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Urodilatin and dopamine: A new interaction in the kidney

Marisa R. Citarella [a], Marcelo R. Choi [a], Mariela M. Gironacci [b], Cecilia Medici [a], Alicia H. Correa [a], Belisario E. Fernández [a,*]

[a] Cátedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, INFIBIOC, IQUIFIB, CONICET, Buenos Aires, Argentina
[b] Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, INFIBIOC, IQUIFIB, CONICET, Buenos Aires, Argentina

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ABSTRACT

Since renal natriuretic peptide urodilatin (URO) exerts similar natriuretic and diuretic actions to those of atrial natriuretic factor (ANF), we hypothesized that URO regulates renal dopamine (DA) availability, contributing to Na^+, K^+-ATPase inhibition. URO (1–100 nM) increased ^{3}H-DA uptake in outer and juxtamedullar renal cortex and medulla slices from Sprague Dawley rats. Hydrocortisone blocked URO-stimulated DA uptake, demonstrating that DA uptake was extraneuronal. The natriuretic peptide receptor type A antagonist anantin blocked URO-dependent increase of ^{3}H-DA uptake, while the natriuretic peptide receptor type C agonist ANF 4–23-amide did not modify URO effect on DA uptake, suggesting that only natriuretic receptors type A are involved. Co-incubation of URO and ANF did not show additive effects on DA uptake. To test whether URO effect involves changes in Na^+, K^+-ATPase activity we performed experiments in renal cortex samples of rats with DA synthesis and neuronal uptake inhibited by carbidopa and nomifensine, respectively. When endogenous DA synthesis was inhibited, URO or DA decreased Na^+, K^+-ATPase activity. URO and DA added together, further decreased Na^+, K^+-ATPase activity showing an additive effect on the sodium pump. Moreover, hydrocortisone reversed URO-DA over-inhibition of the enzyme, confirming that this inhibition is closely related to URO-stimulation on renal DA uptake. URO and DA could act via a common intracellular pathway to decrease sodium and water tubular reabsorption, contributing to its natriuretic and diuretic effects.

1. Introduction

Members of the natriuretic peptide system, such as urodilatin (URO) and atrial natriuretic factor (ANF), are closely involved in the modulation of sodium reabsorption in the kidney, a critical process for maintenance of extracellular volume and for long-term regulation of blood pressure [1]. URO is a peptide of 32 amino acids, isolated from human urine [2]. It is structurally similar to the 28-amino acid ANF, lengthened at the N-terminus of the ANF circulating hormone by Thr-Ala-Pro-Arg [3] and has the same 17 amino-acids loop structure as ANF, closed by a disulphide bridge [4,5]. Thus, URO appears to be produced from the same precursor that produces ANF in the heart but with different post-translational processing to yield the 32 residue peptide [6]. Both natriuretic peptides exert similar actions on the control of renal sodium and water excretion [3,7,8]. URO is a local hormone of renal origin [9] produced mainly in the distal tubule of the nephron and secreted luminally to exert a paracrine effect in the nephron mainly at the inner medullar collecting duct where the peptide inhibits Na^+ entry through amiloride-sensitive sodium channels [10]. Biological actions of URO are mediated by natriuretic peptide receptor type A (NPR-A), which is a guanylate cyclase-coupled receptor previously described for ANF [11], being this receptor expressed along proximal and distal segments of the nephron [1].

The precise regulation of renal sodium excretion depends on an interaction between autocrine, paracrine and endocrine factors, in which dopamine (DA) plays a central role [12,13]. DA causes a large increase in natriuresis that is mainly dependent on the inhibition of both proximal and distal tubular sodium reabsorption [14].

We have previously reported that ANF stimulates DA uptake by renal tubular cells through NPR-A receptors coupled to guanylate cyclase and cGMP as second messenger [15,16]. ANF natriuretic effect is diminished by DA D_1 receptor antagonists, as well as by carbidopa, an inhibitor of DA synthesis [17,18], demonstrating a close relationship between DA and natriuretic peptides, involved in the modulation of renal function.

