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EFFECT OF VASOPRESSIN ON Na^+ KINETICS IN CULTURED RAT VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY: The effect of arginine vasopressin (AVP) on Na^+ kinetics was examined in cultured rat vascular smooth muscle cells (VSMC) and rat renal papillary collecting tubule cells (RPCT) by the direct measurement of intracellular sodium concentration ($[Na^+]i$) using fluorescence dye; SBFI. AVP increased $[Na^+]i$ in a dose-dependent manner at a concentration of 10^{-9} M or higher in rat VSMC but did not affect $[Na^+]i$ in rat RPCT. The calcium (Ca^{2+})-free solution completely blocked the increasing effect of AVP on $[Na^+]i$ in rat VSMC. A Ca^{2+} ionophore, ionomycin ($1 - 2 \times 10^{-6}$ M) increased $[Na^+]i$ both in rat VSMC and RPCT. The Ca^{2+}-free solution abolished the ionomycin-increased $[Na^+]i$ both in rat VSMC and RPCT. These results therefore indicate that after binding the V_1 receptor AVP increases $[Na^+]i$ mediated through an increase in cellular Ca^{2+} uptake in VSMC.

Arginine vasopressin (AVP) binds to the AVP V_1 receptor of vascular smooth muscle cells (VSMC) and activates phospholipase C to produce the two different intracellular second messengers; inositol 1,4,5-trisphosphate (IP_3) and 1,2 diacylglycerol (DAG)(1 - 4). IP_3 mobilizes calcium (Ca^{2+}) from intracellular Ca^{2+} pool and increases cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) accompanied with an increase in cellular Ca^{2+} uptake (1, 3 - 5). An increase in $[Ca^{2+}]i$ stimulates Na^+/Ca^{2+} exchange and Ca^{2+} efflux to return $[Ca^{2+}]i$ to the basal level within a few min (6, 7). DAG stimulates protein kinase C (PKC) activity to enhance Na^+/H^+ exchange, which induces the intracellular alkalinization (8, 9). Vasopressor hormones have been demonstrated to increase cellular $^{22}Na^+$ uptake in rat VSMC (10). On the other hand, after binding the V_2 receptor AVP also mobilizes $[Ca^{2+}]i$ and stimulates adenylate cyclase to produce cellular cAMP in cultured rat renal papillary collecting tubule cells (RPCT)(11). These findings suggest that AVP may enhance cellular Na^+ uptake and increase intracellular sodium concentration ($[Na^+]i$). However, little is known about Na^+ kinetics in response to AVP in rat VSMC and RPCT as compared with Ca^{2+} kinetics.

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There are no suitable methods to measure the vasopressor hormone-induced changes in $[Na^+]i$ in cultured small cells until the developement of new fluorescence indicator, SBFI, for the measurement of intracellular free Na^+ concentration by Tsien et al (12, 13). The availability of fluorescent $[Na^+]i$-sensitive dye provides a powerful tool to study noninvasively rapid changes in $[Na^+]i$ induced by hormones (12, 13). This study was therefore undertaken to examine the effect of AVP on Na^+ kinetics and the interaction between Na^+ and Ca^{2+} kinetics in response to AVP by the direct measurement of $[Na^+]i$ using fluorescence dye in cultured rat VSMC and RPCT.

MATERIALS AND METHODS

Cell culture

Rat VSMC were isolated using the modified method of Chamley et al (4, 14, 15). Briefly, the rat thoracic aortas were dissected from 8 to 10 male Sprague-Dawley rats (200-300 g) and then incubated in Eagle's Minimal Essential Medium (MEM) containing 2 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) for 1 hr at 37 C. After the removal of adventitia and small fragments of the outer membrane, the aortas were minced and incubated in Eagle's MEM containing 2 mg/ml collagenase for 2 hr at 37 C. The freshly isolated cells were resuspended in Eagle's MEM containing 1 μM L-glutamine, 100 U/ml penicillin (PC), 100 μg/ml streptomycin (SM) and 10% fetal calf serum (FCS), pH 7.4. The cells were kept in a humidified incubator under 95% air and 5% CO_2 at 37 C. All experiments were done in subcultured cells from 3 to 10 th passages. The subculture was performed by trypsin-EGTA treatment.

