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The vasoactive peptide urotensin II stimulates spontaneous release from frog motor nerve terminals

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1 The effect of urotensin II (U-II) on spontaneous transmitter release was examined in the frog to see if the biological activity of this vasoactive peptide extended to neural tissues.

2 In normal Ringer solution, frog and human U-II (fU-II and hU-II, respectively) caused concentration-dependent, reversible increases in miniature endplate potential (MEPP) frequency, with hU-II about 22 times more potent than fU-II. hU-II caused a dose-dependent increase in MEPP amplitude, whereas fU-II caused an increase, followed by a decrease with higher concentrations.

3 Increasing extracellular Ca^{2+} three-fold had no effect on the MEPP frequency increase to 25 μM hU-II. Pretreatment with thapsigargin to deplete endoplasmic reticulum Ca^{2+} caused a 61% reduction in the MEPP frequency increase to 25 μM hU-II.

4 Pretreatment with the phospholipase C inhibitor U-73122 caused a 93% reduction in the MEPP frequency increase to 25 μM hU-II and a 15% reduction in the increase in MEPP amplitude. Pretreating with antibodies against the inositol 1,4,5-trisphosphate (IP_3) type 1 receptor using liposomal techniques reduced the MEPP frequency increase by 83% but had no effect on MEPP amplitude.

5 Pretreating with protein kinase C inhibitors (bisindolylmaleimide I and III) had no effect on the response to 25 μM hU-II, but pretreating with protein kinase A inhibitors (H-89 and KT5720) reduced the MEPP frequency increase by 88% and completely abolished the increase in MEPP amplitude.

6 Our results show that hU-II is a potent stimulator of spontaneous transmitter release in the frog and that the effect is mediated by IP_3 and cyclic AMP/protein kinase A.

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Abbreviations: anti-IP_3R_1, monoclonal antibody against the type 1 receptor for IP_3; cADPR, cyclic adenosine diphosphate-ribose; cAMP, cyclic adenosine monophosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; DAG, diacylglycerol; EGTA, ethylene glyco-bis (β-amino ethyl ether) tetraacetic acid; fU-II, frog urotensin II; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide HCl; hU-II, human urotensin II; IP_3, inositol 1,4,5-trisphosphate; MEPP, miniature endplate potential; PKA, protein kinase A; PKC, protein kinase C; SER, smooth endoplasmic reticulum; TG, thapsigargin; U-II, urotensin II

Introduction

Urotensin II (U-II) is a cyclic peptide first isolated from the caudal neurosecretory system of teleost fish and subsequently from frog, rodent and human (see Conlon, 2000). The recent discovery that U-II may be the endogenous ligand for the orphan G-protein-coupled receptor GPR14, which has structural similarity with members of the somatostatin/opioid receptor family (Ames *et al.*, 1999; Liu *et al.*, 1999; Mori *et al.*, 1999; Nothacker *et al.*, 1999), has generated a considerable interest with respect to its biological action(s). Much attention has been directed toward the vascular effects of U-II (Nothacker *et al.*, 1999; Douglas *et al.*, 2000). U-II has been reported to be eight- to 109-fold more potent than endothelin 1 in certain vessels (Douglas *et al.*, 2000).

In addition to its localization in cardiac myocytes and certain vascular tissues, prepro U-II mRNA and/or U-II-immunoreactivity has been detected in ventral horn motoneurons of the spinal cord and cholinergic cranial motoneurons of the brainstem of several species, including frog, human and rat (Chartrel *et al.*, 1996; Coulouarn *et al.*, 1998; Dun *et al.*, 2001). This suggests that the biological activity of U-II may not be limited to the heart and vessels and may conceivably extend to neural tissues. We have examined this possibility by studying the effect of the peptide on spontaneous transmitter release at the frog neuromuscular junction.

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Methods

Preparation and solutions

Frogs (*Rana pipiens*), obtained from J.M. Hazen & Co., Alburg, VT, U.S.A., were decapitated and rapidly

double-pithed, and sciatic–sartorius nerve–muscle preparations isolated. Every effort was made to use the minimum number of animals required for valid statistical analyses and to minimize their suffering. All procedures were reviewed and approved by the University Committee for Animal Care. Muscles were mounted in a 5-ml Sylgard-lined Petri dish bath, which was continuously perfused with Ringer solution using a dual-chambered roller pump. The normal Ringer solution contained (mM): 110 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 2.0 tris(hydroxymethyl)-aminomethane (Tris, to pH 7.2) and 5.6 glucose, while the $3 \times Ca^{2+}$ Ringer solution contained: 104.6 NaCl, 2.5 KCl, 5.4 $CaCl_2$, 2.0 Tris and 5.6 glucose. Peptides were dissolved in either normal or $3 \times Ca^{2+}$ Ringer solution to produce 1 mM stock solutions. Aliquots of 5, 50 or 125 μl were then rapidly dispensed into plastic microcentrifuge containers and frozen. Aliquots were thawed within 2 min before use and (after halting the bath perfusion) delivered locally with a Hamilton syringe. Thus, the concentration of peptide delivered was always 1 mM, and differing final concentrations were achieved by delivering different volumes (5, 50 or 125 μl) to the bath. U-II (human, lot # 414913 and 420065, MW = 1390.56, and frog, lot # 414960, MW = 1519.77) was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA, U.S.A.).

Bisindolylmaleimide I, bisindolylmaleimide III hydrochloride, thapsigargin (TG), (*N*-[2-((*p*-bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide, HCl) (H-89) and KT5720 were from Calbiochem (La Jolla, CA, U.S.A) and monoclonal antibodies against inositol 1,4,5-trisphosphate type 1 receptors (anti-IP_3R_1) were obtained from Research Biochemicals International (Natick, MA, U.S.A.). All other agents, including U-73122 and ethylene glyco-bis (β-amino ethyl ether) tetraacetic acid (EGTA), were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Liposomal techniques

Multilamellar liposomes were prepared with 60 mg ml^{-1} egg phosphatidylcholine (Sigma, type X-E), as previously described (Brailoiu *et al*., 2001). Agents to be incorporated into liposomes, that is, anti-IP_3R_1 or heat-inactivated anti-IP_3R_1 (both 100 μl ml^{-1}), were dissolved in 140 mM KCl solution at pH 6.9. Liposome batches were dialyzed (Sigma dialysis sacs) against control Ringer solution (1/600 v v^{-1}, 150 min) to remove nonincorporated agent, and the Ringer solution changed every 30 min. Liposome suspensions were administered by continuous perfusion after 1/20 v v^{-1} dilution in control Ringer solution. Repeated (10 times) heating (75°C for 30 s) and cooling (4°C for 1 min) were used to inactivate anti-IP_3R_1 antibodies (Kim & Lisman, 2001).