Since URO exerts natriuretic and diuretic actions, we hypothesized that URO regulates renal DA availability and contributes with DA to inhibit Na^+, K^+-ATPase activity. We investigated URO effects on ^{3}H-DA uptake in renal samples and whether this effect involves over-inhibition on Na^+, K^+-ATPase activity.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 250–350 g (from the animal room of the Pathophysiology Department, School of Pharmacy and

* Corresponding author. Cátedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Junín 956, C 1113 AAD Buenos Aires, Argentina. Tel./fax: +54 11 4964 8268.
E-mail address: befernan@ffyb.uba.ar (B.E. Fernández).

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Biochemistry, University of Buenos Aires) were used. The animals were housed in cages, with a 12-h light/dark cycle and controlled temperature and humidity. They were given access to water and food *ad libitum* (Commercial rodents Purina chow, Cooperacion SRL, Argentina).

2.2. Drugs and solutions

The following drugs and solutions were used in the experiments: ^{3}H-Dopamine, 28.0 Ci/mmol of specific activity (New England Nuclear, Boston, MA, USA); urodilatin (95–126), hydrocortisone, nomifensine, anantin, 3-hydroxytyramine hydrochloride, atrial natriuretic peptide (ANF 1-28 rat), des (Gln 18-Ser 19-Gly 20-Leu 21-Gly 22) atrial natriuretic peptide fragment 4–23 amide, imidazol, ATP (adenosine 5′ triphosphate), bovine seroalbumin fraction V of Cohn, Folin reactive were from Sigma-Aldrich, Inc., Saint Louis, Missouri, USA. Carbidopa was gently provided by Dr. Victor Nahmod, Buenos Aires, Argentina. EcoLite, for liquid scintillation was from ICN Pharmaceutical Inc., CA, USA. Standard Krebs bicarbonate (SKB) solution of the following composition (mM) was used as incubation medium: 118 NaCl; 4.7 KCl; 1.2 $MgSO_4.7H_2O$; 1.0 NaH_2PO_4; 2.4 $CaCl_2$; 0.004 EDTA; 11.1 glucose; 0.11 ascorbic acid; 26.0 $NaHCO_3$.

2.3. Procedures

Rats were anesthetized with 10% w/v ethyl urethane (1.3 mg/kg body weight, i.p.). Both kidneys were excised and washed with fresh SKB to remove the residual blood. The renal cortex was dissected from the medulla and then the outer cortex was separated from the juxtamedullar cortex using a small scalpel. After then, slices of the three isolated regions were cut, minced and weighed.

2.4. Determination of ^{3}H-DA uptake

^{3}H-DA uptake was measured as previously described by us [15]. Briefly, tissues were placed in 2.0 mL SKB incubation medium in a Dubnoff incubator and pre-incubated at 37 °C, pH 7.40, bubbled with a gaseous mixture of 95% O_2 and 5% CO_2 for 15 min; nomifensine (50 μM) was added in the medium to avoid neuronal DA uptake. After preincubation, tissues were transferred to fresh SKB and incubated, in similar conditions, with 0.625 μCi/mL of ^{3}H-DA (22.32 nM), 17 μM nomifensine and the different inhibitors for 15 min. After that time, URO was added to the medium and the incubation continued for another 30 min period. Control groups were incubated in the absence of URO. The following experiments were carried out in samples from outer renal cortex.

2.5. URO effects on ^{3}H-DA uptake

A concentration–response curve to URO (1 pM to 100 nM) was performed to examine URO effects on ^{3}H-DA uptake. The following groups were studied: control and incubated with 1, 10 and 100 pM and 1, 10 and 100 nM URO.

A time course curve was carried out to study URO effects on ^{3}H-DA uptake at different times (5, 10, 15, 20 and 30 min). The following groups were studied: control and incubated with 10 nM URO.

To investigate URO effects on ^{3}H-DA uptake in different areas of the kidney, ^{3}H-DA uptake was determined in samples from outer cortex, juxtamedullar cortex and medulla, in the following groups: control and incubated with 10 nM URO.

2.6. Characterization of DA non-neuronal uptake

The effect of URO on ^{3}H-DA uptake was studied in the presence of the non-neuronal uptake blocker hydrocortisone (HC). The following groups were examined: (a) control, and incubated with: (b) 10 nM URO; (c) 100 μM HC or (d) 100 μM HC plus 10 nM URO.

