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Short communication

# Inhibitors of tyrosine phosphatases block angiotensin II inhibition of $Na^+$ pump

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**Abstract**

To determine how angiotensin II inhibits the $Na^+$ pump ($Na^+$, $K^+$–ATPase) in rat zona glomerulosa, we selectively blocked signaling proteins that could be activated by the angiotensin $AT_1$ receptor and known to affect $Na^+$ pump activity. Inhibitors of protein kinase C [calphostin C (1 μM); staurosporine (1 μM)], phospholipase $A_2$ [arachidonyl triflouromethyl ketone (25 μM); quinacrine (75 μM)], diacylgycerol lipase [RHC-80267 (5 μM)], and tyrosine phosphorylation [tyrphostin 47 (100 μM)] had no effect on angiotensin II inhibition of the $Na^+$ pump. On the other hand, inhibitors of tyrosine phosphatases [phenylarsine oxide (5 μM) and 4-bromotetramisole oxalate (100 μM)] blocked angiotensin II inhibition, where as inhibitors of serine/threonine phosphatases [okadaic acid (1 μM) and microcystin (1.5 μM)] did not. Thus, angiotensin II inhibition of the $Na^+$ pump may in part be mediated by a tyrosine phosphatase.


*Keywords:* Ouabain; $Na^+$; $Ca^{2+}$; Aldosterone; Na(+)–K(+)–exchanging ATPase

## 1. Introduction

Stimulation of the angiotensin $AT_1$ receptor by angiotensin II inhibits the $Na^+$ pump ($Na^+$, $K^+$–ATPase) in rat zona glomerulosa by an unknown mechanism that requires the presence of extracellular $Ca^{2+}$ (Hajnoczky et al., 1992). The goal of this study is to begin the identification of signaling proteins that mediate inhibition in order to understand how angiotensin II regulates the $Na^+$ pump and to evaluate how changes in $Na^+$ pump activity alter the secretion of aldosterone (Hajnoczky et al., 1992; Yingst et al., 1999). Such studies will contribute to our understanding of how angiotensin II regulates $Na^+$ transport, which is relevant to the use of angiotensin-converting-enzyme inhibitors and angiotensin II receptor antagonists to treat hypertension and congestive heart failure (Burnier and Brunner, 2000). Our approach was to test the possible involvement of known regulators of the $Na^+$ pump that are activated by the angiotensin $AT_1$ receptor in adrenal glomerulosa cells or by the subsequent increase in intracellular $Ca^{2+}$. The classes of proteins studied include protein kinase C (Efendiev et al., 1999; Tian et al., 1998), those that could generate arachidonic acid (Bharatula et al., 1998), and tyrosine kinases and phosphatases (Bodart et al., 1995; Kapas et al., 1995; Feraille et al., 1999).

## 2. Materials and methods

Okadaic acid and quinacrine were purchased from Sigma (St. Louis, MO). Phenylarsine oxide, 4-bromotetarmisole oxalate, and staurosporine were bought from Fluka (Milwaukee, WI). Tyrphostin 47, arachidonyl triflouromethyl ketone ($AACOCF_3$), and RHC-80267 were acquired from Biomol Research Laboratories (Plymouth Meeting, PA). Microcystin, calphostin C, phorbol-12-myristate-13-acetate, and monensin were procured from Calbiochem (San Diego, CA). PD 098059 was a gift from Parke-Davis (Ann Arbor, MI). $^{86}Rb$ was ordered from NEN Research Products (Boston, MA). Losartan was a gift from Merck (Rahway, NJ).

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A stock solution of 500 μM monensin was prepared in 0.5% dimethyl sufoxide (DMSO) at 37°C. Calphostin C, staurosporine, quinacrine, microcystin, tyrphostin 47, 4-bromotetramisole oxalate, and phenylarsine oxide were dissolved in DMSO such that the final DMSO concentrations in the assay ranged from 0.04% to 0.2%. $AACOCF_3$ and okadaic acid were prepared in absolute ethanol, resulting in ethanol concentration in the assay of 0.1%. All drugs were added to the assay 15–30 min before the addition of $^{86}Rb$.

