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Natriuretic effect of bufalin in isolated rat kidneys involves activation of the Na^+-K^+-ATPase-Src kinase pathway

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Arnaud-Batista FJ, Costa GT, de Oliveira IMB, Costa PPC, Santos CF, Fonteles MC, Uchôa DE, Silveira ER, Cardi BA, Carvalho KM, Amaral LS, Pôças ESC, Quintas LEM, Noël F, Nascimento NRF. Natriuretic effect of bufalin in isolated rat kidneys involves activation of the Na^+-K^+-ATPase-Src kinase pathway. *Am J Physiol Renal Physiol* 302: F959–F966, 2012. First published January 11, 2012; doi:10.1152/ajprenal.00130.2011.—Bufadienolides are structurally related to the clinically relevant cardenolides (e.g., digoxin) and are now considered as endogenous steroid hormones. Binding of ouabain to Na^+-K^+-ATPase has been associated, in kidney cells, to the activation of the Src kinase pathway and Na^+-K^+-ATPase internalization. Nevertheless, whether the activation of this cascade also occurs with other cardiotonic steroids and leads to diuresis and natriuresis in the isolated intact kidney is still unknown. In the present work, we perfused rat kidneys for 120 min with bufalin (1, 3, or 10 μM) and measured its vascular and tubular effects. Thereafter, we probed the effect of 10 μM 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4amine (PP2), a Src family kinase inhibitor, and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (UO126), a highly selective inhibitor of both MEK1 and MEK2, on bufalin-induced renal alterations. Bufalin at 3 and 10 μM profoundly increased several parameters of renal function in a time- and/or concentration-dependent fashion. At a concentration that produced similar inhibition of the rat kidney Na^+-K^+-ATPase, ouabain had a much smaller diuretic and natriuretic effect. Although bufalin fully inhibited the rat kidney Na^+-K^+-ATPase in vitro, its IC_{50} (33 ± 1 μM) was threefold higher than the concentration used ex vivo and all its renal effects were blunted by PP2 and UO126. Furthermore, the phosphorylated (activated) ERK1/2 expression was increased after bufalin perfusion and this effect was totally prevented after PP2 pretreatment. The present study shows for the first time the direct diuretic, natriuretic, and kaliuretic effects of bufalin in isolated rat kidney and the relevance of Na^+-K^+-ATPase-mediated signal transduction.

bufadienolides; sodium excretion; extracellular volume regulation; signal transduction; renal physiology

BUFADIENOLIDES ARE CARDIOTONIC steroids structurally related to the clinically relevant cardenolides (e.g., digoxin). Recently, some bufadienolides, now considered as steroid hormones, were also detected in mammals (2, 19, 23) and their (patho) physiological significance has started to be unveiled. Several works point out that the production of endogenous Na^+-K^+-ATPase ligands, including bufadienolides, is associated to hypertension and salt loading and the resulting natriuretic response is achieved by renal Na^+-K^+-ATPase inhibition and vasoconstriction due to vascular Na^+-K^+-ATPase inhibition (4, 5, 11, 13, 36).

Likewise, bufalin-like immunoreactivity was correlated to increased systolic pressure in rats and humans (28). Nevertheless, this putative natriuretic effect of cardiotonic steroids is still disputed as in the case of bufalin, one of the major components of the toad venom-prepared traditional Chinese medicine called Ch'an Su, where data are conflicting. Pamnani et al. (30) reported that bufalin increased urinary volume, excretion of sodium and potassium after intravenous infusion in rats. In contrast, equimolar doses of ouabain produced significantly smaller effects on urinary volume and sodium excretion (30). On the other hand, bufalin decreased glomerular filtration rate (GFR), urinary flow (UF), and urinary sodium excretion (ENa^+) in male Sprague-Dawley rats that received intravenous infusion of bufalin (31). Eliades et al. (12) reported that intravenous infusion of bufalin to dogs did not cause significant diuresis, natriuresis, or alteration in renal blood flow. The conflicting results obtained with bufalin in vivo are probably due to differences in animal species/strain, dose range, route of administration, and/or experimental conditions (conscious vs. anesthetized rats; uniphrectomized vs. intact animals). More importantly, the in vivo evaluation of renal effects of bufadienolides may be misleading due to their potent systemic vascular effects allied to extrarenal regulatory mechanisms (hormonal, neuronal) and putative hepatic biotransformation.

It is now well-established that binding of cardiotonic steroids to Na^+-K^+-ATPase can also activate several signal transduction pathways, e.g., a downstream phosphorylation cascade via Src kinase-Ras-Raf-ERK1/2 pathway, which can be independent on Na^+-K^+-ATPase ion pumping activity (21, 22, 37, 40). Indeed, the interaction between the α subunit of Na^+-K^+-ATPase and Src kinase family forms a functional signaling complex (21, 37). The activation of this signaling pathway in the basolateral membrane of renal epithelial cells has been shown to induce caveolin-1- and/or clathrin-dependent endocytosis of Na^+-K^+-ATPase (24, 25) and decrease the expression and activity of the Na^+/H^+ exchanger isoform 3 (NHE3) in the apical membrane, reducing sodium reabsorption (9, 29). The regulation of the NHE3 trafficking and subsequent inhibition of its activity occurred independent of intracellular Na^+ concentration alterations (9). Whether the activation of

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this cascade leading to diuresis and natriuresis operates in the isolated intact kidney is still unknown. Although similar in structure, the different cardiotonic steroids exhibit diverse biological responses (11) so that these effects observed with ouabain cannot be necessarily extrapolated to bufalin.