2.7. Additive effects of URO and ANF on DA uptake

To test possible ANF-URO additive effects, ^{3}H-DA uptake was determined in the following groups: (a) control, and incubated with: (b) 10 nM URO; (c) 100 nM ANF; (d) 10 nM URO plus 100 nM ANF.

2.8. Identification of URO receptor

To analyze if NPR-A was involved in URO effects on ^{3}H-DA uptake, the following groups were studied: (a) control, and incubated with: (b) 10 nM URO; (c) 10 nM anantin (specific NPR-A receptor blocker); (d) 10 nM anantin plus 10 nM URO.

To analyze if the NPR-C was involved in URO effects on ^{3}H-DA uptake, the following groups were studied: (a) control, and incubated with: (b) 10 nM URO; (c) 10 nM ANF 4–23-amide (specific NPR-C receptor agonist); d) 10 nM ANF 4–23-amide plus 10 nM URO.

At the end of the incubation period, the tissues were washed with cold SKB solution, along 3 periods of 5 min each one, and then homogenized with 2.5 ml of 10% trichloroacetic acid. The homogenates were centrifuged at 1700 *g* at 4 °C for 30 min and tritium activity in the supernatants was determined by usual scintillation counting method. Results of ^{3}H-DA uptake are expressed as d.p.m./g of fresh tissue.

2.9. Effects of URO and DA on Na^+, K^+-ATPase activity

To test whether the increase in renal DA produced by URO-stimulated DA uptake is associated with changes in Na^+, K^+-ATPase

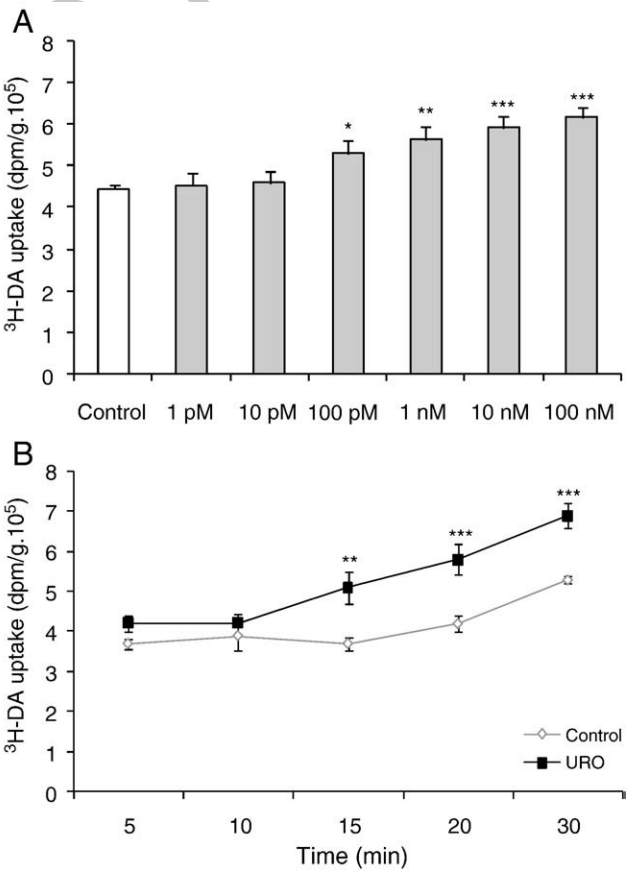

Fig. 1. A. Effects of increasing concentrations (1 pM–100 nM) of urodilatin (URO) on ^{3}H-dopamine (^{3}H-DA) uptake in experiments carried out *in vitro* in isolated outer renal cortex. ^{3}H-DA uptake is presented as dpm/g±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with control. Number of samples: 8–14. B. Effects of 10 nM URO on the time-course curve of ^{3}H-DA uptake in experiments carried out *in vitro* in isolated outer renal cortex samples, between 5 and 30 min. ^{3}H-DA uptake is presented as dpm/g±SEM. **$p<0.01$; ***$p<0.001$ compared with control. Number of samples: 6–15.

