± 6.4 nM (n=9) (Fig. 1).

In cultured HAEC exposed to two consecutive superfusion of U-II (100 nM), the second superfusion consistently caused a much smaller increase in $[Ca^{2+}]_i$ as compared to that produced by the first application; a representative experiment is shown in Fig. 2A. The first and second administration produced an averaged increase in $[Ca^{2+}]_i$ of 463 ± 8 nM (n=23) and 216 ± 7 nM (n=23), respectively (Fig. 2B).

$[Ca^{2+}]_i$ in static HAEC

Under static conditions, U-II (100 nM) added directly to cultured HAEC did not result in a significant change of $[Ca^{2+}]_i$ in any of the cells tested (n= 76). Spontaneous Ca^{2+}oscillations occurred in 14 out of 161 HAEC examined (8.7%). Addition of U-II (100 nM) abolished oscillations in all of the 14 cells analyzed; a representative example of actual recordings from three cells displaying oscillations is shown in Fig. 3.

Effects of U-II on ATP- and thrombin-induced $[Ca^{2+}]_i$ in static state

IP_3 has been shown to be one of the signaling pathways involved in Ca^{2+}oscillations [49]. ATP and thrombin are known to mobilize Ca^{2+} in endothelial cells through the IP_3 pathway. The following experiments were conducted to test the hypothesis that U-II abolishes Ca^{2+} oscillations by modulating the IP_3 pathway. Under static conditions, ATP (10 μM) caused a fast and transitory increase of $[Ca^{2+}]_i$ ($\Delta F/F_0$, Fig. 4A1, black trace, n=27). Pretreating the HAEC with U-II (100 nM) did not significantly alter the ATP-induced increase in $[Ca^{2+}]_i$ either in Ca^{2+}-containing (Fig. 4A1, red trace) or Ca^{2+}-free saline (Fig. 4A2, red trace, n=35). U-II was added to the chamber one minute before ATP and for the duration of ATP administration.

U-II (100 nM, red trace) reduced the thrombin-induced increase in $[Ca^{2+}]_i$ (Fig. 4B1 and 4B2, black trace, n=33). This effect was more evident in Ca^{2+}-free saline (Fig. 4B2, n=29), as U-II inhibited thrombin-induced $[Ca^{2+}]_i$ increase by 19 ± 1% in Ca^{2+}-containing saline and by 37 ± 1.3 % in Ca^{2+}-free saline (Fig. 4C1 and 4C2). The traces represent the mean $\Delta F/F_0$ ± S.E.M.

Discussion

Endothelial cells have a major role in regulating the diameter of the blood vessels and their adaptation to hemodynamic demands [45]. Urotensin II, the most potent vasoconstrictor agonist yet identified, was first reported to produce an endothelium-dependent relaxation and endothelium-independent contractions of rat aorta [32]. Significant differences in the vascular response to U-II have been reported [15,26]. For example, U-II is an endothelium-dependent vasodilator in mesenteric and coronary arteries in the rat, as well as in the capillaries of the ear, but not in the basilar artery [11,52]. The relaxant responses are attributed to a release of nitric oxide and endothelium-derived hyperpolarizing factors [3,11,62].

Intracellular calcium acts as a second messenger and serves a critical role in regulating the activity of endothelial cells. The vascular endothelium responds to several hormones and chemical signals via changes in cytosolic Ca^{2+}, with subsequent activation of Ca^{2+}-dependent signaling mechanisms [35]. U-II reportedly mobilizes Ca^{2+} by different mechanisms in different types of cell. For example, the effect of U-II was abolished by thapsigargin, indicating the participation of endoplasmic reticulum Ca^{2+} pools in rhabdomyosarcoma cell line [27] as well as in frog motor nerve terminals [12]. In rat, rabbit and cat blood vessels [2,32,55,56,61] and in rat cultured astrocytes [16], the effect of U-II was inhibited by the phospholipase C inhibitor U-73122, indicating the involvement of phospholipase-C/IP_3 pathways. In contrast, U-II elevated $[Ca^{2+}]_i$ largely by facilitating Ca^{2+} entry through plasmalemmal Ca^{2+} channels in rat spinal motoneurons [30].