The present study was conducted to compare the effects of bufalin on renal Na^+-K^+-ATPase activity in vitro and its direct effects on renal function in the ex vivo isolated, perfused kidney. This model keeps renal function free of the interference of humoral factors, neurotransmitters, and blood-borne vasoactive substances. In addition, we evaluated the impact of the Na^+-K^+-ATPase-Src kinase-ERK1/2 signal on bufadienolide effect.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (250–300 g) obtained from the Central Animal House of the Federal University of Ceará were used. The rats were maintained under the conditions of constant temperature (22 ± 2°C) and relative humidity (55 ± 10%), in 12:12-h light-dark cycles. The animals were fed with standard animal chow (Purina) and had free access to drinking water. All animals were randomized into groups. The study protocols were approved by the Institutional Ethics

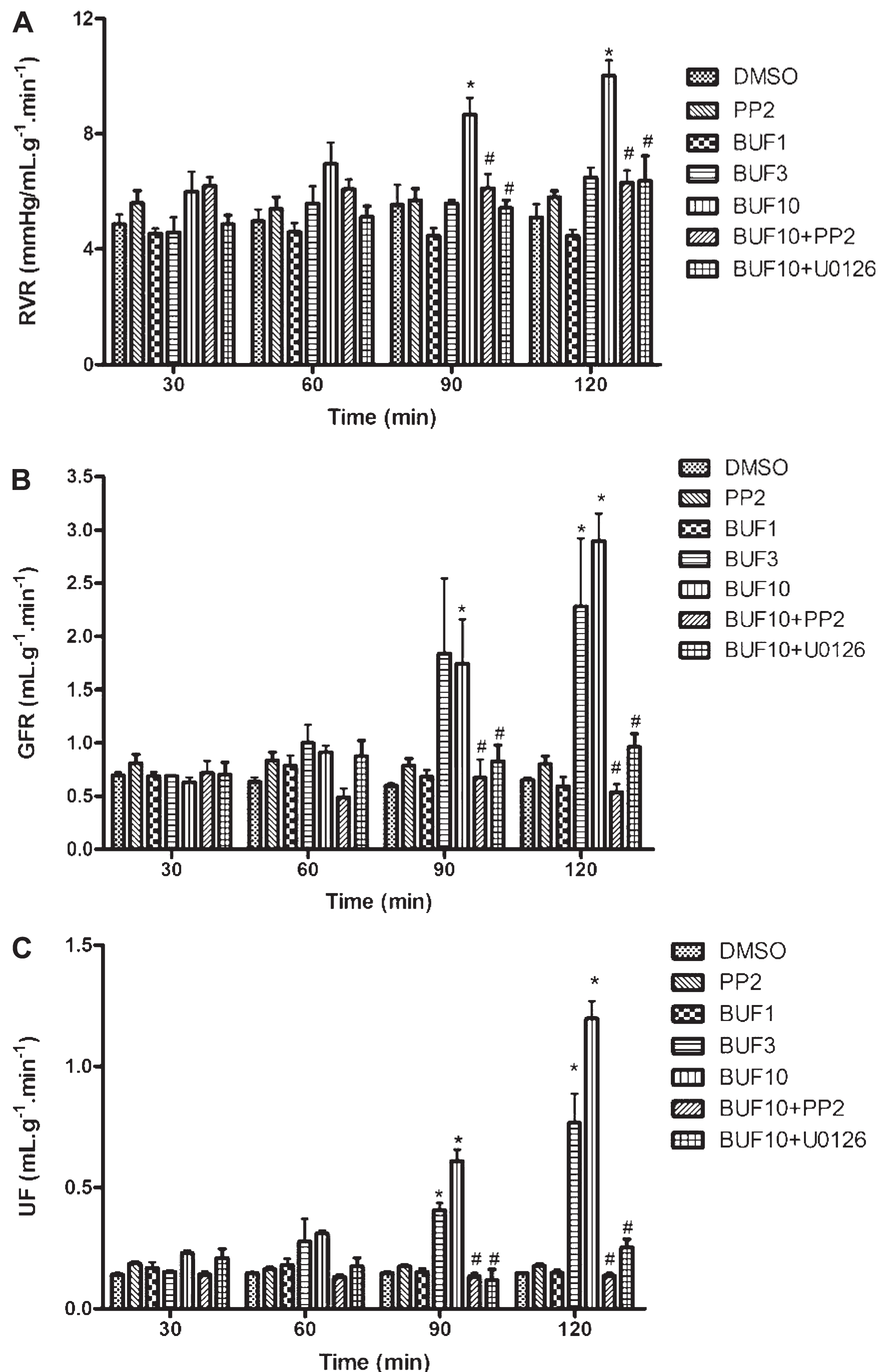

Fig. 1. Effect of bufalin (BUF), PP2 (a relatively specific Src kinase inhibitor), and U0126 (a highly selective inhibitor of both MEK1 and MEK2) on the renal vascular resistance (RVR; *A*), glomerular filtration rate (GFR; *B*), and urinary flow (UF; *C*) of perfused rat kidneys. BUF (1, 3, or 10 μM) was added after a 30-min period of controlled perfusion and its effect was measured 30, 60, and 90 min later. BUF10+PP2: PP2 (10 μM) was perfused 30 min before 10 μM bufalin. BUF10+UO126: UO126 (10 μM) was perfused 30 min before 10 μM bufalin. *$P < 0.05$ vs. DMSO. #$P < 0.05$ vs. BUF10, ANOVA followed by Bonferroni.