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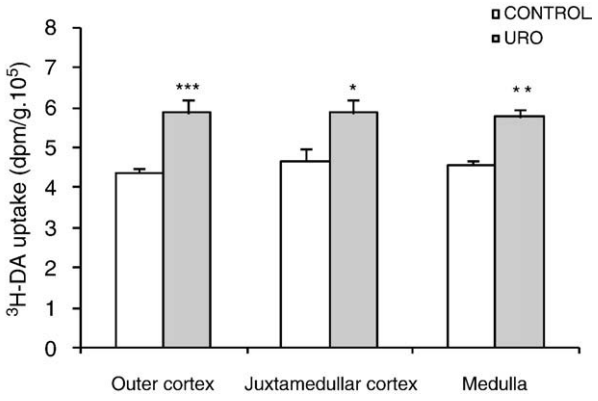

Fig. 2. Effects of 10 nM URO on ^{3}H-DA uptake in experiments carried out *in vitro* in isolated outer or juxtamedullar renal cortex or medulla samples. ^{3}H-DA uptake is presented as dpm/g±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with control. Number of samples: 8–14.

activity, experiments were performed in the presence of nomifensine (to avoid neuronal DA uptake) and carbidopa (to avoid DA synthesis). Carbidopa was administered *in vivo* (200 μg/kg, i.p., 24 and 2 h before animal sacrifice) and *in vitro* (100 μM in the medium, along the preincubation and incubation periods). URO effects were tested in the presence and in the absence of radiounlabeled DA and the non-neuronal DA uptake blocker HC.

The following groups were studied: (a) Control; and incubated with: (b) 100 μM carbidopa; c) 1 μM DA plus 100 μM carbidopa; d) 10 nM URO plus 100 μM carbidopa; e) 100 μM HC plus 100 μM carbidopa; f) 10 nM URO plus 1 μM DA and 100 μM carbidopa and g) 100 μM HC plus 10 nM URO plus 1 μM DA and 100 μM carbidopa.

Tissues were incubated for 30 min as described above and then homogenized (1:10 weight/volume) in 25 mM imidazole-1 mM EDTA-0.25 M sucrose solution and centrifuged at 5.000 rpm at 4 °C for 15 min. Na^+, K^+-ATPase activity was assayed in the supernatant as previously described [16]. ATPase activity was measured by colorimetric determination of released orthophosphate [19,20]. Protein was determined by the method of Lowry et al. [21]. Results are expressed as percentage of Na^+, K^+-ATPase activity, considering control values as 100%.

2.10. Statistical analysis

All values are expressed as mean±S.E.M. Data were processed using Graph Pad In Stat Software (San Diego, CA, USA). The Student's *t*-test, one way analysis of variance (ANOVA) and the Tukey test were

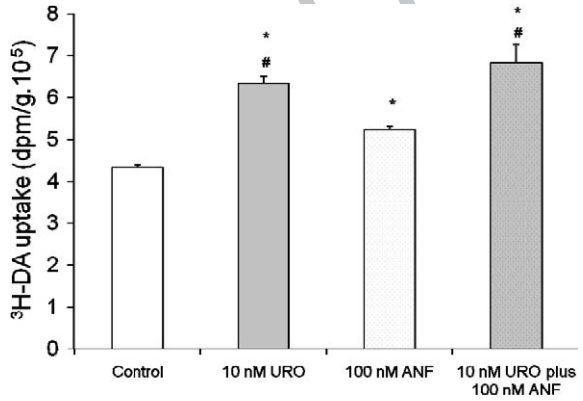

Fig. 3. Effects of 10 nM URO on 3H-DA uptake in experiments carried out *in vitro* in isolated outer renal cortex samples in the presence and in the absence of 100 nM ANF. ^{3}H-DA uptake is presented as dpm/g±SEM. *$p<0.001$ compared with control; $^{\#}p<0.001$ vs ANF. Number of cases: 7–12.