Electrophysiologic procedures

Miniature endplate potentials (MEPPs) were recorded using conventional microelectrode (3 M KCl, 5–15 MΩ resistance) techniques similar to those previously described (Brailoiu & Miyamoto, 2000). Selection of recordings was made from impalements that showed large MEPP size (>0.3 mV), good signal-to-noise ratio (baseline peak-to-peak noise <0.1 mV) and high and stable muscle resting membrane potential (>−80 mV, with <3 mV decline during the control period). Resting potentials ranged between −80 and −90 mV in different fibers. Experiments were conducted at the ambient room temperature (21–22°C), and only one continuous single junction recording was carried out on each muscle. Preparations were equilibrated for at least 30 min before use. Signals were fed into a high impedance preamplifier (A-M Systems, Carlsborg, WA,U.S.A.) and viewed on a R5103N oscilloscope (Tektronix, Beaverton, OR, U.S.A.). Signal-to-noise ratio was increased with a band-pass filter (0 – 1 kHz) and boosted for interfacing with a data acquisition unit with 1 MHz digitization frequency (RC Electronics, Goleta, CA, U.S.A.). MEPPs were recorded with a modified videocassette recorder (AM Vetter, Rebersburg, PA, U.S.A.) for off-line analysis.

Analysis of data

MEPP amplitudes (100 samples for each time point) were measured using stored digitized data and a grid template on a flat screen monitor. A fast sweep speed (2 ms cm^{-1}) was used to facilitate visual discrimination of MEPP peaks at the higher frequencies. In the case of overlapping MEPPs, the amplitude was measured as the point of overlap to the peak signal. This became problematic at frequencies >50 s^{-1}, which necessitated approximation in these instances. To minimize the effects of junction-to-junction variation, data for each experiment were expressed as percents of values at time zero, and results from six single experiments averaged (plots show means ±s.e.m.). Analysis of statistical differences was made by comparing each point with points obtained in control Ringer (paired Student's *t*-test and one-way ANOVA) or by comparing points between two curves (paired Student's *t*-test), with $P<0.05$ indicating significant differences.

Results

Effect of frog urotensin II (fU-II) in normal Ringer solution

Superfusion of fU-II (1, 10 and 25 μM) to nerve–muscle preparations produced dose-dependent increases in MEPP frequency (0, 202 and 283%, respectively) (Figure 1a). The effect of fU-II on MEPP amplitude was more complex: there was an increase with 1 μM but a smaller increase with 10 μM and a significant decrease with 25 μM (Figure 1b). There was no change in muscle resting potential during administration of the peptide.

Effect of human urotensin II (hU-II) in normal Ringer solution

Exposure of muscles to hU-II (1, 10 and 25 μM) caused marked, concentration-dependent increases in MEPP frequency and amplitude. For example, 1 and 25μM hU-II produced MEPP frequency increases of 300 and 5900%, respectively, over control (Figure 2a, with logarithmic ordinate). The increase in MEPP amplitude was, by comparison, much less dramatic but nonetheless significant (Figure 2b, with linear ordinate). In both cases, there was an increase and peak within a few minutes, followed by a gradual decline despite the continued presence of hU-II. However, the time course of rise and fall for MEPP frequency was more rapid than that for MEPP amplitude, for example, the peak effect

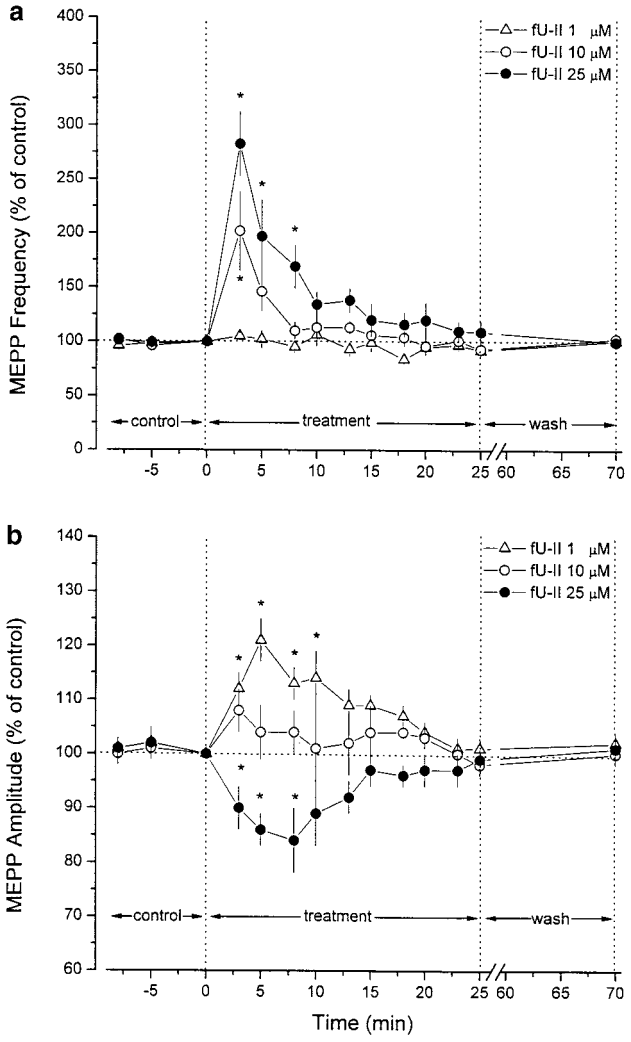

Figure 1 Effect of fU-II on MEPP frequency and amplitude in normal Ringer solution. Data are expressed as per cents of the values at time zero, and the results from six continuous single-junction experiments averaged. (a) Plots of effects of 1, 10 and 25 μM fU-II on MEPP frequency are superimposed for comparison. The peak effect occurs at 3 min for 10 and 25 μM fU-II. MEPP frequencies: 100% = 0.936 s^{-1} (1 μM fU-II), 0.886 s^{-1} (10 μM) and 0.925 s^{-1} (25 μM). (b) Effects of 1, 10 and 25 μM fU-II on MEPP amplitude. The peak effect on MEPP amplitude (at time = 5 – 7 min) is delayed compared to the peak effect for MEPP frequency, and the time course of decay is slower. Each point represents the mean $\pm$ s.e.m. Asterisks denote statistically significant differences ($P < 0.05$) from control.