Rat RPCT were cultured by the method of our previous studies (16), modified from the study of Grenier et al (17). The kidneys removed from 8 male Sprague-Dawley rats weighing 150 to 175 g were placed in sterile Krebs-Ringer buffered saline (KRB). Renal papillary tissues were dissected out and minced with 1 mg/ml collagenase (type II, Worthington Biochemicals) using a shape blade. The minced renal papillary tissues were kept at 37 C for 1.5 hr. During this incubation period the tissues were gently shaken every 30 min. After the exposure to hypotonic solution the cells were dissolved in KRB containing 10% bovine serum albumin and then were centrifuged at 500 x g for 4 min. The pellets were resuspended in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% FCS, 100 U/ml PC, and 100 μg/ml SM. On the second day of culture the medium was changed to 99% DMEM containing 1% FCS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 x 10^{-8} M hydrocortisone, 5 x 10^{-8} M T_3, 100 U/ml PC, and 100 μg/ml SM. The cultured cells were subjected to the following studies on day 5 of culture.

In the studies for the measurement of $[Na^+]i$ the cells were cultured on thin glass slides (13 mm in diameter; Matsunami Kogyo Co., Osaka, Japan).

Measurement of $[Na^+]i$

The rat VSMC and RPCT grown on thin glass slides were rinsed twice with physiological saline solution (PSS) [140 mM NaCl, 4.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4] and KRB, respectively and loaded with 10 μM sodium-binding benzofuran isophthalate acetoxymethyl esters (SBFI/AM; Molecular Probes, Inc., Eugene, OR) for 3 hr at 37 C (12, 13, 18). SBFI/AM was dissolved in PSS or KRB containing 0.02% pluronic F-127, a nonionic surfactant. After the loading period, the cells were rinsed with PSS or KRB and then placed in a 1 x 1-cm quartz cuvette in a fluorescence spectrophotometer (CAF-100, Japan Spectrometer Co., Tokyo, Japan).

The complete hydrolysis of SBFI/AM to SBFI was judged by changes in the excitation and emission spectra. AVP (Sigma, St. Louis, MO) or ionomycin (Calbiochem-Boehringer, La Jolla, CA) was added to the cells after the measurement of basal $[Na^+]i$. The relation between AVP- or ionomycin-induced $[Na^+]i$ changes and extracellular Na^+ concentration was examined by using extracellular Na^+-free solution supplemented with 140 mM choline chloride. The Ca^{2+}-free solution containing 0.1 or 1 mM ethylene glycol-bis [(β -aminoethylether)-N,N'-tetraacetic acid (EGTA)] was also used to examine the relation between AVP- or ionomycin-induced $[Na^+]i$ changes and extracellular Ca^{2+} concentration. The dual wavelength excitation method for the measurement of SBFI fluorescence was used. The fluorescence was monitored at 500 nm with exitation wavelengths of 340 and 380 nm in the ratio mode. $[Na^+]i$ was calibrated by equilibrating $[Na^+]i$ with the extracellular Na^+ concentration using 1 x 10^{-6} M gramicidine. The reference standard solution was made from appropriate mixtures of Na^+ and K^+ which were ajusted to 135 mM. The $[Na^+]i$ was determined by the relation between the ratio and the authentic $[Na^+]i$ (12, 13, 18).

Statistical analysis

The results were expressed as the mean±SEM. The unpaired Student's t-test and an analysis of multiple variance using Scheffe's method were used for statistical comparison. A P value of less than 0.05 was considered significant.