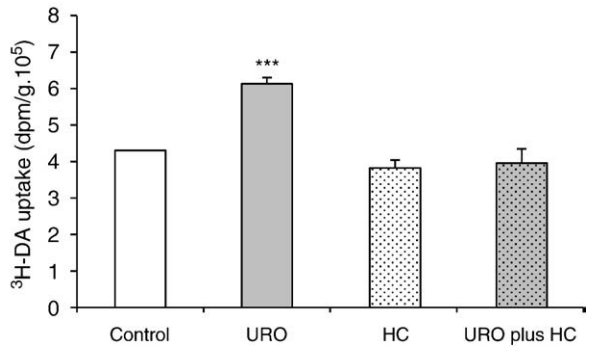

Fig. 4. Effects of 10 nM URO on ^{3}H-DA uptake in experiments carried out *in vitro* in isolated outer renal cortex samples in the presence or absence of 100 μM hydrocortisone (HC). ^{3}H-DA uptake is presented as dpm/g±SEM. ***$p<0.001$ compared with control or HC or URO plus HC. Number of samples: 6–9.

performed when it corresponded. *P* values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effects of URO on ^{3}H-DA uptake

Fig. 1A shows the effects of increasing concentrations of URO (1 pM to 100 nM) on ^{3}H-DA uptake in renal outer cortex. 100 pM to 100 nM

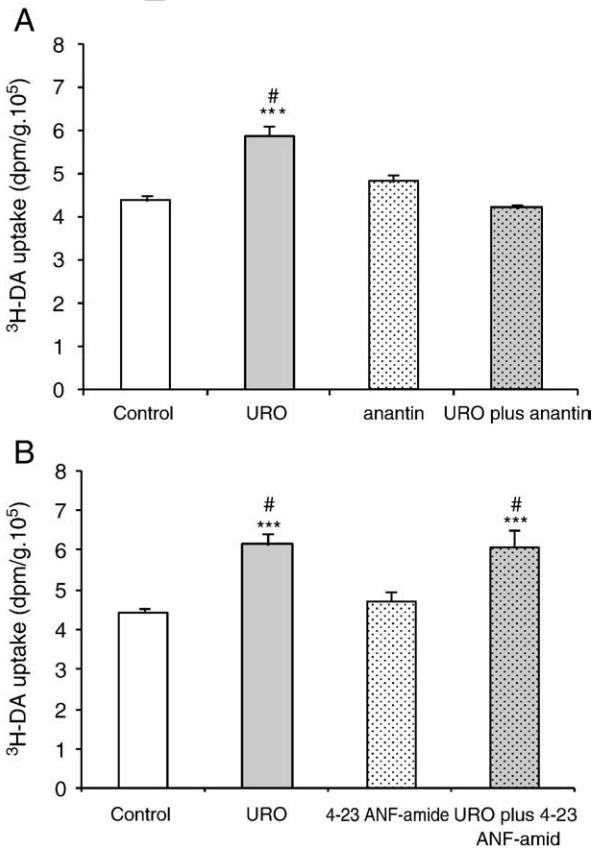

Fig. 5. A. Effect of 10 nM URO in the presence or absence of 10 nM anantin on ^{3}H-DA uptake in experiments carried out *in vitro* in isolated outer renal cortex samples. ^{3}H-DA uptake is presented as dpm/g±SEM. $^{\#}p<0.01$ compared with anantin. ***$p<0.001$ compared with control or 10 nM URO plus anantin. Number of samples: 6–8. B. Effect of 10 nM URO in the presence or absence of 10 nM ANP 4–23-amide on ^{3}H-DA uptake in experiments carried out *in vitro* in isolated outer renal cortex samples. ^{3}H-DA uptake is presented as dpm/g±SEM. $^{\#}p<0.05$ compared with ANP 4–23-amide. ***$p<0.001$ compared with control. Number of samples: 6–8.