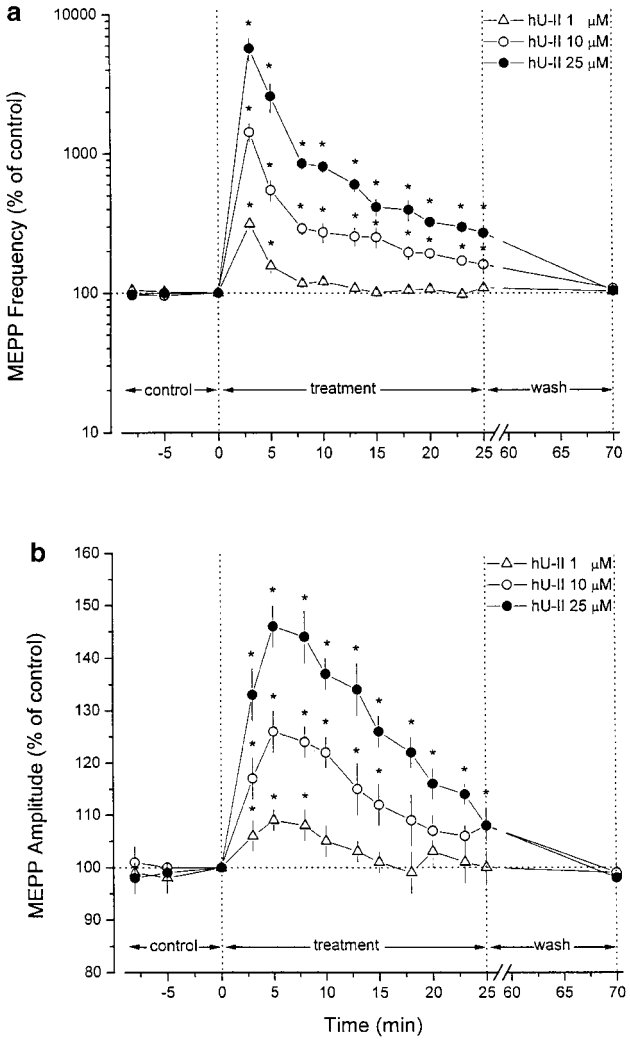

Figure 2 Effect of hU-II on MEPP frequency and amplitude in normal Ringer solution. (a) Plots of effects of 1, 10 and 25 μM hU-II on MEPP frequency are superimposed for comparison (note the logarithmic ordinate). The peak effect occurs at 3 min for all concentrations of hU-II. MEPP frequencies: 100% = 0.916 s^{-1} (1 μM hU-II), 0.961 s^{-1} (10 μM hU-II) and 1.058 s^{-1} (25 μM hU-II). (b) Effects of 1, 10 and 25 μM hU-II on MEPP amplitude. Again, the peak effect on MEPP amplitude is delayed (at time = 5 – 7 min) compared to the effect on MEPP frequency, and the time course of decay is slower. Each point represents the mean $\pm$ s.e.m. from six experiments. Asterisks denote statistically significant differences ($P < 0.05$) from control.

for MEPP frequency occurred at 3 min (Figure 2a), whereas that for MEPP amplitude occurred at 5 min (Figure 2b). Both frequency and amplitude returned to control levels after 45 min of wash. There was no change in muscle resting potential during administration of the peptide. Analysis of MEPP amplitudes using cumulative frequency plots showed distinct shifts to the right for each of the six experiments with 25 μM hU-II (Figure 3). This indicated that the increases in MEPP amplitude were not artifacts due to erroneous measurement of a number of summed events or due to increased frequency of giant MEPPs.

Comparison of the results in Figures 1a and 2a showed that hU-II was about 22 times more potent than fU-II in increasing MEPP frequency. In view of this marked difference, we decided to use the more potent hU-II in ensuing studies.

Effect of hU-II in elevated Ca^{2+} Ringer solution (3 × normal Ca^{2+})

Increases in transmitter release, such as that produced by hU-II, may be mediated by an influx of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and/or release of Ca^{2+} from internal stores (Silinsky, 1985; Berridge, 1993). To examine if the increase in MEPP frequency was altered by increases in $[Ca^{2+}]_o$, we repeated the 25 μM hU-II experiments in 3 × Ca^{2+} Ringer solution. Despite the three-fold increase in $[Ca^{2+}]_o$, the increase in MEPP frequency was

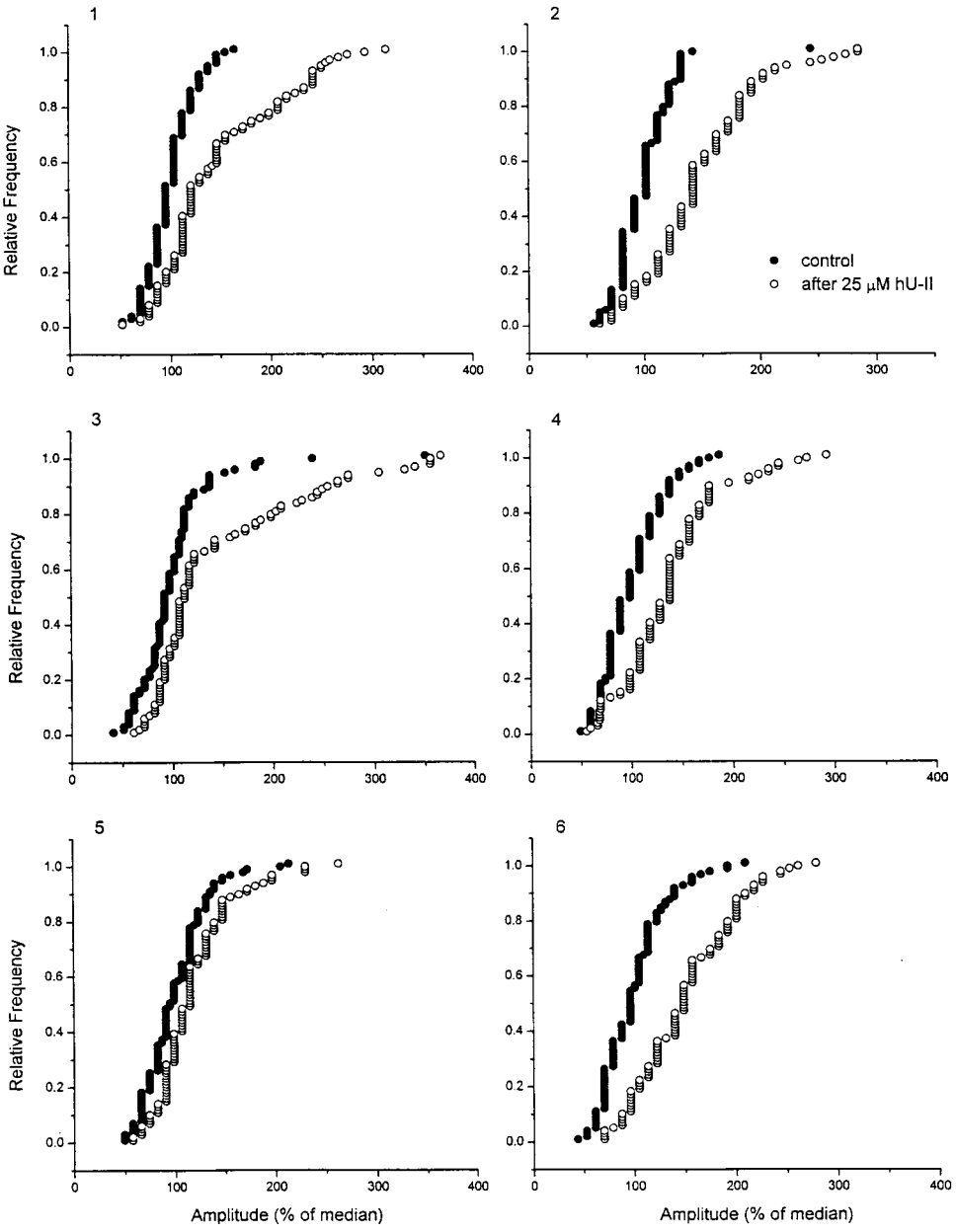

Figure 3 Cumulative MEPP amplitude-relative frequency plots in the absence (filled circles) and presence (clear circles) of 25 μM hU-II. Panels 1 – 6 show results of the six single-junction experiments with hU-II. Abscissas represent MEPP amplitudes as per cents of the median MEPP amplitude obtained at time = 0 min in control solution. Ordinates represent the cumulative relative frequency of 100 measurements for each curve (each symbol represents one MEPP). Results in the presence of hU-II were obtained during the maximal effect at time = 5 min. In all cases, there is a clear shift to the right from the control curves, which indicates an increase in MEPP amplitude with hU-II.