### 2.1. Adrenal cell preparation

Adrenal capsules, containing the zona glomerulosa, were obtained from female Sprague–Dawley rats weighing 200–224 g. Glomerulosa cells were collagenase dispersed as previously described (Craven et al., 1988) in medium 199 (Gibco, Grand Island, NY) containing modified Earle's salts (130 mM NaCl, 4.0 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$), 10 mM HEPES, Na salt, 0.2% bovine serum albumin, and no bicarbonate at pH 7.4. Following dispersion the cells were incubated for 2 h to allow for recovery and continuously gassed with 100% $O_2$.

### 2.2. $Na^+$ pump activity

The activity of the $Na^+$ pump was measured as ouabain-sensitive $^{86}Rb$ uptake over a period of linear uptake as previously described (Hajnoczky et al., 1992). Quadruplicate samples were run in the presence and absence of 1 mM ouabain. Experiments were performed in a total volume of 150 μl with 100,000 cells and from 1 to 3 μCi of $^{86}Rb$ in a 96 well plate (Millipore, Cat. N. MADPN6550) maintained at 37°C on top of heated sand. Ouabain or buffer was added to all cells at time zero. Monensin at a final concentration of 10 μM was added at 20 min. Angiotensin II was added at a final concentration of 100 nM at 25 min. The flux was begun by adding $^{86}Rb$ at 30 min and ended at 35 min by rapid filtration of the cells onto membranes using a Millipore Multiscreen Assay System followed by six washes (~300 μl/wash) with ice cold medium 199. The membranes (containing the washed cells) were punched out and placed in 0.5 ml of 1% sodium dodecyl sulfate (SDS) to which scintillation fluid was added. The filter blank in this assay was $< 0.015\%$ of the total counts.

### 2.3. Statistics

Each data point is the mean and S.E. of quadruplicate samples of an individual experiment. Each experiment is representative of at least three similar experiments. The data were analyzed by a one-way analysis of variance. Groups were compared using a Bonferroni multiple comparisons test. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

In the studies presented here, $Na^+$ pump activity was maximally inhibited using 100 nM angiotensin II (Hajnoczky et al., 1992). The inhibitory effect of angiotensin II was completely reversed by 10 μM losartan (data not shown), confirming that inhibition was mediated by angiotensin $AT_1$ receptor (Hajnoczky et al., 1992).

### 3.1. Protein kinase C

High concentrations of calphostin C and staurosporine, which should inhibit all known isoforms of protein kinase C (Mizuno et al., 1995), had no effect on angiotensin II inhibition of $Na^+$ pump activity (Table 1A and B). Likewise, acute treatment with 0.1–1.0 μM phorbol-12-myristate-13-acetate did not affect $Na^+$ pump activity (data not shown). Thus, these data do not support the concept that angiotensin II inhibits the $Na^+$ pump through activation of protein kinase C.

### 3.2. Phospholipase $A_2$ and arachidonic acid

Neither $AACOCF_3$, a potent inhibitor ($K_i < 5 \times 10^{-5}$ mol fraction) of the 85 kDa cytoplasmic phospholipase $A_2$ (Street et al., 1993), nor quinacrine (mepacrine), a non-specific inhibitor of $Ca^{2+}$-dependent phospholipase $A_2$ activity (Hofmann et al., 1982), blocked the inhibitory effect of angiotensin II (Table 1C and D). However, arachidonic acid can also be produced by diacylglycerol lipase acting on diacylglycerol produced by the activation of phospholipase C by angiotensin II. We, therefore, tested the effects of RHC-80267 (5 μM), which inhibits diacyl-