RESULTS

AVP increased $[Na^+]i$ in a dose-dependent manner at a concentration of 1 x 10^{-9} M or higher in rat VSMC (Fig. 1a and 2) but did not affect $[Na^+]i$ in rat RPCT (Fig. 1b) (Basal, 15.7 ± 0.7 mM, 1 x 10^{-7} M AVP, 15.9 ± 0.8 mM, n=5, n.s.). Basal $[Na^+]i$ levels were 13.3 ± 1.2 and 15.9 ± 0.8 mM (n=6) in rat VSMC and RPCT, respectively. An AVP-induced increase in $[Na^+]i$ in rat VSMC was occurred within about 20-30 sec and reached the peak level 5 min after the addition of AVP, and then gradually declined. But it remained higher levels than basal $[Na^+]i$ level for at least 15 min (Fig. 1a). Since the cellular action of vasopressor hormones has been suggested to be associated with an increase in cellular Ca^{2+} uptake, the

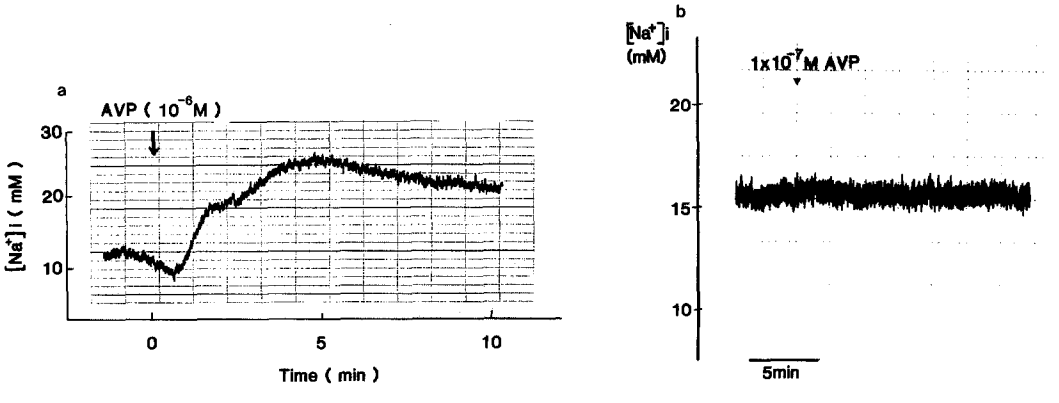

Figure 1a. Effect of AVP on $[Na^+]i$ in cultured rat VSMC.
Figure 1b. No effect of AVP on $[Na^+]i$ in rat RPCT.