indistinguishable from that observed in normal Ringer solution (Figure 4, results from Figure 2a superimposed for purposes of comparison). This was confirmed by quantitative analysis, which showed no significant differences ($P>0.05$) between the magnitude and time course of the response obtained in normal Ringer and that obtained in $3\times Ca^{2+}$ Ringer.

These findings notwithstanding, it could be argued that the effect of 25 μM hU-II in normal Ca^{2+} Ringer was near maximal, so that further stimulation would bring about little or no increment in the response. This was unlikely since 50 μM hU-II produced an MEPP frequency increase that exceeded that produced by 25 μM hU-II but was too high (overlapping MEPPs) to adequately quantify (two experiments). Another way of examining the dependence of hU-II action on $[Ca^{2+}]_o$ was to conduct the experiments in zero $[Ca^{2+}]_o$. However, since incubation in zero $[Ca^{2+}]_o$ would yield highly equivocal results because of depletion of internal Ca^{2+} stores (Zerbes *et al*., 1998), we opted to see if the response to hU-II was altered by selective depletion of smooth endoplasmic reticulum (SER) stores of Ca^{2+}.

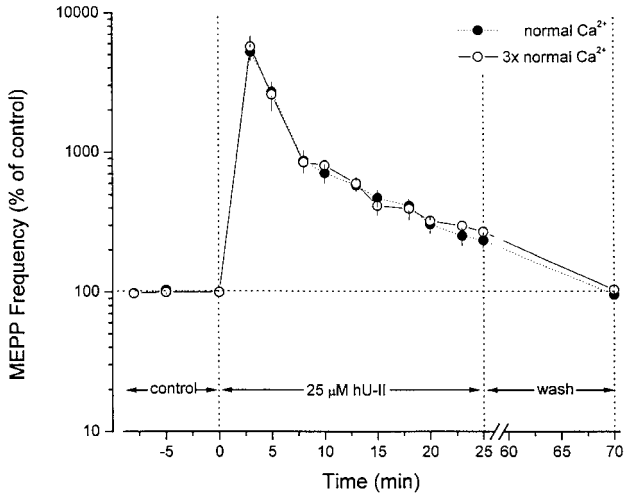

Figure 4 Effect of 3× normal extracellular Ca^{2+} on the MEPP frequency increase to 25 μM hU-II. Experimental protocol is identical to that in Figure 2 except for use of 3× Ca^{2+} Ringer in place of normal Ringer solution. Results in normal Ringer (filled circles) and those in 3× Ca^{2+} Ringer (clear circles) are superimposed for purposes of comparison. Note the logarithmic ordinate. Each point represents mean ± s.e.m. from six experiments. There is no significant difference in the MEPP frequency response (peak, time course) to 25 μM hU-II obtained in 3× Ca^{2+} Ringer vs normal Ringer solution ($P>0.05$, paired *t*-test).

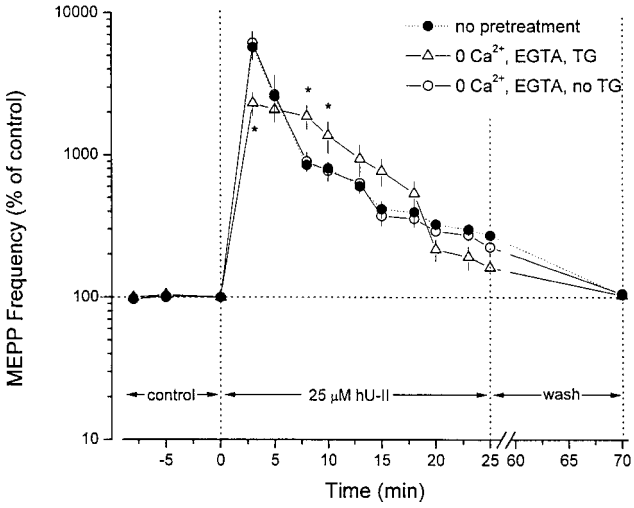

Figure 5 Effect of TG pretreatment on the MEPP frequency increase to 25 μM hU-II. Protocol is identical to that in Figure 2, except for 30 min pretreatment in Ringer containing zero Ca^{2+}, 2.5 mM EGTA and 1 μM TG, followed by 30 min wash in normal Ringer solution. Control experiments in preparations pretreated with zero Ca^{2+}, 2.5 mM EGTA but no TG (clear circles) show no difference in magnitude or time course of effect from results obtained in untreated preparations (filled circles). In TG-treated muscles, the peak increase in MEPP frequency (clear triangles) is reduced 61% from the peak effect in untreated muscles. Note the logarithmic ordinate. Each point represents the mean ± s.e.m. from six experiments. Asterisks denote statistically significant differences ($P<0.05$, paired *t*-test) from corresponding points in untreated muscles.

Response to hU-II in preparations pretreated with TG

Functional deletion of SER in nerve terminals was produced with TG, which inhibits Ca^{2+}-ATPase in SER (Takemura *et al.*, 1989) and abolishes Ca^{2+} release to inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1993) and cyclic adenosine diphosphate-ribose (cADPR) (Lee, 2001). Muscles were incubated in Ca^{2+}-free Ringer plus 2.5 mM EGTA and 1 μM TG for 30 min, followed by restoration of muscles to normal Ca^{2+}-containing Ringer for another 30 min. Subsequent application of hU-II (25 μM) produced a peak MEPP frequency increase of only 2324% (open triangles in Figure 5). To test the possibility that the incubation procedure itself may have reduced the response to hU-II, control experiments were performed on muscles incubated in Ca^{2+}-free Ringer plus 2.5 mM EGTA but without TG. Application of 25 μM hU-II in this case produced a time course of effect (clear circles in Figure 5) that was statistically indistinguishable from that seen in untreated preparations (filled circles in Figure 5). There was also no alteration in the hU-II-induced increase in MEPP amplitude following pretreatment with TG (results not shown). Accordingly, it could be concluded that the incubation procedure was not a confounding factor and that pretreatment with TG attenuated the hU-II-induced increase in MEPP frequency by 61%.