Table 1
The effect of angiotensin II on sodium pump activity in the presence and absence of inhibitors of protein kinases, protein phosphatases, and phospholipase $A_2$

| | Control | + Angiotensin II[a] | Inhibitor |
|---|---|---|---|
| A | 4590 ± 212 | 1431 ± 97 | – |
| | 3872 ± 75 | 2036 ± 263 | + Calphostin (1 μM) |
| B | 4343 ± 215 | 1945 ± 120 | – |
| | 4465 ± 414 | 2350 ± 36 | + Staurosporine (1 μM) |
| C | 4554 ± 136 | 2411 ± 135 | – |
| | 4672 ± 177 | 2084 ± 112 | + $AACOCF_3$ (25 μM) |
| D | 5940 ± 155 | 3355 ± 79 | – |
| | 4686 ± 192 | 1994 ± 161 | + Quinacrine (75 μM) |
| E | 6714 ± 372 | 2391 ± 206 | – |
| | 6557 ± 361 | 2442 ± 275 | + Okadaic acid (1 μM) |
| F | 5548 ± 235 | 1691 ± 57 | – |
| | 4854 ± 277 | 2514 ± 112 | + Microcystin (1.5 μM) |

The values shown are the mean and S.E. of ouabain-sensitive $^{86}Rb$ uptake (cpm).
[a] ≤ 0.05 relative to corresponding controls.

glycerol lipase with an $IC_{50}$ of 5 μM (Moriyama et al., 1999). However, RHC-80267 had no effect (data not shown). Thus, our data do not suggest that arachidonic acid produced by diacylglycerol or phospholipase $A_2$ mediates angiotensin II inhibition of the $Na^+$ pump.

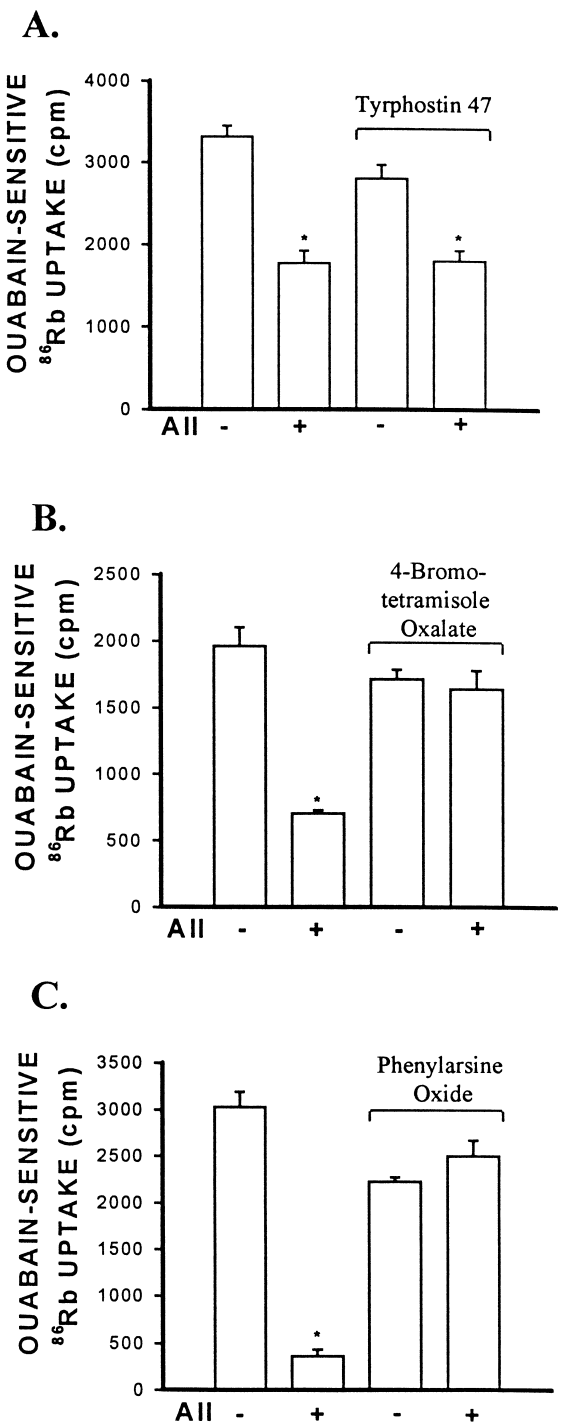


Fig. 1. Effect of angiotensin II on sodium pump activity in the presence and absence of: (A) tyrphostin 47 [100 μM]; (B) 4-bromotetramisole oxalate [100 μM]; and (C) phenylarsine oxide (5 μM). $^*P < 0.05$ relative to corresponding control.