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Committee of the Ceará State University and are in accordance with the guidelines of National Institutes of Health on the use and care of animals for experimentation.

Drugs, reagents, and bufalin purification. 3-(4-Chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4amine (PP2) was purchased from Calbiochem (San Diego, CA). The reagents for standard solutions were of analytical grade obtained from either Sigma (St. Louis, MO) or Reagen (Rio de Janeiro, RJ, Brazil). Bufalin was isolated from the skin secretion of *Rhinella jimi* (formerly *Bufo paracnemis*) as previously described (27). The fractions were purified to homogeneity by HPLC on a preparative Shim-Pack PREP-ODS column (30 cm × 2.5) eluted isocratically (9 ml/min) with acetonitrile/water (1:2.5) and analyzed at 214 nm to detect different bufadienolides as well as other biologically active substances from this venom. The peak eluted at 48 min was recovered and chromatographed again to estimate its purity (>94%; the online version of this article contains supplemental data; see Supplemental Fig. S1).

The structure of 3,14-dihydroxy-5β-bufa-20,22-dienolide (bufalin) was unambiguously determined by spectrometric techniques such as infrared, mass spectrometry, and a combination of one- and two-dimensional nuclear magnetic resonance (NMR) methods (as an example, see H1 NMR spectral data in Supplemental Fig. S2). The bufadienolide was diluted in DMSO so that the final concentration of this solvent in the perfusate of the kidney was 0.05%.

Kidney perfusion and membrane preparation. The animals were anesthetized with pentobarbital sodium (50 mg/kg ip) and thereafter, 3 ml of 20% manitol were injected via the left femoral vein to make it easier to perform the cannulation of the urether. After incision in the linea alba, the right kidney was isolated and the right urether was clamped and cannulated with a PE-20 tube for urine collection in preweighed vials. The superior mesenteric and renal arteries were dissected and after clamping of the superior mesenteric artery, this vessel was cannulated and a stainless catheter (30 × 10 adapted needle) was introduced and displaced until the renal artery. The kidney was perfused in situ to avoid interruption of flow and then was transferred to the perfusion system. The perfusate consisted of a modified Krebs-Henseleit solution (MKHS) with the following composition (in mM): 118 NaCl, 1.2 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4.7H_2O$, 2.5 $CaCl_2.2H_2O$, and 25 $NaHCO_3$. Bovine serum albumin (6% wt/vol) was added to the MKHS and dialyzed for 48 h at 4°C against 10 Vol of MKHS. Immediately before the beginning of each perfusion, urea (0.1% wt/vol), inulin (0.05% wt/vol), and glucose (0.05% wt/vol) were added to the solution and pH was adjusted to 7.4. The total volume of perfusate solution was 100 ml and the flow rate was adjusted to achieve a perfusion pressure ~100 mmHg, as measured by an aneroid manometer. This was normally attained by using a flow rate ~20 ml/min. The solution was warmed to 37°C, the pH adjusted to 7.4, and gassed by means of an artificial lung (sylastic membrane) with 95% O_2-5% CO_2. Besides the manometer, a bubble trapper, a 1.2-μm millipore filter, and a flowmeter (Gilmont, Barrington, IL) were coupled to the system. A 30-min perfusion period was observed as an equilibration period. The perfusion pressure and