With respect to the HAEC, our result indicates that U-II induced an elevation of $[Ca^{2+}]_i$ under flow but not under static state. Similarly, rat aortic adventitial segments exposed to U-II release nitric oxide upon continuous shaking [41]. Elevation of endothelial cell $[Ca^{2+}]_i$ may be achieved by Ca^{2+} entry via Ca^{2+} channels in the plasma membrane and/or by Ca^{2+} release from intracellular stores [1]. In shear stress, U-II caused a concentration-dependent elevation of $[Ca^{2+}]_i$ mediated by Ca^{2+}entry through plasmalemmal Ca^{2+} channels as well as Ca^{2+} release from intracellular Ca^{2+}stores. In large arteries, the average wall shear stress is between 1 to 20 dyne/cm^2. At curves and bifurcations, peak wall shear stress may be as high as 100 dyne/cm^2. Immediate (milliseconds to seconds) responses to shear stress include increases in ionic conductance [40,48], intracellular Ca^{2+} [57,58] and IP3 [8,46]. As a corollary, U-II may facilitate the shear stress-induced increase of $[Ca^{2+}]_i$ and/or IP_3. In the case of consecutive administration of U-II to HAEC, the response to the second administration of U-II was smaller than the first response, implying the occurrence of desensitization. This result is similar to that reported in rat vasculature [15], but different from that of spinal neurons [30]. An alternative interpretation would be that the internal pool of Ca^{2+} contributing to the overall U-II-induced Ca^{2+} increase was only partially refilled at the time interval between applications.

Ca^{2+} oscillations, which are probably initiated by Ca^{2+} release from intracellular pools rather than Ca^{2+} entry from the extracellular medium, have been demonstrated in a population of cultured endothelial cells [45]. A second novel observation made in our study is that U-II not only did not raise $[Ca^{2+}]_i$ but abolished Ca^{2+} oscillations in HAEC under static conditions.

In endothelial cells, IP_3 is the most common pathway leading to an elevation of $[Ca^{2+}]_i$. At concentrations up to 10 μM, ATP acting on P2Y purinergic receptors raised $[Ca^{2+}]_i$ and activated Gq/G11 phospholipase C pathways [53,60]. Thrombin is another potent agonist that

elevates $[Ca^{2+}]_i$ in endothelial cells by different mechanisms, including Ca^{2+} influx [23]. Thrombin signaling in the endothelium is mediated by a family of G protein–coupled receptors known as protease-activated receptors (PARs) [22]. In aortic endothelial cells, activation of PAR-2 or P2Y receptors elevates Ca^{2+} through phospholipase C/IP_3 pathways subsequent to activation of $G_{q/11}$ [37]. Under static conditions, pretreatment of HAEC with U-II (100 nM) did not affect ATP-induced $[Ca^{2+}]_i$ elevation either in normal or Ca^{2+}-free saline, indicating that the peptide does not interfere with phospholipase C/IP_3 pathways. In contrast, U-II pretreatment significantly reduced thrombin-induced $[Ca^{2+}]_i$ mobilization. Since the ATP response is not affected, U-II may directly modulate PAR-2, thereby affecting the coupling with Gq protein in HAEC.

A possible explanation for the differences observed between U-II-induced effects in shear stress vs static state is that the affinity of U-II to its receptors may vary in different microenvironment. Alternatively, there is evidence that peptides may be active when internalized into the cytoplasm [6,13,33]. Hence, we cannot exclude a possible differential regulation of calcium homeostasis in endothelial cells by activated intracellular U-II receptors.

In conclusion, our result shows that, depending on the condition under which the experiment is conducted, U-II can exert multiple effects on human aortic endothelial cells.

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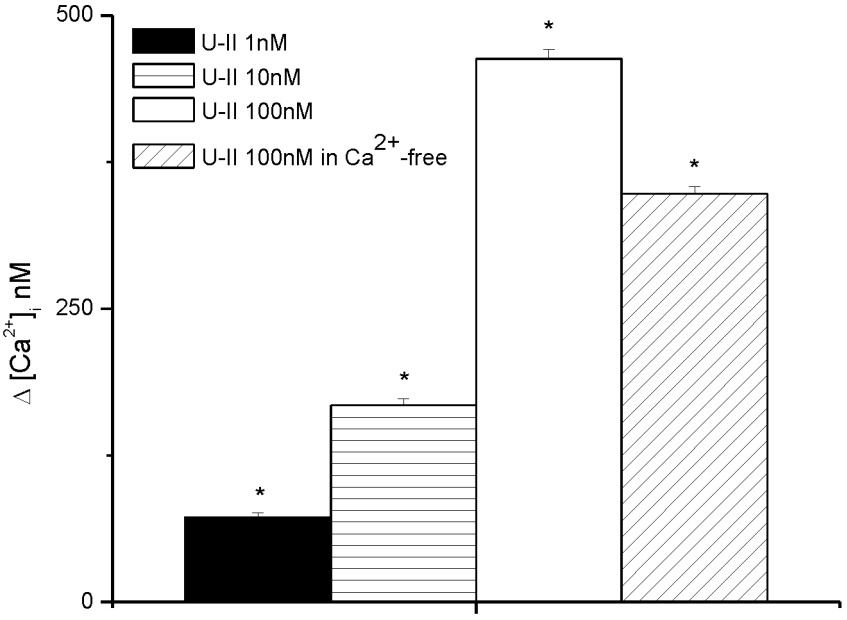

Fig. 1.