### 3.3. Tyrosine phosphorylation

Angiotensin II inhibition was effectively blocked by both 4-bromotetramisole oxalate and phenylarsine oxide (Fig. 1B and C) which inhibit tyrosine phosphatases (Kovach et al., 1992; Scher et al., 1998; Liao et al., 1991). Tyrphostin 47, which inhibits tyrosine kinase activity (Gazit et al., 1989), did not block or reduce angiotensin II inhibition of the $Na^+$ pump, although it did tend to reduce the basal flux in the absence of the hormone (Fig. 1A). This reduction was statistically significant in one out of three experiments performed (data not shown). In contrast, neither okadaic acid nor microcystin, which inhibit many serine/threonine phosphatases (Bialojan and Takai, 1988; MacKintosh et al., 1990), altered inhibition of the $Na^+$ pump by angiotensin II (Table 1E and F). Thus, these data suggest that tyrosine phosphorylation plays a role in mediating inhibition of the $Na^+$ pump by angiotensin II.

## 4. Discussion

These studies are the first to implicate tyrosine phosphorylation in the mechanism by which angiotensin II inhibits the $Na^+$ pump in any cell. Specifically, they suggest that angiotensin II could inhibit the $Na^+$ pump by activating a tyrosine phosphatase. Our inhibitor studies do not implicate either protein kinase C or the production of arachidonic acid in the mechanism of $Na^+$ pump inhibition, although both are stimulated by the angiotensin $AT_1$ receptor in the zona glomerulosa and are known to regulate the $Na^+$ pump in other cells.

A role for tyrosine phosphorylation in the mechanism by which hormones regulate $Na^+$ pump activity is a novel concept suggested by the recent observation that insulin both stimulates $Na^+$ pump activity and increases the phosphorylation of $Tyr^{10}$ in the $\alpha$ subunit of the $Na^+$ pump in the proximal tubule (Feraille et al., 1999). There have been no studies to determine if angiotensin II increases the phosphorylation of the $Na^+$ pump in the zona glomerulosa. Thus, the target of phosphorylation could either be the $Na^+$ pump itself, a protein that interacts with the $Na^+$ pump, or part of the signaling pathway that alters $Na^+$ pump activity.

In addition to inhibiting tyrosine phosphatase activity, both phenylarsine oxide and 4-bromotetramisole have other effects, as do all other tyrosine phosphatase inhibitors, including orthovanadate (Morioka et al., 1998). For instance, 4-bromotetramisole inhibits alkaline phosphatase activity and phenylarsine oxide inhibits receptor internalization (Hunyady et al., 1991). What they have in common is their ability to inhibit tyrosine phosphatase activity, an observation that strengthens our conclusion.

In summary, these studies are the first to suggest that changes in tyrosine phosphorylation could be part of the mechanism by which angiotensin II inhibits the $Na^+$ pump

in the zona glomerulosa. As such, they will be useful in guiding future work. Direct biochemical conformation of the role of tyrosine phosphorylation and identification of the proposed phosphatase and its protein target will require additional research.

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## References

Bharatula, M., Hussain, T., Lokhandwala, M.F., 1998. Angiotensin II $AT_1$ receptor/signaling mechanisms in the biphasic effect of the peptide on proximal tubular $Na^+$, $K^+$–ATPase. Clin. Exp. Hypertens. 20, 465–480.

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. 256, 283–290.

Bodart, V., Ong, H., DeLean, A., 1995. A role for protein tyrosine kinase in the steroidogenic pathway of angiotensin II in bovine zona glomerulosa cells. J. Steroid Biochem. Mol. Biol. 54, 55–62.

Burnier, M., Brunner, H.R., 2000. Angioensin II receptor antagonists. Lancet 355, 637–645.

Craven, T.G., Kem, D.C., Schiebinger, R.J., 1988. Atrial natriuretic peptides: the role of phenylalanine on biological activity. Endocrinology 122, 826–830.

Efendiev, R., Bertorello, A.M., Pedemonte, C.H., 1999. PKC-beta and PKC-zeta mediate opposing effects on proximal tubule $Na^+$, $K^+$–ATPase activity. FEBS Lett. 456, 45–48.