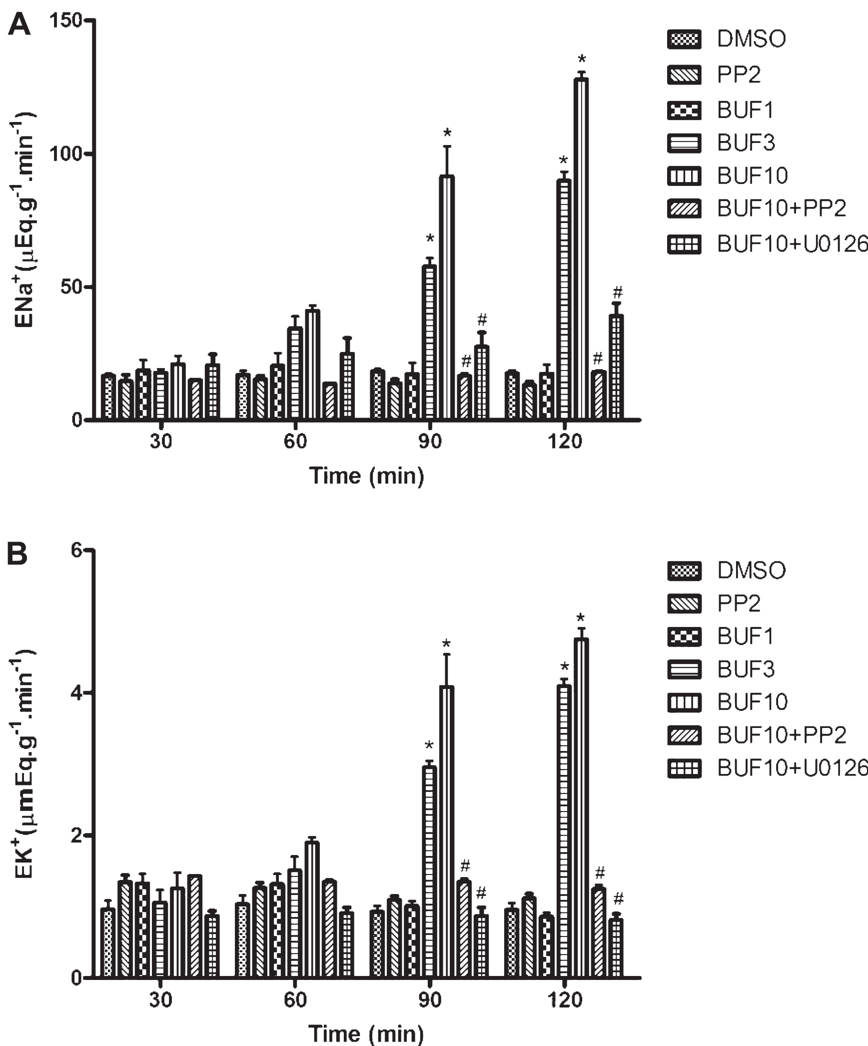

Fig. 2. Effect of BUF (1, 3, or 10 μM) on renal sodium (*A*) and potassium (*B*) excretion and the effect of 30-min period pretreatment with 10 μM PP2 (a relatively specific Src kinase inhibitor) or 10 μM UO126 (a highly selective inhibitor of both MEK1 and MEK2). *$P < 0.05$ vs. DMSO. #$P < 0.05$ vs. BUF10, ANOVA followed by Bonferroni.

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flow rate were recorded continuously. All the experiments lasted 120 min divided in four periods of 30 min: from 0 to 30 min (*t30*), from 31–60 min (*t60*), from 61 to 90 min (*t90*), and from 91 to 120 min (*t120*). During these 120 min, the first 30 are used as an internal quality control. DMSO (0.05% in MKHS), bufalin, or ouabain was added after the end of the 30-min control period. When pharmacological inhibitors were used, they were added in the perfusion system at *time 0*, i.e., 30 min before addition of bufalin. The glomerular filtration rate was determined by the inulin clearance, using the method described by Walser et al. (39) as modified by Fonteles et al. (14). Inulin was measured by a colorimetric method after direct acid hydrolysis with acetic acid and chloridric acid and by using diphenylamine as color reagent. Sodium and potassium concentrations in both urine and perfusate were analyzed by means of a flame photometer (International Laboratories, model 443 IL). Osmolality of the perfusate and urine was measured by means of a vapor pressure osmometer (Vapro model 5100c, Advanced Instruments, Needham Heights, MA).

After the end of the perfusion period, the kidney was rapidly frozen in liquid nitrogen and stored at −80°C. Crude membrane preparations