Ca^{2+} responses induced by urotensin-II (U-II) in human aortic endothelial cells. Addition of U-II (1, 10, 100 nM) to perfusing saline increased $[Ca^{2+}]_i$ by an additional 72 ± 4 (n=16), 168 ±5 (n=12) and 463 ± 8.4 nM (n=15), respectively. In a Ca^{2+}-free saline, U-II (100 nM) induced a transitory increase in $[Ca^{2+}]_i$ by 348 ± 6.4 nM (n=9). The asterisk denotes statistically significant difference as compared to control.

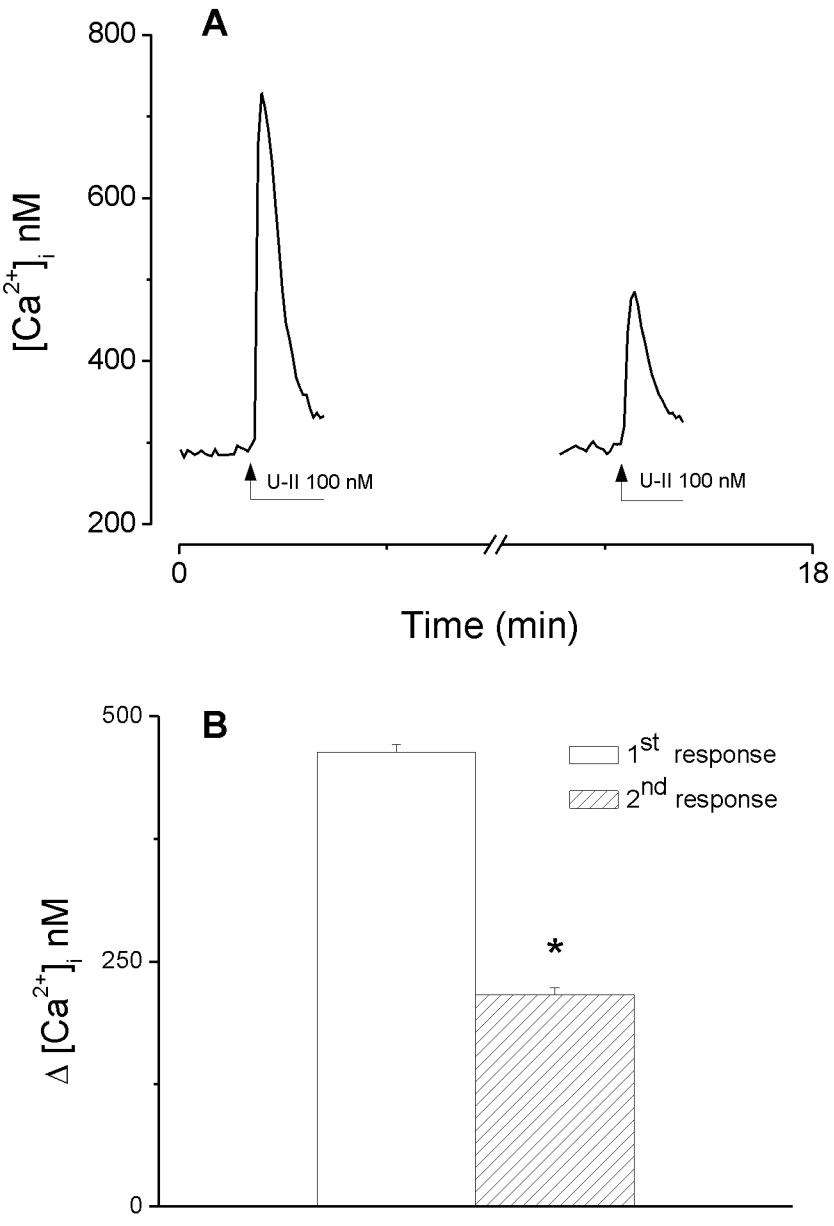

Fig. 2.

Ca^{2+} responses induced by two consecutive administrations of urotensin-II (U-II). A, Actual traces of two consecutive responses produced by superfusion of U-II (100 nM); the second superfusion consistently caused a much smaller increase in $[Ca^{2+}]_i$ as compared to that produced by the first application. B, Comparison of the first and second response produced by U-II (100 nM): the first administration produced an increase in $[Ca^{2+}]_i$ by 463 ± 8 nM, whereas the second administration produced an increase by 216 ± 7 nM (n=23). The asterisk denotes statistically significant difference as compared to the first response.