Effect of hU-II in preparations pretreated with U-73122

The results with TG suggested that the effect of hU-II on MEPP frequency was partially dependent on Ca^{2+} release from SER. This could occur if hU-II stimulated the formation of IP_3 (cf. Saetrum Opgaard *et al.*, 2000), which in turn activated Ca^{2+} release from SER (Berridge, 1993). To test this, we pretreated muscles for 30 min with U-73122 (1 μM), a phospholipase C inhibitor (Smith *et al.*, 1990), to block the formation of IP_3. Subsequent addition of hU-II (25 μM) produced an MEPP frequency increase of only 400% (clear circles in Figure 6a). When compared to the expected 5900% increase in untreated preparations (filled circles in Figure 6a), this meant that pretreatment with U-73122 reduced the effect of hU-II by 93%. Pretreatment with U-73122 also caused a 15% reduction in the effect of 25 μM hU-II on MEPP amplitude (Figure 6b). However, the time course of effect (increase, peak, decline) was not altered.

Effect of hU-II in preparations pretreated with antibodies against type 1 IP_3 receptors (anti-IP_3R_1)

To further examine if IP_3 might be involved in the response to hU-II, we tested the effect of 25 μM hU-II in preparations pretreated with monoclonal antibodies against the IP_3 type 1 receptor. Antibodies were delivered into the nerve terminal by encapsulating them in liposomes and pretreating preparations for 1 h with the liposomal suspension, followed by washing with normal Ringer for 30 min. Control experiments were performed on preparations pretreated with liposomes that contained heat-inactivated anti-IP_3R_1 (see Methods). Application of 25 μM hU-II to preparations treated with anti-IP_3R_1 resulted in a peak MEPP frequency increase of only 1000% (clear triangles in Figure 7), significantly less than that seen in untreated (filled circles in Figure 7) or heat-inactivated anti-IP_3R_1-treated muscles (clear circles in Figure 7), that is, the peak response to hU-II was reduced 83% with anti-IP_3R_1 treatment. Interestingly, the time course of effect was almost

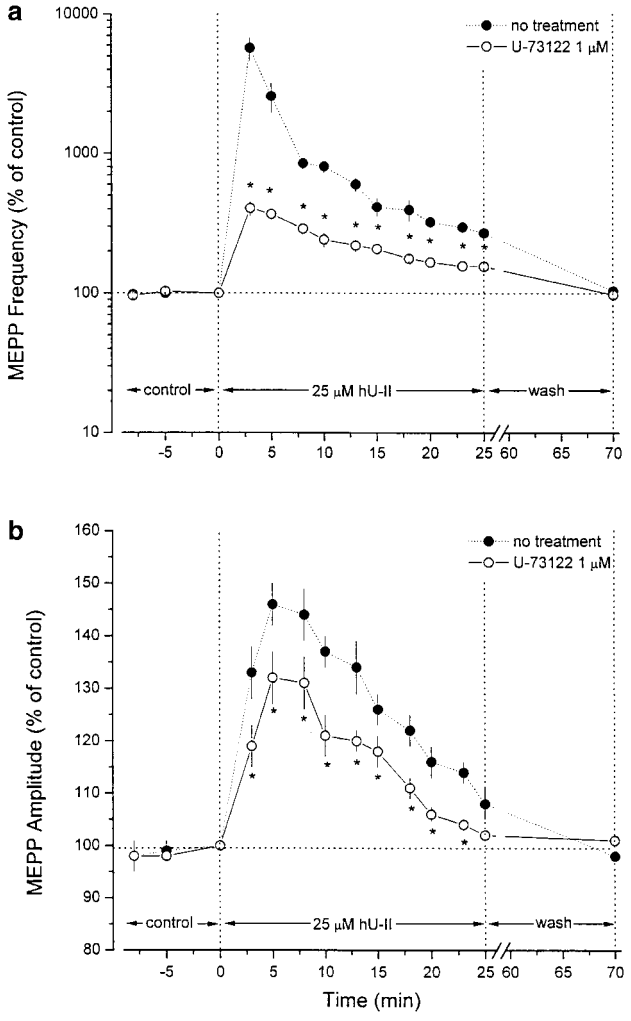

Figure 6 Effect of pretreatment with the phospholipase C inhibitor U-73122 on the MEPP frequency increase to 25 μM hU-II. Protocol is identical to that for Figure 2 except for 30 min pretreatment with 1 μM U-73122. (a) Administration of hU-II to treated muscles produces an MEPP frequency increase (clear circles) that is only 7% of the response obtained in untreated preparations (filled circles), that is, the effect of hU-II is reduced by 93%. Note the logarithmic ordinate. (b) Pretreatment with U-73122 causes a small (15%) but significant reduction in the MEPP amplitude increase to hU-II. For (a) and (b), each point represents the mean $\pm$ s.e.m. from six experiments. Asterisks denote statistically significant differences ($P<0.05$, paired t-test) from corresponding points for muscles not pretreated with U-73122.

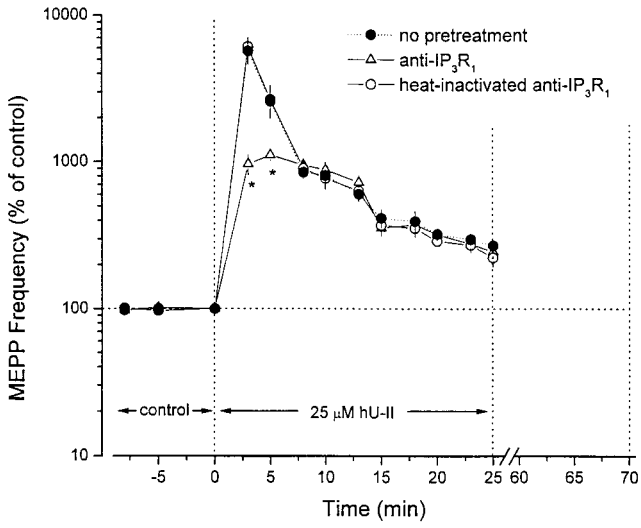

Figure 7 Effect of IP_3 type 1 receptor block on the MEPP frequency increase to 25 μM hU-II. Preparations were pretreated with antibodies against the IP_3 type 1 receptor (anti-IP_3R_1) by encapsulating either anti-IP_3R_1 or heat-inactivated anti-IP_3R_1 in liposomes (100 μl ml^{-1}) and perfusing muscles with the liposomal suspension for 1 h followed by 30 min of wash. The magnitude and time course of effect with heat-inactivated anti-IP_3R1 (clear circles) is indistinguishable from that in untreated preparations (filled circles). The peak increase to hU-II is reduced 83% (logarithmic ordinate) in preparations treated with anti-IP_3R_1 antibodies (clear triangles). Note that the points for all the three curves are almost identical for times >7.5 min. Each point represents the mean $\pm$ s.e.m. from six experiments. Asterisks denote statistically significant differences ($P<0.05$, paired t-test) from corresponding points in untreated muscles.

identical for all three curves at times >7.5 min. This could mean that the residual effect, after subtracting out the contribution of the type 1 IP_3 receptor, might be because of a mediator other than IP_3. Pretreatment with anti-IP_3R_1 or heat-inactivated anti-IP_3R_1 had no effect on the hU-II-induced increase in MEPP amplitude (results not shown).