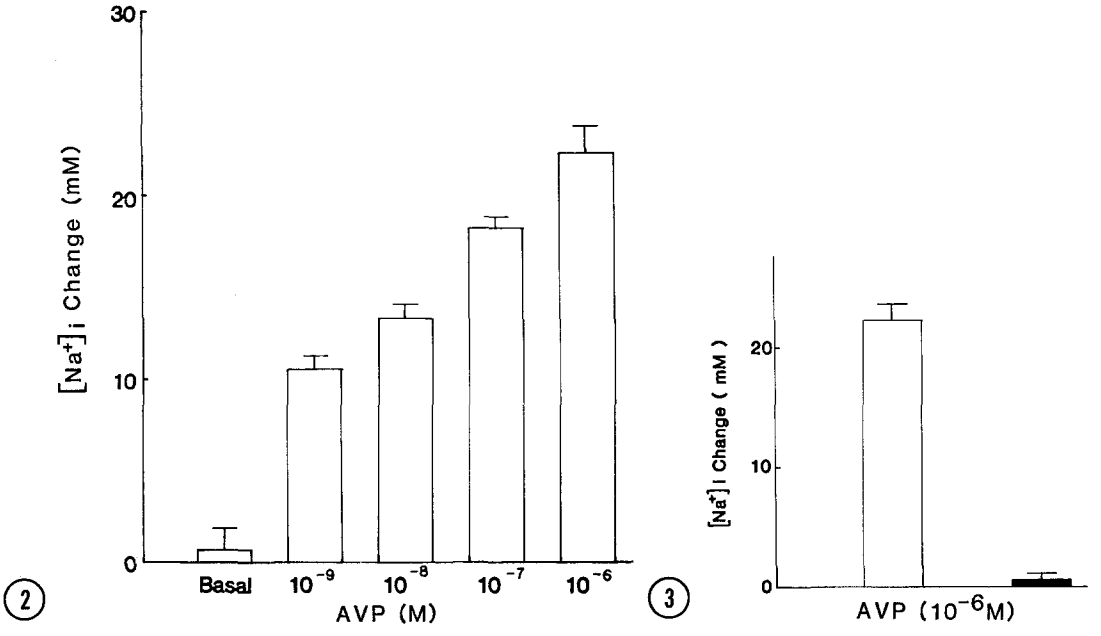

Figure 2. Dose-dependent effect of AVP on increment in $[Na^+]i$ in cultured rat VSMC.

Figure 3. Effect of extracellular Ca^{2+} concentration ($[Ca^{2+}]e$) on AVP-induced increment in $[Na^+]i$ in cultured rat VSMC. Vehicle, 2 mM $[Ca^{2+}]e$ (open bar) and 0 mM $[Ca^{2+}]e$ containing 0.1 mM EGTA (closed bar). n=6.

relation between an AVP-induced increase in $[Na^+]i$ and extracellular Ca^{2+} concentration ($[Ca^{2+}]e$) was also examined to investigate whether the increasing effect of AVP on $[Na^+]i$ was dependent on an increase in cellular Ca^{2+} uptake. The Ca^{2+}-free solution containing 0.1 mM EGTA also abolished an AVP-induced increase in $[Na^+]i$ (Fig. 3). Furthermore, a Ca^{2+} ionophore, ionomycin, was used to examine the relation between $[Na^+]i$ and $[Ca^{2+}]e$. Ionomycin ($1 - 2 \times 10^{-6}$ M) increased $[Na^+]i$ both in rat VSMC and RPCT, respectively (Table 1 and Fig. 4). The Ca^{2+}-free solution containing 0.1 or 1 mM EGTA completely abolished the ionomycin-increased $[Na^+]i$ both in rat VSMC and RPCT (Table 1 and Fig. 4).

Table 1. Effect of extracellular Ca^{2+} on ionomycin-induced increment in $[Na^+]i$ in cultured rat VSMC

	$[Na^+]i$ (mM)		
	Basal	10^{-6} M ionomycin	P
Control	12.2 ± 0.7	27.6 ± 1.5	<0.01
Ca^{2+}-free medium containing 0.1 mM EGTA	12.5 ± 0.7	11.3 ± 0.8*	n.s.

n=5, *P<0.01 vs the control group.

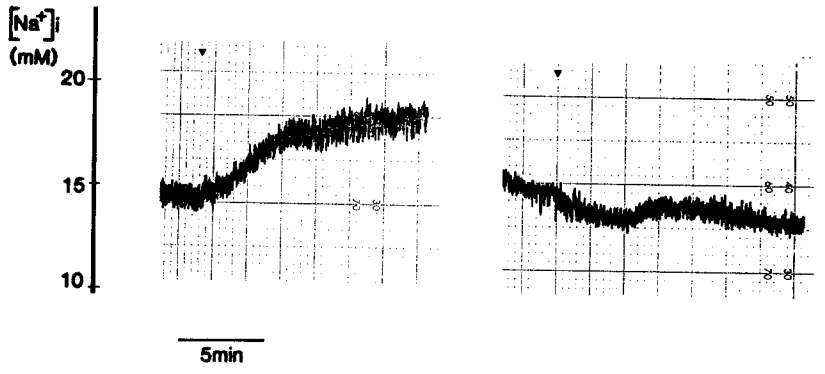

Figure 4. Effect of extracellular Ca^{2+} concentration ($[Ca^{2+}]e$) on ionomycin-induced increase in $[Na^+]i$ in rat RPCT. Vehicle, 2 mM $[Ca^{2+}]e$ (left side) and 0 mM $[Ca^{2+}]e$ containing 1 mM EGTA (right side). Arrows show the addition of 2×10^{-6} M ionomycin.