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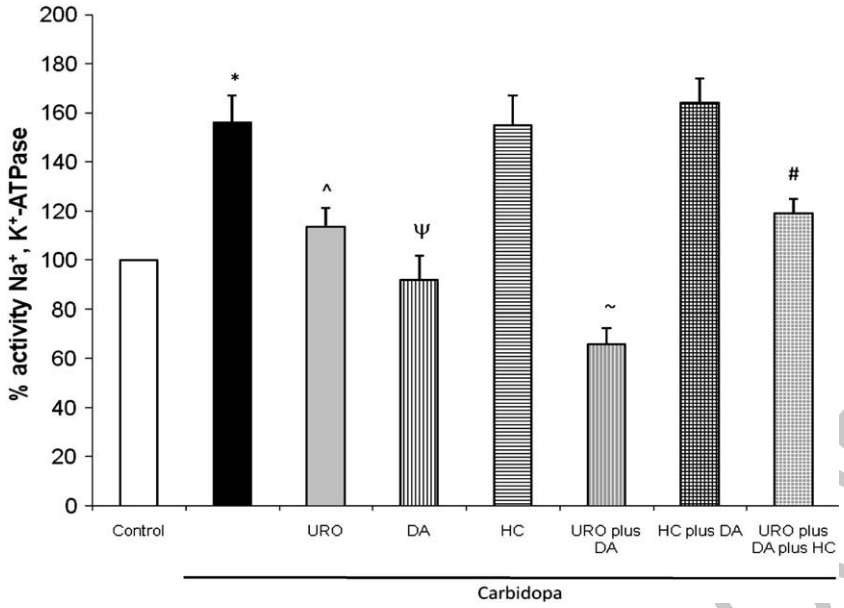

Fig. 6. Effects of URO, dopamine (DA) and hydrocortisone (HC) on Na^+, K^+-ATPase activity calculated as percentage of Na^+, K^+-ATPase activity of control values ± S.E.M in outer renal cortex. The experiments were carried out in the absence (control) or in the presence of carbidopa. *$p<0.01$ compared with control; $^{\Psi}p<0.01$ compared with carbidopa alone; ^$p<0.05$ compared with carbidopa alone; $^{\sim}p<0.05$ compared with URO. $^{\#}p<0.05$ compared with HC plus DA. Number of samples: 6–11.

URO caused a significant increase in ^{3}H-DA uptake. The increase in DA uptake caused by 10 nM URO was more significant ($p<0.001$) than that of 100 pM ($p<0.05$) or 1 nM URO ($p<0.01$); thus 10 nM was chosen to carry out the studies.

Time course of ^{3}H-DA uptake between 5 and 30 min is shown in Fig. 1B. 10 nM URO increased DA uptake at 15 min and this effect lasted up to 30 min. Therefore, we carried out further studies on ^{3}H-DA uptake with 30 min-incubation period.

URO (10 nM) increased ^{3}H-DA uptake not only in renal outer cortex but also in renal juxtamedullar cortex and medulla (Fig. 2).

Since ANF increases ^{3}H-DA uptake in renal cortex [15], we studied whether both peptides added together potentiate their response on DA uptake. Fig. 3 illustrates that 10 nM URO exhibited more potency than 100 nM ANF to stimulate renal DA uptake (46.7 ± 3.9% and 21.0 ± 1.7% of increase in DA uptake with respect to control, respectively). URO and ANF simultaneously co-incubated did not show significant additive effects (57.8 ± 10.2% increase in DA uptake with respect to control).

3.2. Characterization of renal extraneuronal uptake

Fig. 4 shows the effects of the extraneuronal amine uptake blocker, 100 μM HC on renal ^{3}H-DA uptake, in the presence and in the absence of 10 nM URO. 100 μM HC by itself did not affect renal DA uptake but blunted the increasing effects of 10 nM URO on renal DA uptake, suggesting that URO modified extraneuronal DA uptake.

3.3. Identification of URO receptor

The natriuretic receptor subtypes coupled to the stimulatory activity of URO on DA uptake in renal cortex were studied. The selective NPR-A receptor blocker, 10 nM anantin, blocked 10 nM URO effect on DA uptake (Fig. 5A), suggesting the involvement of NPR-A receptors. In contrast, the specific NPR-C agonist, 10 nM ANF 4–23-amide, neither altered DA uptake nor modified the stimulatory effect of URO on DA uptake (Fig. 5B).