Effect of hU-II in preparations pretreated with blockers of protein kinase C

The above results suggested that IP_3 was, at least in part, involved in the effect of hU-II. If so, then generation of IP_3 should be accompanied by the formation of diacylglycerol (DAG), which could in turn activate protein kinase C (PKC). To see if PKC was involved in the MEPP frequency increase with hU-II, we pretreated preparations for 30 min with two relatively specific blockers of PKC, namely, bisindolylmaleimide I and III (0.1 μM). However, subsequent application of 25 μM hU-II, in the continued presence of the inhibitors, produced MEPP frequency increases that were not significantly different from the responses seen in untreated muscles. There was also no change in the MEPP amplitude increase induced by hU-II (results not shown). This indicated that PKC was probably not involved.

Effect of hU-II in preparations pretreated with blockers of protein kinase A

Recent findings have suggested that the effect of hU-II may involve protein kinase A (PKA) (Filipeanu *et al.*, 2002). To test this possibility, we pretreated preparations for 30 min with either of two specific blockers of PKA, namely, H-89 (50 nM) and KT5720 (1 μM). Subsequent application of 25 μM hU-II, in the continued presence of the inhibitors, produced MEPP frequency increases of only 700% above control (Figure 8a), that is, the response to hU-II was reduced 88%. In addition, pretreatment with the PKA inhibitors caused a complete abolition of the MEPP amplitude increase to hU-II (Figure 8b). This indicated that PKA was involved in the hU-

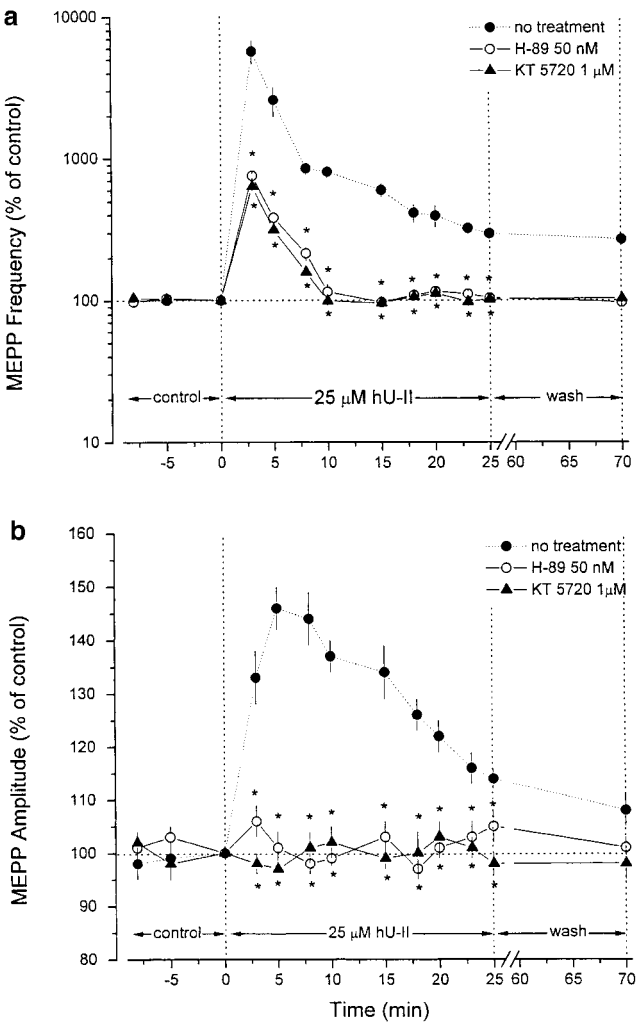

Figure 8 (a) Effect of pretreatment with the PKA inhibitors, H-89 and KT5720, on the MEPP frequency increase to 25 μM hU-II. Protocol is identical to that for Figure 2 except for 30 min pretreatment with either 50 nM H-89 (clear circles) or 1 μM KT5720 (filled triangles). For both PKA inhibitors, administration of hU-II to treated muscles produces an MEPP frequency increase that is only 12% of the response obtained in untreated preparations, that is the effect of hU-II is reduced by 88%. Note the logarithmic ordinate. (b) Effect of PKA inhibitors on the MEPP amplitude increase to 25 μM hU-II. Pretreatment with PKA inhibitors causes a complete reversal (clear circles, filled triangles) of the expected increase in MEPP amplitude to 25 μM hU-II (filled circles). Note the linear ordinate. For (a) and (b), each point represents the mean $\pm$ s.e.m. from six experiments, and asterisks denote statistically significant differences ($P<0.05$, paired t-test) from corresponding points in untreated muscles.

II-induced increase in both MEPP frequency and MEPP amplitude.

Effect of sequential exposure to hU-II

As noted above, the marked increase in MEPP frequency with hU-II was not sustained despite continuous exposure to the peptide (Figure 2a). We used two ways to test if this might be because of tachyphylaxis. In the first approach, we administered 1 μM hU-II followed 15 min later (at a time when the MEPP frequency increase had returned to control) by 25 μM

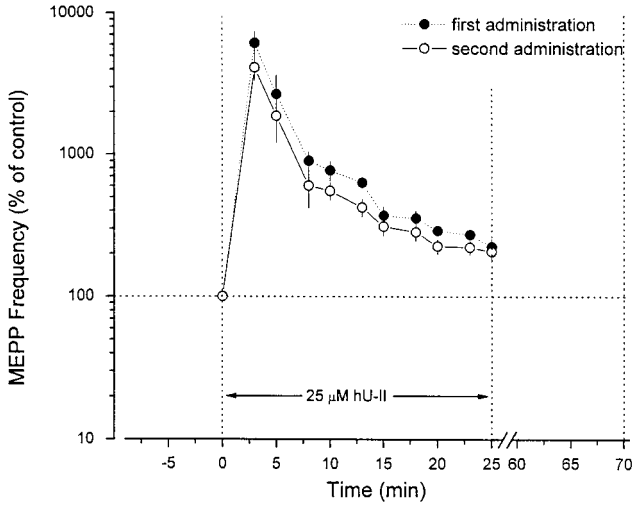

Figure 9 Effect of sequential administration of hU-II on the MEPP frequency response. Experimental protocol is identical to that described in Figure 2 except that continuous intracellular recordings were maintained for two exposures to 25 μM hU-II separated by 10 min of wash. Results of the first and second administrations are superimposed to examine for differences in magnitude and time course of MEPP frequency increase. Results show only a modest, nonsignificant ($P>0.05$, paired t-test) reduction in the response from the second exposure to hU-II. Each point represents the mean $\pm$ s.e.m. from six experiments.

hU-II. In five of six experiments, pre-exposure to 1 μM hU-II caused no reduction in the MEPP frequency increase to 25 μM hU-II. In the second approach, preparations were challenged with 25 μM hU-II for 25 min. This was followed by 10 min of wash with normal Ringer, then a second exposure to hU-II (25 μM) for 25 min. In this case, the second exposure to hU-II produced a time course of effect (clear circles in Figure 9) that mimicked that produced by the first exposure (filled circles in Figure 9), with only a slight, nonsignificant reduction in the overall response. The findings from both approaches suggested that the gradual decrease in MEPP frequency with exposure to hU-II was probably not because of loss of responsiveness of the frog motor nerve. These results, however, are in marked contrast to the tachyphylaxis seen in the contractile response of rat aorta on multiple exposures to hU-II (Camarda *et al.*, 2002).