DISCUSSION

Flame photometory, ^{22}Na tracer experiments , Na^+-sensetive microelectrodes and nuclear magnetic resonance of ^{23}Na are whereby used to measure intracellular Na^+. Flame photometory and $^{22}Na^+$ tracer experiments measure total not free Na^+ and destructive assays. Na^+-sensetive microelectrodes are limited to relatively large cells. Nuclear magnetic resonance is nondestructive but demands large quantities of tissues and long acquisition time. Therefore, these techniqus are not available to measure the hormone-induced changes in $[Na^+]i$ in cultured rat VSMC. The new fluorescent Na^+ indicator, SBFI, developed by Tsien et al (12, 13) has provided a nondestructive, fast responding method for measuring $[Na^+]i$. The fluorescent measurement of $[Na^+]i$ enables us to examine the interaction between the AVP-induced Na^+ and Ca^{2+} kinetics and present the first observation about the interaction between these two kinetics in response to AVP in cultured rat VSMC in this paper.

The present findings have shown that AVP causes an increase in $[Na^+]i$ mediated through the V_1 receptor and this effect of AVP is closely associated with an increase in cellular Ca^{2+} uptake induced by AVP per se. The experimental evidence from the present investigation includes the following observations; 1) AVP increased $[Na^+]i$ in rat VSMC but did not in rat RPCT; 2) The Ca^{2+}-free solution in the presence of 140 mM $[Na^+]e$ abolished the AVP-increased $[Na^+]i$ in rat VSMC.

Previous studies have shown that after AVP binding the V_1 receptor of VSMC, AVP activates phopholipase C to produce IP_3 and DAG (1 - 4). Recent studies from our laboratory have also demonstrated that after AVP binding to the V_2 receptor of rat RPCT, AVP mobilizes $[Ca^{2+}]i$ and activates the adenylate cyclase to increase cellular cyclic AMP production (

11, 19). The present study has shown that AVP increased $[Na^+]i$ in rat VSMC but did not in RPCT. Taken together, AVP increases $[Na^+]i$ mediated through the V_1 receptor.

It has been suggested that vasopressor hormones activate Na^+/H^+ exchange in a PKC-independent or dependent manner to increase cellular Na^+ uptake (8, 20). The PKC-independent activation of Na^+/H^+ exchange has been reported to be associated with an increase in cellular Ca^{2+} uptake (20). An AVP-increased $[Ca^{2+}]i$ is composed of both an increase in cellular Ca^{2+} uptake and the IP_3-mediated mobilization of $[Ca^{2+}]i$ (1, 3 - 5). Therefore, the relation between an AVP-increased $[Na^+]i$ and extracellular Ca^{2+} was examined in this study. The Ca^{2+}-free solution in the presence of 140 mM $[Na^+]e$ completely abolished this increasing effect of AVP on $[Na^+]i$ and the AVP-induced intracellular alkalinization (unpublished data). The AVP-induced intracellular alkalinization results from the activation of Na^+/H^+ exchange in HCO_3-free buffer in rat VSMC (8, 9). A PKC activator, phorbol 12-myristate 13-acetate (PMA) did not affect $[Na^+]i$ in rat VSMC (unpublished data). These finding suggests that in the presence of extracellular Na^+ the AVP-induced increase in $[Na^+]i$ is dependent on cellular Ca^{2+} mobilization, particularly on an increase in cellular Ca^{2+} uptake, to stimulate the PKC-independent activation of Na^+/H^+ exchange. This proposal was supported by the results of a Ca^{2+} ionophore, ionomycin. Ionomycin incerased $[Na^+]i$ both in rat VSMC and RPCT and the Ca^{2+}-free solution abolished the ionomycin-induced increase in $[Na^+]i$ both in rat VSMC and RPCT.