3.4. URO and DA effects on Na^+, K^+-ATPase activity

To test the possibility that URO-stimulated DA uptake may modulate Na^+, K^+-ATPase activity, we assayed the effect of URO and DA, added alone and together, on the enzyme activity. To rule out any influence from endogenous DA on the enzyme activity, endogenous DA was inhibited with carbidopa. Fig. 6 shows that when renal DA synthesis was inhibited by carbidopa, Na^+, K^+-ATPase activity increased as compared with controls and when DA or URO were added alone, the enzyme activity tended to diminish. Although, when DA and URO were added simultaneously, a significant decrease in Na^+, K^+-ATPase was observed. On the other hand, the addition of HC did not modify *per se* Na^+, K^+-ATPase activity as compared with carbidopa treated group, but the corticoid reversed URO-DA over inhibition of the enzyme.

4. Discussion

Our study shows that URO increases renal DA uptake through NPR-A receptors activation, resulting in a decreased Na^+, K^+-ATPase activity. In this way, URO may elicit its natriuretic and diuretic effects. In accord, we have previously demonstrated that ANF, another compound of this natriuretic peptides system, causes a stimulatory action on renal DA uptake [15]. The URO-stimulated DA uptake was faster and more potent than ANF, similarly to the renal excretory effect [10]. This could be due to the fact that ANF is easily degraded by endopeptidase EC 24.11, whereas URO is more resistant to the action of this enzyme [22]. Therefore, URO may be more relevant in regulating the natriuresis and diuresis [1,6]. In contrast to the stimulating action on DA uptake elicited by ANF [15] or URO (present results), ANG II, as a physiological antagonist of ANF, inhibits tubular DA uptake in the kidney through AT_1 receptors, leading to a decrease in dopaminergic activity and reinforcing ANG II hypertensive effects [23].

URO increased renal DA uptake at 15 min of incubation and this stimulation lasted up to 30 min period (present results). There are two sources of DA in the kidney: neuronal and extraneuronal. The extraneuronal source includes DA uptaken from the blood and the tubular fluid, while the neuronal source involves DA synthesized from L-Dopa [14,24,25]. Renal DA is located mainly in tubular cells, in the blood stream and in dopaminergic nervous endings. Our study was carried out in washed tissues to eliminate circulating DA and in the presence of nomifensine to avoid neuronal DA uptake by sympathetic nerve endings. Since no report demonstrated the presence of DA in others extraneuronal sites, such as the vessels wall, glomerular and mesangial cells and interstitial cells in comparable amount with

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tubular DA content, we can conclude that in our experimental conditions renal tubules may be the only structures involved in DA uptake.

The present study demonstrates that the URO-stimulated renal DA uptake is coupled to NPR-A receptors activation since anantin, that did not modify *per se* DA uptake, inhibited URO effects. In accord, we previously showed that ANF increases DA uptake in renal cortex through NPR-A receptors stimulation [15]. URO and ANF, added together, did not exhibit additive effects, showing that both natriuretic peptides share the same mechanism of action on DA uptake, which depends on NPR-stimulation [18]. Renal DA uptake was confirmed as non-neuronal monoamine uptake since HC inhibited URO enhancing effects on DA uptake. By the other hand, NPR-C are not involved in URO-induced increase of DA uptake in renal outer cortex, since the ANF analogue, ANF 4–23-amide that binds to NPR-C, did not alter URO effect.

URO increased DA uptake in outer and juxtamedullar cortex as well in medulla by the same magnitude, according the same concentration used in the experiments and to NPR-A presence and URO localization in these areas, although the highest concentrations of URO were found in distal segments of the nephron, predominantly located at the renal medulla [26]. Then, further experiments were carried out only in outer cortex.