Discussion

The principal observation made in this study is that both fU-II and hU-II accelerate spontaneous transmitter release at the frog motor nerve terminal. This means that the biological activity of U-II is not limited to cardiovascular tissues. The amino-acid sequence of U-II is well conserved across different species, and the cyclic ring in particular, where the biological activity resides, is identical from teleost to human (Coulouarn *et al.*, 1999). The observation that both the fU-II and the hU-II increase neurosecretion is consistent with the idea that the cyclic ring is the biologically active region. The finding that hU-II is more potent than fU-II at the frog motor nerve terminal is unexpected. However, it is not without precedent, if one considers, for example, the greater potency of salmon calcitonin over human calcitonin in humans (Marcus, 2001).

Nonetheless, the concentrations of hU-II employed in these studies are relatively high for a peptide, which indicates that the effect in the frog may be pharmacologic rather than physiologic. It is possible that a physiologic effect may be seen in mammals, since the threshold effect in rat spinal cord neurons occurs at nanomolar concentrations (Filipeanu *et al.*, 2002).

Our results show that the increase in MEPP frequency with 25 μM hU-II is not significantly changed by a three-fold increase in $[Ca^{2+}]_o$. This suggests that the response is not markedly dependent on influx of $[Ca^{2+}]_o$ and may instead be mediated by Ca^{2+} release from internal stores. In this regard, the action of hU-II may be similar to that of neurotrophin-3, which is independent of extracellular Ca^{2+} (He *et al.,* 2000). One possibility is that hU-II may stimulate the formation of inositol phosphates, as already demonstrated in rabbit aorta (Saetrum Opgaard *et al*., 2000), which may in turn increase MEPP frequency (Brailoiu & Miyamoto, 2000) by releasing Ca^{2+} from SER (Berridge, 1993). An involvement of SER is suggested by the results with TG, that is, depletion of SER with TG produces a 61% reduction in the MEPP frequency increase to hU-II (Figure 5). An involvement of IP_3 is supported by the results that show a 93% decrease in effect after treatment with the phospholipase C inhibitor U-73122 (Figure 6) and an 83% reduction in effect after blockade of intracellular IP_3 type 1 receptors (Figure 7).

However, the less-than-complete block with TG, U-73122 and anti-IP_3R_1 may indicate, in addition to IP_3, the participation of another intermediary. DAG and cyclic AMP (cAMP) are two agents that mediate protein phosphorylation and may be involved in transmitter release. Activation of PKC by DAG is probably not involved in the effect of hU-II, since the response to hU-II is not altered by the PKC inhibitors, bisindolylmaleimide I and III. A more likely possibility is activation of PKA by cAMP, since the response to hU-II is reduced 88% by blockers of PKA (Figure 8a). This is consistent with the finding that blockers of PKA, but not blockers of PKC, significantly attenuate the $[Ca^{2+}]_i$ increase produced by hU-II in rat spinal cord neurons (Filipeanu *et al.*, 2002).

The effect of U-II on MEPP amplitude is less straightforward. hU-II causes a dose-dependent increase in MEPP amplitude (Figure 2b), whereas fU-II causes an increase at low doses and a decrease in MEPP amplitude at higher doses (Figure 1b). An increase in MEPP amplitude is seen with liposomal delivery of IP_3 or with membrane-permeable analogues of cAMP (Van der Kloot & Branisteanu, 1992) but not with cADPR (Brailoiu & Miyamoto, 2000), nicotinic acid adenine dinucleotide phosphate (Brailoiu *et al.*, 2001), sphingosine-1-phosphate (Brailoiu *et al.*, 2002) or membrane-permeable analogues of DAG (Van der Kloot & Branisteanu, 1992). Accordingly, the increase in MEPP amplitude with hU-II is consistent with effects mediated by IP_3 and cAMP. An action of hU-II to stimulate formation of IP_3 and cAMP (and activation of PKA) may explain several of the present findings, that is, (1) the reversal of the hU-II-induced increase in MEPP amplitude by blockers of PKA; (2) the lack of effect of anti-IP_3R_1 on the hU-II-induced increase in MEPP amplitude (since activation of PKA would still occur even though IP_3R_1 are blocked); and (3) the slower time course of MEPP amplitude increase compared to MEPP frequency increase (if the former depends on delayed activation of PKA). Finally, the reason for the small effect of U-73122 on MEPP amplitude (Figure 6b) is unclear but may be because of nonspecific actions of the agent (Pulcinelli *et al.*, 1998).

In contrast to the situation in many central neurons, few peptides have been reported in vertebrate ventral horn motoneurons. The only known peptide that has been demonstrated in a population of motoneurons is calcitonin gene-related peptide (Skofitsch & Jacobowitz, 1985). Viewed in this context, U-II would be the second peptide identified in vertebrate motoneurons. Liu *et al.* (1999) have reported the presence of GRP14, which appears to be the receptor for U-II, in motoneurons and skeletal muscle. This suggests that U-II may have a function at the motor nerve terminal to enhance neurosecretion, perhaps in an autocrine fashion. The mechanism appears to involve, at least in part, release of Ca^{2+} from intracellular stores. This is consistent with recent studies in which U-II is able to increase $[Ca^{2+}]_i$ in the absence of extracellular calcium (Filipeanu *et al.*, 2002) and to mobilize Ca^{2+} in COS-7 cells transiently transfected with rat GRP14/pIRESpuromycin (Liu *et al.*, 1999).

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References

AMES, R.S., SARAU, H.M., CHAMBERS, J.K., WILLETTE, R.N., AIYAR, N.V., ROMANIC, A.M., LOUDEN, C.S., FOLEY, J.J., SAUERMELCH, C.F., COATNEY, R.W., AO, Z., DISA, J., HOLMES, S.D., STADEL, J.M., MARTIN, J.D., LIU, W.S., GLOVER, G.I., WILSON, S., MCNULTY, D.E., ELLIS, C.E., ELSHOURBAGY, N.A., SHABON, U., TRILL, J.J., HAY, D.W., OHLESTEIN, H.H., BERGSMA, D.J., & DOUGLAS, S.A. (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature,* **401,** 282 – 286.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature,* **361,** 315 – 325.