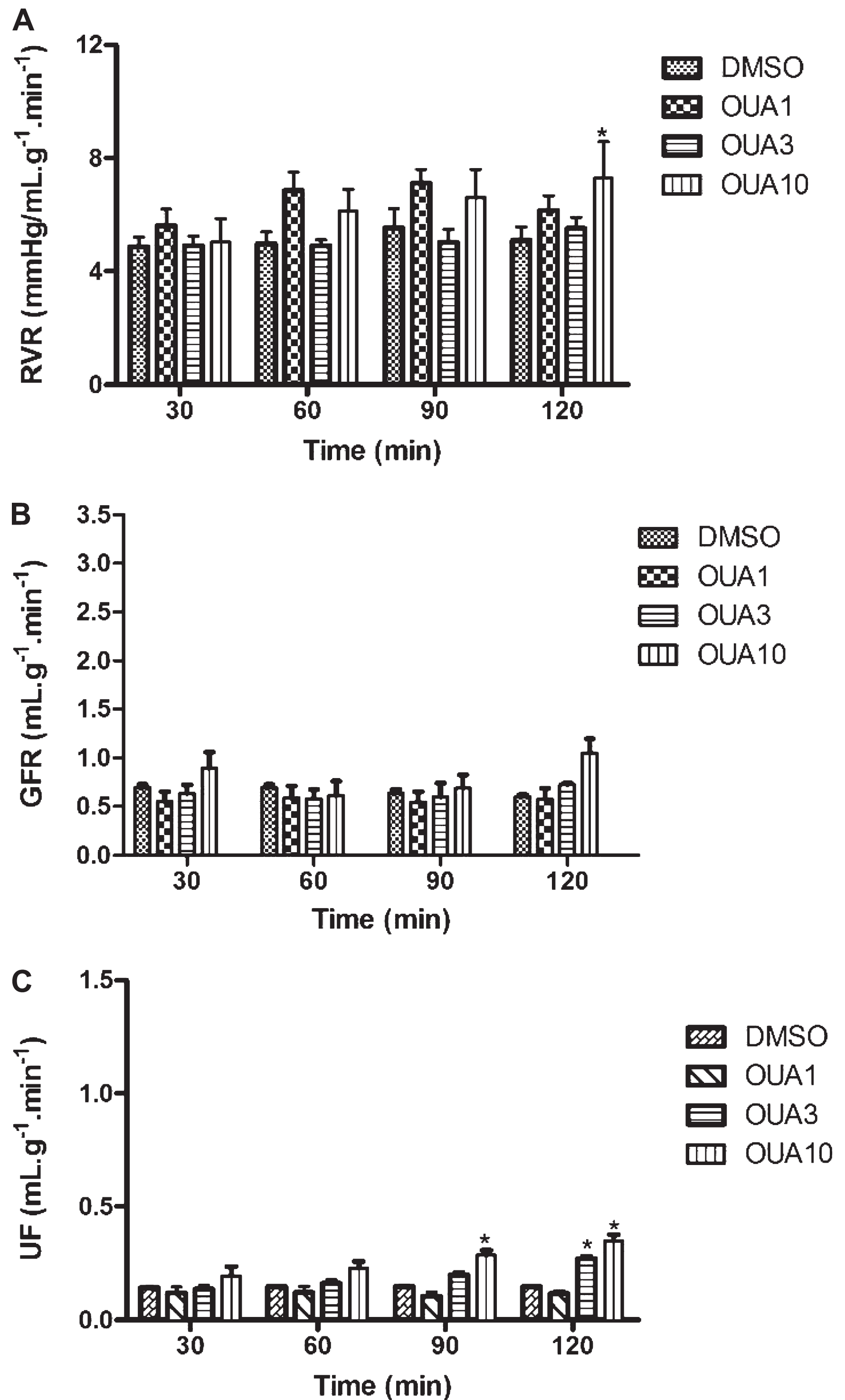

Fig. 3. Effect of ouabain (OUA) on the RVR (*A*), GFR (*B*), and UF (*C*) of perfused rat kidneys. OUA (1, 3, or 10 μM) was added after a 30-min period of controlled perfusion and its effect was measured 30, 60, and 90 min later. *$P < 0.05$ vs. DMSO, ANOVA followed by Bonferroni.

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were performed according to Quintas et al. (33), with modifications. Briefly, kidneys were minced and homogenized in Ultra-Turrax (9,500 rpm × 10 s and two steps of 13,500 rpm × 10 s) in a solution containing (in mM) 250 sucrose, 10 EGTA, 1 EDTA, 0.5 DTT, 1 PMSF, and 20 mM Tris·HCl buffer (pH 7.2), plus 15 μg/ml aprotinin, 2 μg/ml antipain, followed by additional homogenization in Dounce apparatus and ultracentrifugation (100,000 g_{av} × 60 min). The pellets were resuspended with the same buffer and stored at −80°C until use for Western blot analysis.

Na^+-K^+-ATPase preparation from rat kidney. To evaluate the direct effect of bufalin on Na^+-K^+-ATPase activity in vitro, adult male Wistar rats were killed by decapitation and their kidneys were rapidly excised and stored at −80°C. Preparations enriched in Na^+-K^+-ATPase were obtained by chaotropic treatment with 2 M KI for 1 h and 0.1% DOC (sodium deoxicholate) overnight, followed by differential centrifugation (26). The protein concentration was measured using bovine serum albumin as the standard, as previously described (34).

Inhibition of Na^+-K^+-ATPase activity. The Na^+-K^+-ATPase activity was determined by the Fiske and Subbarow method with slight modifications (32). The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain (ouabain-resistant activity). The maximal Na^+-K^+-ATPase activity was ~60 μmol Pi·mg $protein^{-1}·h^{-1}$, corresponding to 70% of the total ATPase activity. The quantity of protein was adjusted to hydrolyze no more than 10–15% of the substrate during the incubation period. The reaction was started by addition of kidney preparation, incubated at 37°C for 2 h, in a total volume of 0.5 ml. Unless otherwise stated, the incubation was performed in the presence of (in mM) 84 NaCl, 3 KCl, 3 $MgCl_2$, 1.2 $ATPNa_2$, 1 EGTA, 10 sodium azide, and 20 maleic acid-Tris buffer (pH 7.4). Inhibition curves were obtained in the presence of increasing concentrations of bufalin.

Western blot. Samples were resolved by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. After staining the membranes with Ponceau red to confer homogeneous transfer, they were incubated (in Tris-buffered saline containing 0.1% Tween 20) with 5% nonfat dry milk for 1 h, followed by the primary (anti-phospho or -total ERK1/2, 1:1,000 dilution; Cell Signaling Technology) and secondary (horseradish peroxidase-conjugated anti-rabbit, 1:2,000 dilution; Promega) antibodies, for 1 h each (34). Proteins were detected using chemiluminescence kit (Supersignal; Termo Fisher Scientific) by exposure to CL-Xposure films (Termo Fisher Scientific) and blots were analyzed by Image J software (NIH).

Statistical analysis. Results are shown as means ± SE of n experiments for each group. Differences between groups were analyzed by using the ANOVA followed by the Bonferroni test. The level of significance was set at $P < 0.05$.

The inhibition curves were fitted using a computerized nonlinear regression analysis of the untransformed data (Prism, GraphPad Software), assuming a single population of enzymes.