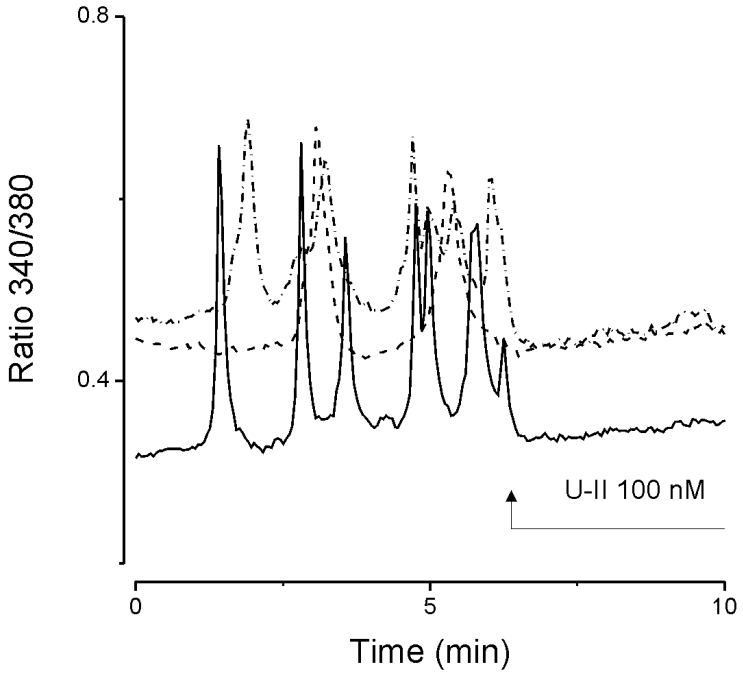

Fig. 3.

Effects of urotensin II (U-II) on Ca^{2+} oscillations. U-II (100 nM) abolished spontaneous Ca^{2+} oscillations in HAEC. Actual recordings from three different cells (solid line, dashed line and dot-dash line) exhibiting Ca^{2+} oscillations are shown.

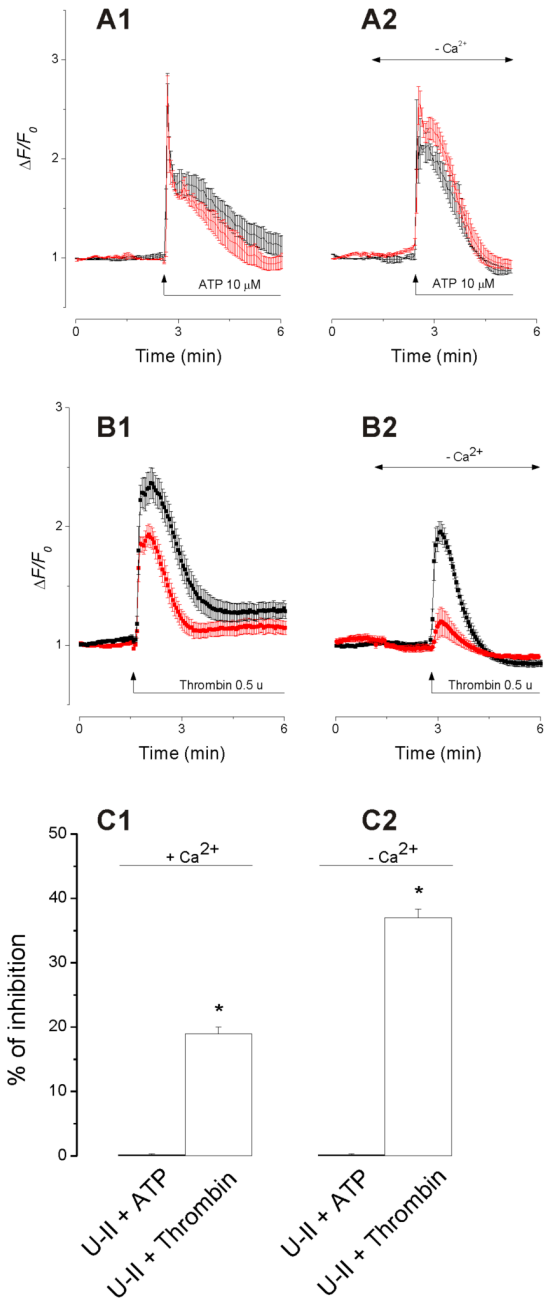

Fig. 4.

Effect of urotensin-II (U-II, 100 nM) on ATP- and thrombin-induced increase in $[Ca^{2+}]_i$. A1 and A2, administration of U-II (red trace) did not significantly affect the ATP-induced increase in $[Ca^{2+}]_i$ (black trace) in Ca^{2+}-containing or in Ca^{2+}-free saline; traces represent mean $\Delta F/F_0 \pm$ S.E.M. B1 and B2, administration of U-II (red trace) reduced the thrombin-induced (black trace) increase in $[Ca^{2+}]_i$ in Ca^{2+}-containing and Ca^{2+}-free saline. C1 and C2, comparison of the effect of U-II on ATP- and thrombin-induced increase in $[Ca^{2+}]_i$ in saline with and without Ca^{2+}.