DA coordinates the effects of natriuretic and antinatriuretic agents for the maintenance of sodium homeostasis and normal blood pressure [17]. DA acts as an intrarenal natriuretic agent by inhibiting Na^+, K^+-ATPase which is present in high concentration in the basolateral membrane of all tubular cells [27,28]. We have demonstrated that ANF, through stimulation of DA uptake, favors DA intracellular accumulation which in turn results in an over inhibition of renal Na^+, K^+-ATPase activity [16]. To examine whether URO stimulating effect on DA uptake was able by itself to modify Na^+, K^+-ATPase activity, the effects of endogenous renal DA and neuronal DA were discarded by inhibiting renal DA synthesis by carbidopa and neuronal DA uptake by nomifensine. Our study shows that renal Na^+, K^+-ATPase activity increased when DA synthesis was inhibited by carbidopa, in agreement with the decrease of DA availability. On the other hand, when exogenous DA was added, the activity of the enzyme decreased, according with the restored DA availability. URO or DA alone caused a decrease in sodium pump activity, but when they were present together the decreased Na^+, K^+-ATPase activity was even greater than when they were alone, showing an additive effect on the sodium pump. To assess if URO-induced inhibition of Na^+, K^+-ATPase activity is related to URO stimulated non-neuronal DA uptake, additional experiments were performed in the presence of HC, a known inhibitor of non-neuronal uptake, plus the DA synthesis inhibitor carbidopa to avoid any influence from endogenous DA. HC modified Na^+, K^+-ATPase activity as carbidopa did, but reversed URO-DA over inhibition of the enzyme, confirming that this inhibition is closely related to renal URO-stimulation DA uptake.

The biological effects of DA in the kidney are mediated by renal tubular DA-D_1 receptors and the consequently increase of cyclic adenosine guanosine-3′, 5′-monophosphate (cAMP) and protein kinase A (PKA) activation. DA-D_1 receptors are mainly intracellulary located in basal conditions, although, these receptors can be recruited to the plasma membrane, either by D_1 agonists or by the increase of intracellular DA availability [12,28]. It was also shown that ANF and its second messenger, cGMP, cause a rapid translocation of D_1 receptors to the plasma membrane [12]. Thus, an increase in cAMP or cGMP is the proposed mechanism for homologous and the heterologous, respectively, sensitization of D_1 receptors. The recruited D_1 receptors are functional in terms of G protein coupling, cAMP accumulation, and Na^+, K^+-ATPase inhibition [29]. Therefore, the increasing of URO-dependent uptake of DA, as well as cGMP induced generation by URO may favor D_1 receptors recruitment to the plasma membrane helping to sustain the response to DA and providing a general mechanism by which peptide hormones can regulate sodium homeostasis indirectly via sensitization of DA receptors [30].

URO effects on DA uptake may be mediated by different mechanisms such as increase of transporters, changes in cell membrane potential and/or alteration in the carrier affinity. Several organic cation transporters (OCTs) have been cloned and characterized including electrogenic and electroneutral OCTs named OCT 1-3 and OCTN 1-3, respectively [31,32]. Some of them have been identified in the kidney and are able to transport DA along all the nephron segments, including outer and juxtamedullar cortex and medulla [33]. OCTs are under the control of PKA, PKC and also PKG, since many putative phosphorylation sites can be identified in their intracellular domain [26,32,34]. Our results show that basal DA uptake was similar in the three segments studied suggesting that all the machinery necessary to incorporate DA into the renal cells is present in those zones. DA inhibition on renal transport of sodium can be observed in proximal tubules, thick ascendant segment of Henle loop and cortical and medullar collector tubules [35,36]. Previous filtered or secreted DA by the proximal tubules into the tubular flow may be re-uptaken by OCT2 at distal tubules [37]. In addition, Grundemann et al. [38] demonstrated that OCT2 is able to transport tritiated DA. Moreover, the inhibition of renal transporters by GBR 12909, induced antidiuresis and antinatriuresis, in agreement with inhibition of DA effects on sodium reabsorption [33]. So, we cannot disregard that URO may stimulate OCTs which in turn may uptake DA. Further experiments have to be done to test this hypothesis.

In summary, URO binds to NPR-A receptors like ANF and is able to inhibit proximal tubule Na^+, K^+-ATPase activity, acting as a paracrine factor [1]. DA and URO may act in concert to inhibit tubular sodium reabsorption, as a consequence of increased DA uptake, resulting in over inhibition of Na^+, K^+-ATPase activity. In this way, URO reproduces the mechanisms of sodium transport regulation triggered by ANF. Further studies must be performed in order to determine the intracellular events involved in URO-DA relationship signaling.

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