RESULTS

Effect of bufalin on renal function in the ex vivo isolated, perfused kidney. After the 20-min equilibration period, all functional parameters of the control perfused kidney remained stable throughout the experiment and were not changed by addition of the vehicle (0.05% DMSO in MKHS, $n = 5$). The

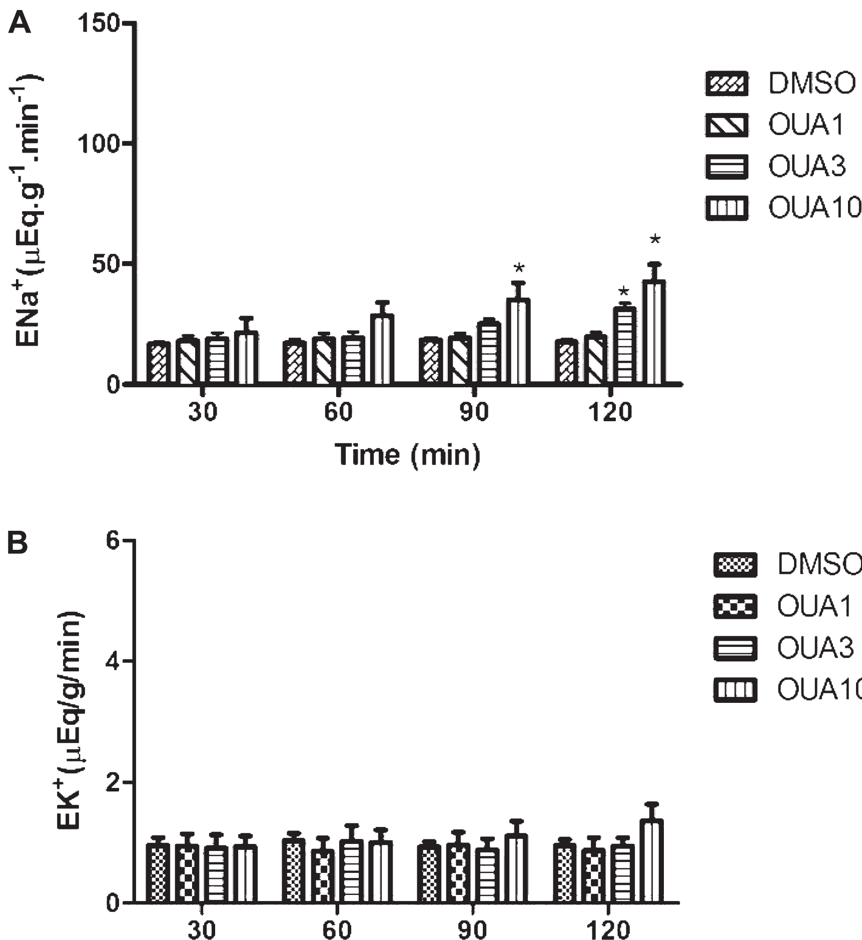

Fig. 4. Effect of OUA (1, 3, or 10 μM) on renal sodium (*A*) and potassium (*B*) excretion. *$P < 0.05$ vs. DMSO, ANOVA followed by Bonferroni.

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addition of 1 μM bufalin, after the 30-min equilibrium period, did not affect GFR, UF, and ENa^+ throughout the experiment (Figs. 1 and 2). On the other hand, perfusion with 3 and 10 μM bufalin increased renal vascular resistance (RVR; Fig. 1*A*), GFR (Fig. 1*B*), UF (Fig. 1*C*), ENa^+ (Fig. 2*A*), and urinary potassium excretion (EK^+; Fig. 2*B*) in a time- and/or concentration-dependent fashion. Compared with the vehicle, the highest increases occurred 90 min after bufalin addition (i.e., $t = 120$ min). For instance, 10 μM bufalin led to increments around two- (RVR, Fig. 1*A*), five- (GFR, Fig. 1*B*, and EK^+, Fig. 2*B*), and eightfold (UF, Fig. 1*C*, and ENa^+, Fig. 2*A*). Ouabain, a classical cardenolide used as comparator, has also a diuretic and natriuretic effect in our conditions (Figs. 3 and 4) but its intensity is much less than the one produced by bufalin, even when comparing concentrations that produced similar inhibition of the rat kidney Na^+-K^+-ATPase, i.e., 10 μM ouabain vs. 3 μM bufalin.

Effect of bufalin on rat kidney Na^+-K^+-ATPase activity in vitro. Cardiotonic steroids are known as specific inhibitors of Na^+-K^+-ATPase and their effect on mammalian kidney has been considered responsible for their natriuretic effect (4, 5, 13). To measure the direct inhibitory effect of bufalin on the rat renal Na^+-K^+-ATPase, we performed a concentration-effect curve using a purified preparation routinely used in our laboratory in diverse situations to assess ouabain potency (26, 32). Bufalin inhibited the rat kidney Na^+-K^+-ATPase in a concentration-dependent manner, with an IC_{50} of 33 ± 1 μM (Fig. 5), a value 2.5-fold lower than for ouabain tested in the same condition (86 ± 9 μM; Fig. 5). Note that the same IC_{50} for bufalin was achieved when using crude membrane preparations from the kidneys employed in our functional ex vivo experiments (IC_{50} in μM): nonperfused (33 ± 12), PP2-perfused (35 ± 14), bufalin-perfused (30 ± 10), and bufalin+PP2-perfused (35 ± 5).