Feraille, E., Carranza, M.L., Gonin, S., Beguin, P., Pedemonte, C., Rousselot, M., Caverzasio, J., Geering, K., Martin, P.Y., Favre, H., 1999. Insulin-induced stimulation of $Na^+$, $K^+$–ATPase activity in kidney proximal tubule cells depends on phosphorylation of the alpha-subunit at Tyr-10. Mol. Biol. Cell 10, 2847–2859.

Gazit, A., Yaish, P., Gilon, C., Levitzki, A., 1989. Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. J. Med. Chem. 32, 2344–2352.

Hajnoczky, G., Csordas, G., Hunyady, L., Kalapos, M.P., Balla, T., Enyedi, P., Spat, A., 1992. Angiotensin-II inhibits $Na^+/K^+$ pump in rat adrenal glomerulosa cells: possible contribution to stimulation of aldosterone production. Endocrinology 130, 1637–1644.

Hofmann, S.L., Prescott, S.M., Majerus, P.W., 1982. The effects of mepacrine and p-bromophenacyl bromide on arachidonic acid release in human platelets. Arch. Biochem. Biophys. 215, 237–244.

Hunyady, L., Merelli, F., Baukal, A.J., Balla, T., Catt, K.J., 1991. Agonist-induced endocytosis and signal generation in adrenal glomerulosa cells. A potential mechanism for receptor-operated calcium entry. J. Biol. Chem. 266, 2783–2788.

Kapas, S., Purbrick, A., Hinson, J.P., 1995. Role of tyrosine kinase and protein kinase C in the steroidogenic actions of angiotensin II, alpha-melanocyte-stimulating hormone and corticotropin in the rat adrenal cortex. Biochem. J. 305, 433–438.

Kovach, J.S., Svingen, P.A., Schaid, D.J., 1992. Levamisole potentiation of fluorouracil antiproliferative activity mimicked by orthovanadate, an inhibitor of tyrosine phosphatase. J. Nat. Cancer Inst. 84, 515–519.

Liao, K., Hoffman, R.D., Lane, M.D., 1991. Phosphotyrosyl turnover in insulin signaling. Characterization of two membrane-bound pp15 protein tyrosine phosphatases from 3T3-L1 adipocytes. J. Biol. Chem. 266, 6544–6553.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264, 187–192.

Mizuno, K., Noda, K., Ueda, Y., Hanaki, H., Saido, T.C., Ikuta, T., Kuroki, T., Tamaoki, T., Hirai, S., Osada, S., 1995. UCN-01, an anti-tumor drug, is a selective inhibitor of the conventional PKC subfamily. FEBS Lett. 359, 259–261.

Morioka, M., Fukunaga, K., Kawano, T., Hasegawa, S., Korematsu, K., Kai, K., Hamada, J., Miyamoto, E., Ushio, Y., 1998. Serine/threonine phosphatase activity of calcineurin is inhibited by sodium orthovanadate and dithiothreitol reverses the inhibitory effect. Biochem. Biophys. Res. Commun. 253, 342–345.

Moriyama, T., Urade, R., Kito, M., 1999. Purification and characterization of diacylglycerol lipase from human platelets. J. Biochem. 125, 1077–1085.

Scher, B.M., Wei, X.J., Waxman, S., Scher, W., 1998. Dephosphorylation of Vav is associated with the induction of mouse erythroleukemia cell differentiation: effects of orthovanadate and levamisole. Int. J. Oncol. 12, 1307–1313.

Street, I.P., Lin, H.K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N.M., Huang, Z., Weech, P.K., Gelb, M.H., 1993. Slow- and tight-binding inhibitors of the 83-kDa human phospholipase Ap2. Biochemistry 32, 5935–5940.

Tian, Y., Smith, R.D., Balla, T., Catt, K.J., 1998. Angiotensin II activates mitogen-activated protein kinase via protein kinase C and Ras/Raf-1 kinase in bovine adrenal glomerulosa cells. Endocrinology 139, 1801–1809.

Yingst, D.R., Davis, J., Krenz, S., Schiebinger, R.J., 1999. Insights into the mechanism by which inhibition of NaK-ATPase stimulates aldosterone secretion. Metabolism 48, 1167–1171.