In conclusion, the present results suggest that the AVP-increased $[Na^+]i$ results from the PKC-independent activation of Na^+/H^+ exchange modulated by an increase in cellular Ca^{2+} uptake after binding the V_1 receptor of vascular smooth muscle cells. These findings are the first observation about the vasopressor hormones-induced cellular Na^+ kinetics in vascular smooth muscle cells by the direct measurement of $[Na^+]i$ using the fluorescent technique and provide new potential insights into the relation between the AVP-induced Na^+ and Ca^{2+} kinetics in vascular smooth muscle cells.

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REFERENCES

1. Nabika, T., Velletri, P. A., Lovenberg, W., and Beaven, M. A. (1985) J. Biol. Chem. 260, 4661 - 4670.
2. Grillone, L. R., Clark, M. A., Godfrey, R. W., Stassen, F., and Crooke, S. T. (1988) J. Biol. Chem. 263, 2658 - 2663.

3. Monck, J. R., Reynolds, E. E., Thomas, A. P., and Williamson, J. R. (1988) J. Biol. Chem. 263, 4569 - 4575.
4. Okada, K., Caramelo, C., Tsai, P., and Schrier, R. W. (1990) J. Clin. Invest. (in press)
5. Burgess, G. M., Godfrey, P. P., Mckinney, J. S., Berridge, M. J., Irvine, R. F., and Putney, J. W. Jr. (1984) Nature 309, 63 - 66.
6. Blaustein, M. P. (1988) J. Cardiovasc. Pharmacol. 12 (Suppl. 5), S56 - S68.
7. Nabel, E. G., Berk, B. C., Brock, T. A., and Smith, T. W. (1988) Circ. Res. 62, 486 - 493.
8. Berk, B. C., Aronow, M. S., Brock, T. A., Cragoe, E. Jr, Gimbrone, M. A. Jr, and Alexander, R. W. (1987) J. Biol. Chem. 262, 5057 - 5064.
9. Hatori, N., Fine, B. P., Nakamura, A., Cragoe, E. Jr, and Aviv, A. J. Biol. Chem. (1987) 262, 5073 - 5078.
10. Smith, J. B., and Brock, T. A. (1983) J. Cell Physiol. 114, 284 - 290.
11. Ishikawa, S., Okada, K., and Saito, T. (1988) Endocrinology 123, 1376 - 1384.
12. Minta, A., and Tsien, R. Y. (1989) J. Biol. Chem. 264, 19449 - 19457.
13. Harootunian, A. T., Kao, J. P. Y., Eckert, B. K., and Tsien, R. Y. (1989) J. Biol. Chem. 264, 19458 - 19467.
14. Chamley, J. H., Campbell, G. R., and McDonnel, J. D. (1977) Cell Tissue Res. 177, 503 - 522.
15. Caramelo, C., Okada, K., Tsai, P., and Schrier, R. W. (1989) Am. J. Physiol. 256, F875 - F881.
16. Ishikawa, S., Saito, T., and Kuzuya, T. (1985) J. Endocrinol. 107, 15 - 22.
17. Grenier, F. C., Rollins, T. E., and Smith, W. L. (1981) Am. J. Physiol. 241, F94 - F104.
18. Ishikawa, S., Okada, K., and Saito, T. (1990) Endocrinology (in press)
19. Ishikawa, S., and Saito, T. (1990) Kidney Int. 37, 1060 - 1066.
20. Little, P. J., Weissberg, P. L., Cragoe, E. J. JR, and Bobik, A. (1988) Prog. Biochem. Pharmacol. 23, 142 - 149.