Effect of Src kinase-Ras-Raf-ERK1/2 inhibition on the functional alterations induced by bufalin in perfused rat kidneys. To evaluate whether the novel Na^+-K^+-ATPase signal transduction pathway (phosphorylation cascade via Src kinase-Ras-Raf-ERK1/2) was involved in the bufalin effects observed in our model of perfused rat kidney, two critical kinases implicated in that cascade were studied, i.e., Src and ERK1/2. PP2 (10 μM), the widely used inhibitor of the Src family of protein kinases (6), was perfused before 10 μM bufalin and completely prevented bufalin-evoked renal effects (Figs. 1 and 2). PP2 alone had no significant influence on renal parameters (Figs. 1 and 2). Although PP2 is generally considered as a selective inhibitor of Src family tyrosine kinases, it is not specific so that we decided to test the effects of another inhibitor of this pathway. Figures 1 and 2 show that administration of a highly selective inhibitor of both MEK1 and MEK2 (10 μM U0126) before 10 μM bufalin completely prevented the bufalin-evoked renal effects. Moreover, when ERK1/2 was investigated in these kidneys, we observed a clear increase of the phosphorylated (activated) ERK1/2 expression after bufalin perfusion. Furthermore, this increase was totally prevented when the kidneys were pretreated with PP2 (Fig. 6).

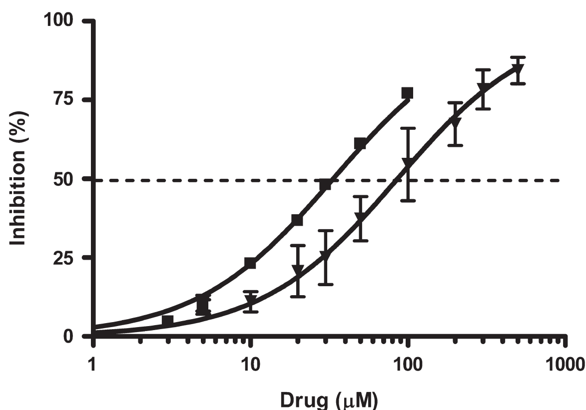

Fig. 5. Effects of BUF and OUA on rat kidney Na^+-K^+-ATPase activity. The effect of BUF (■) and OUA (▾) on Na^+-K^+-ATPase activity was tested in vitro using a partially purified preparation of kidneys from untreated rats. Results were expressed as percent of the inhibition measured in the presence of 1 mM OUA (means ± SE) and were obtained from 3 experiments performed in triplicate. Curves were drawn using the parameters fitted by nonlinear regression analysis with the model of sigmoidal concentration-response curve (Imax fixed at 100%).

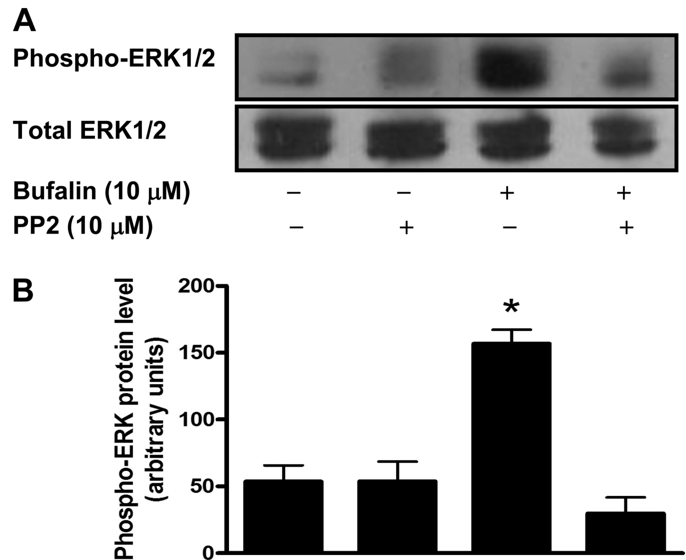

Fig. 6. Effects of BUF on ERK1/2 phosphorylation in rat kidney. The effect of BUF on ERK1/2 expression was measured ex vivo by performing immunoblots of phosphorylated and total ERK1/2 in isolated, perfused rat kidneys treated or not with BUF and the Src kinase inhibitor PP2. *A*: representative Western blot. *B*: densitometric data for the phosphorylated ERK1/2. *P = 0.0019 (n = 3), ANOVA followed by Tukey.

DISCUSSION

Endogenous bufadienolides are thought to contribute to sodium excretion during conditions of salt overload by their natriuretic properties and the primary underlying mechanism is supposed to be the inhibition of the renal Na^+-K^+-ATPase, resulting in decreased sodium transport of the renal tubular ultrafiltrate into the peritubular blood (5, 11, 13, 36), but a case-by-case evaluation is necessary since the different cardiotonic steroids exhibit diverse biological responses (11).