BRAILOIU, E., COOPER, R.L. & DUN, N.J. (2002). Sphingosine 1-phosphate enhances spontaneous transmitter release at the frog neuromuscular junction. *Br. J. Pharmacol.,* **136,** 1093 – 1097.

BRAILOIU, E. & MIYAMOTO, M.D. (2000). Inositol trisphosphate and cyclic adenosine diphosphate-ribose increase quantal transmitter release at frog motor nerve terminals: possible involvement of smooth endoplasmic reticulum. *Neuroscience,* **95,** 927 – 931.

BRAILOIU, E., MIYAMOTO, M.D. & DUN, N.J. (2001). Nicotinic acid adenine dinucleotide phosphate enhances quantal neurosecretion at the frog neuromuscular junction: possible action on synaptic vesicles in the releasable pool. *Mol. Pharmacol.,* **60,** 718 – 724.

CAMARDA, V., RIZZI, A., CALÒ, G., GENDRON, G., PERRON, S.I., KOSTENIS, E., ZAMBONI, P., MASCOLI, F. & REGOLI, D. (2002). Effects of human urotensin II in isolated vessels of various species; comparison with other vasoactive agents. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **365,** 141 – 149.

CHARTREL, N., CONLON, J.M., COLLIN, F., BRAUN, B., WAUGH, D., VALLARINO, M., LAHRICHI, S.L., RIVIER, J.E. & VAUDRY, H. (1996). Urotensin II in the central nervous system of the frog *Rana ridibunda*: immunohistochemical localization and biochemical characterization. *J. Comp. Neurol.,* **364,** 324 – 339.

CONLON, J.M. (2000). Singular contributions of fish neuroendocrinology to mammalian regulatory peptide research. *Regul. Pept.,* **93,** 3 – 12.

COULOUARN, Y., JEGOU, S., TOSTIVINT, H., VAUDRY, H. & LIHRMANN, I. (1999). Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett.,* **457,** 28 – 32.

COULOUARN, Y., LIHRMANN, I., JEGOU, S., ANOUAR, Y., TOSTIVINT, H., BEAUVILLAIN, J.C., CONLON, J.M., BERN, H.A. & VAUDRY, H. (1998). Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 15803 – 15808.

DOUGLAS, S.A., SULPIZIO, A.C., PIERCY, V., SARAU, H.M., AMES, R.S., AIYAR, N.V., OHLSTEIN, E.H. & WILLETTE, R.N. (2000). Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br. J. Pharmacol.,* **131,** 1262 – 1274.

DUN, S.L., BRAILOIU, G.C., YANG, J., CHANG, J.K. & DUN, N.J. (2001). Urotensin II-immunoreactivity in the brainstem and spinal cord of the rat. *Neurosci. Lett.,* **305,** 9 – 12.

FILIPEANU, C.M., BRAILOIU, E., DUN, S.L. & DUN, N.J. (2002). U-II regulates intracellular calcium in dissociated rat spinal cord neurons. *J. Neurochem.,* **83,** 879 – 884.

HE, X.-P., YANG, F., XIE, Z.-P. & LU, B. (2000). Intracellular Ca^{2+} and Ca^{2+}/calmodulin-dependent kinase II mediate acute potentiation of neurotransmitter release by neurotrophin-3. *J. Cell. Biol.,* **149,** 783 – 791.

KIM, C.H. & LISMAN, J.E. (2001). A labile component of AMPA receptor-mediated synaptic transmission is dependent on microtubule motors, actin, and *N*-ethylmaleimide-sensitive factor. *J. Neurosci.,* **21,** 4188 – 4194.

LEE, H.C. (2001). Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu. Rev. Pharmacol. Toxicol.,* **41,** 317 – 345.

LIU, Q., PONG, S.S., ZENG, Z., ZHANG, Q., HOWARD, A.D., WILLIAMS, JR D.L., DAVIDOFF, M., WANG, R., AUSTIN, C.P., MCDONALD, T.P., BAI, C., GEORGE, S.R., EVANS, J.F. & CASKEY, C.T. (1999). Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem. Biophys. Res. Commun.,* **266,** 174 – 178.

MARCUS, R. (2001). Agents affecting calcification and bone turnover: calcium, phosphate, parathyroid hormone, vitamin D, calcitonin, and other compounds. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 10th edn., ed. Hardman, J.G., Limbird, L.L. & Gilman, A.G. pp. 1715 – 1743. New York: McGraw Hill.

MORI, M., SUGO, T., ABE, M., SHIMOMURA, Y., KURIHARA, M., KITADA, C., KIKUCHI, K., SHINTANI, Y., KUROKAWA, T., ONDA, H., NISHIMURA, O. & FUJINO, M. (1999). Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem. Biophys. Res. Commun.,* **265,** 123 – 129.

NOTHACKER, H.P., WANG, Z., MCNEILL, A.M., SAITO, Y., MERTEN, S., O'DOWD, B., DUCKLES, S.P. & CIVELLI, O. (1999). Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat. Cell Biol.,* **1,** 383 – 385.

PULCINELLI, F.M., GRESELE, P., BONUGLIA, A.M. & GAZZANIGA, P.P. (1998). Evidence for separate effects of U73122 on phospholipase C and calcium channels in human platelets. *Biochem. Pharmacol.,* **56,** 1481 – 1484.

SAETRUM OPGAARD, O., NOTHACKER, H., EHLERT, F.J. & KRAUSE, D.N. (2000). Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur. J. Pharmacol.,* **406,** 265 – 271.

SILINSKY, E.M. (1985). The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmacol. Rev.,* **37,** 81 – 132.

SKOFITSCH, G. & JACOBOWITZ, D.M. (1985). Calcitonin gene-related peptide: detailed immunohistochemical distribution in the central nervous system. *Peptides,* **6,** 721 – 745.

SMITH, R.J., SAM, L.M., JUSTEN, J.M., BUNDY, G.L., BALA, G.A. & BLEASDALE, J.E. (1990). Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J. Pharmacol. Exp. Ther.,* **253,** 688 – 697.

TAKEMURA, H., HUGHES, A.R., THASTRUP, O. & PUTNEY JR, J.W. (1989). Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool and not an inositol phosphate regulates calcium fluxes at the plasma membrane. *J. Biol. Chem.,* **264,** 12266 – 12271.

VAN DER KLOOT, W. & BRANISTEANU, D.D. (1992). Effects of activators and inhibitors of protein kinase A on increases in quantal size at the frog neuromuscular junction. *Pflügers Arch.,* **420,** 336 – 341.

ZERBES, M., BUNN, S.J. & POWIS, D.A. (1998). Histamine causes Ca^{2+} entry via both a store-operated and a store-independent pathway in bovine adrenal chromaffin cells. *Cell Calcium,* **23,** 379 – 386.

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