Here, we showed for the first time that bufalin exerted a profound diuretic and natriuretic effect in the isolated, perfused rat kidney, a model free of the interference of humoral factors, neurotransmitters, and blood-borne vasoactive substances and that this effect is higher than for ouabain. Qualitatively similar results were obtained by Brownlee et al. (7) and Pamnani et al. (30), but in vivo, so that they might be at least partially related

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to the hypertensive and inotropic effects of these cardiac steroids. In our model, 3 μM bufalin induced a significant increase in sodium excretion with increased GFR but with no increase in RVR. At 10 μM, the perfusion pressure was increased as well as sodium excretion and both GFR and RVR, as also observed after high concentrations of angiotensin II in the isolated kidney (17). Therefore, increased filtration, increased distal delivery, and decreased tubular reabsorption are probably involved in the bufalin natriuretic effect. Despite the cortical blood flow and GFR being kept under narrow limits of variation, under a wide range of perfusion pressure modification, the renal papilar blood flow is not autoregulated and with increased perfusion pressure the vasa recta capilar pressure and the interstitial pressure also increase leading to decreased sodium reabsorption (10). Bufalin induced similar increases in GFR at the concentration of 3 and 10 μM but there was a significantly more pronounced natriuresis with the concentration of 10 μM, which promoted higher RVR increase. The increased RVR associated with bufalin perfusion could be interpreted as a more prominent vasoconstrictor effect on postglomerular capillaries than in preglomerular capillaries. This is reinforced by the fact that ouabain, which only induced a transient increase in perfusion pressure, did not induce such increases in GFR and RVR. Also, an increased distal delivery is probably related to the kaliuresis induced by bufalin since this effect is not observed in the ouabain-perfused kidneys. The smaller increase of sodium excretion exerted by ouabain (2- vs. 7-fold induced by bufalin) is probably dependent mainly on tubular mechanisms.

Early works demonstrated a positive correlation between natriuresis and inhibition of renal Na^+-K^+-ATPase by ouabain (18, 38). Here, we showed that bufalin inhibits rat kidney Na^+-K^+-ATPase activity in vitro with an IC_{50} of 33 μM, a value consistent with the well-known low affinity of the kidney Na^+-K^+-ATPase isoform of this species (1). Interestingly, however, is that Na^+-K^+-ATPase inhibition and natriuresis did not occur in the same range of concentrations indicating that other mechanisms besides direct enzyme inhibition are involved in bufalin-induced effects. This assumption is highy supported by our observation that ouabain produced a much smaller diuretic and natriuretic effect than bufalin when comparing concentrations that produced the same level of Na^+-K^+-ATPase inhibition in the rat kidney. Novel roles of Na^+-K^+-ATPase have been intensely investigated for which its interaction with the nonreceptor tyrosine kinase Src has a key role for activating an entire phosphorylation cascade in which one of the main components is ERK1/2 (8, 21, 37). Through this mechanism, cardiotonic steroids induce basolateral Na^+-K^+-ATPase and apical NHE3 endocytoses in cultured kidney cells (9, 24, 25, 29) and this is attributed to work in vivo for urinary sodium excretion. Our results demonstrate that in isolated rat kidney this signal transduction system is stimulated by bufalin (revealed by ERK1/2 phosphorylation) and, more importantly, that it is critical for the renal effects produced by such bufadienolide (revealed by Src and MEK pharmacological blockade). Although the model (hepatocellular carcinoma) is quite different, the activation of ERK1/2 by bufalin has already been demonstrated (20).

As the vasoactive effect of cardiotonic steroids has been traditionally explained by Na^+ pump inhibition (3), little is known about the importance of Na^+-K^+-ATPase signal transduction. Although the existence of Na^+-K^+-ATPase-Src pathway has been demonstrated in rat A7r5 cells (15, 16) and human umbilical artery endothelial cells (35), to our knowledge, this is the first time that this signaling cascade is related to a functional vascular effect (increase of RVR, also blocked by PP2 and UO126).

Bufalin has not been irrefutably characterized in mammals, but bufalin derivatives as well as bufalin-like immunoreactive factors were detected (19, 23, 28). Our findings reveal that bufalin ex vivo acts as a natriuretic compound and Na^+-K^+-ATPase-mediated signal transduction might be considered the primary mechanism but whether there is a physiological or physiopathological significance in mammals, where endogenous cardiotonic steroids seem to circulate at nanomolar concentration, is still unknown.

In conclusion, the present study shows for the first time the direct diuretic, natriuretic, and kaliuretic effects of bufalin in isolated rat kidney and the relevance of Na^+-K^+-ATPase-mediated Src kinase-Ras-Raf-ERK1/2 pathway.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.J.A.-B., G.T.C., I.M.B.O., P.P.C., C.F.S., D.E.U., B.A.C., L.S.A., and E.S.P. performed experiments; F.J.A.-B., G.T.C., I.M.B.O., P.P.C., C.F.S., D.E.U., E.R.S., B.A.C., K.M.C., L.S.A., E.S.P., L.E.M.Q., and N.R.N. analyzed data; F.J.A.-B., G.T.C., I.M.B.O., P.P.C., M.C.F., D.E.U., E.R.S., B.A.C., K.M.C., L.S.A., E.S.P., L.E.M.Q., F.N., and N.R.N. interpreted results of experiments; F.J.A.-B., G.T.C., P.P.C., C.F.S., D.E.U., K.M.C., L.S.A., and E.S.P. prepared figures; M.C.F., L.E.M.Q., F.N., and N.R.N. edited and revised manuscript; E.R.S., K.M.C., L.E.M.Q., F.N., and N.R.N. conception and design of research; L.E.M.Q. and N.R.N. drafted manuscript; F.N. and N.R.N. approved final version of manuscript.

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