L-Arginine inhibits vasopressin-stimulated mesangial cell Ca\(^{2+}\)

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Balsam, Leah, and Nasrin Nikbakht. L-Arginine inhibits vasopressin-stimulated mesangial cell Ca\(^{2+}\) Am. J. Physiol. 275 (Cell Physiol. 44): C352–C357, 1998. — L-Arginine (L-Arg) affects various parameters that modulate the progression of renal disease. These same factors (e.g., glomerular filtration rate, changes in mesangial cell (MC) tension, and production of NO) are all controlled at least in part by changes in MC intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}). We therefore evaluated the effect of L-Arg on MC [Ca\(^{2+}\)]. We found that L-Arg inhibits the vasopressin-stimulated rise in MC [Ca\(^{2+}\)] both in rat and murine cell cultures. This effect does not appear to be due to metabolism of L-Arg to either NO or L-ornithine (L-Orn). Blocking the metabolism of L-Arg with N\textsuperscript{\textasteriskcentered}monomethyl-L-arginine, an NO synthase inhibitor, or with 20 mM L-valine (L-Val), an inhibitor of Orn metabolism, does not reverse the inhibition. However, other cationic amino acids, as well guanidine, the functional group of L-Arg, all inhibit the vasopressin-stimulated rise in [Ca\(^{2+}\)], consistent with a structural basis for this effect. We conclude that 1) L-Arg inhibits vasopressin-stimulated murine and rat MC [Ca\(^{2+}\)] rise, 2) this inhibition is not mediated by metabolism of L-Arg to either NO or L-Orn, and 3) the effect of L-Arg is due to its cationic functional group, guanidine.

THE PROGRESSION OF RENAL disease is modulated by L-Arg (17, 19–21). The effects of L-Arg on renal function are multiple, as it affects diverse functions such as the ultrafiltration coefficient (K\(_f\)) and glomerular filtration rate (20, 21), the production of transforming growth factor-\(\beta\) (TGF-\(\beta\); a prosecretory agent; Ref. 17), and formation of NO (13, 18). A unifying mechanism for these various effects may be a modulation by L-Arg of mesangial cell (MC) intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}). MC [Ca\(^{2+}\)] controls MC contraction and relaxation (5). These changes in MC tension influence the K\(_f\) and glomerular filtration rate (24) as well as production of TGF-\(\beta\) (7). MC Ca\(^{2+}\) is also necessary for the production of NO via a constitutive activation of NO synthase (28).

We therefore decided to investigate the effect of L-Arg on MC [Ca\(^{2+}\)]. We measured the influence of L-Arg on both baseline and stimulated [Ca\(^{2+}\)] in rat MC and murine MC (MMC). To stimulate [Ca\(^{2+}\)], we used Arg-vasopressin (AVP), an agonist known to increase [Ca\(^{2+}\)] (5); induce MC contraction (2); and release NO from various tissues (15, 26). We determined that L-Arg does inhibit vasopressin-stimulated [Ca\(^{2+}\)]. This is due to a charge effect related to the cationic structure of L-Arg rather than to its metabolism.

METHODS

We performed our studies in an immortalized MMC line. To rule out any species-specific effects, we repeated some of the experiments in a commonly used glomerular MC culture model, the Sprague-Dawley rat MC.

Cell Culture

Immortalized, differentiated MMC were obtained as a gift from Dr. Eric Neilson (University of Pennsylvania, Philadelphia, PA). They originated from normal mice whose cells were transformed with non-capssid-forming simian virus 40 to establish a permanent cell line. Previous studies have demonstrated that this cell line exhibits many features of differentiated MC: the cells expressed the Thy 1.1 antigen and stained positive with antibodies against desmin, vimentin, and collagen type I and IV but failed to bind antibody directed against the proximal tubular nephritogenic 3M 1 antigen (29). The cells in our studies were grown in DMEM with 5% FCS and 1% penicillin and streptomycin. The cells were subcultured when confluent.

Rat MC were isolated from 150-g Sprague-Dawley rats maintained on tap water and Purina rat chow ad libitum, as previously described (23). The kidneys were removed under pentobarbital anesthesia, and the rats were killed. The cortex was separated from the medulla, minced with a razor blade, and placed through a sequential sieving process. Collagenase was added to release the MC from the surrounding matrix. The cells were grown in culture medium consisting of RPMI 1640 supplemented initially by 20% FCS, with 1% penicillin and streptomycin. Once confluent, ~15–20 days after glomerular isolation, the MC were then subcultured at 2- to 3-wk intervals until the third subculture. They were subsequently fed with medium supplemented with 10% FCS and subcultured when confluent, approximately every 7–10 days. For measurement of [Ca\(^{2+}\)], passages 3–10 were used. MC were verified by the presence of positive immunofluorescence using mouse actin antibodies and negative staining for ED1, a marker for macrophages (1).

Measurement of [Ca\(^{2+}\)]

[Ca\(^{2+}\)]\textsubscript{i} was determined by fluorescence spectrophotometry (PTI Deltascan), using the intracellular fluorescent indicator fura 2-AM (11, 27). The cells were grown on 25-mm coverslips in sterile petri dishes. When they were 70–90% confluent, they were used for the measurement of [Ca\(^{2+}\)]. First, unloaded cells were measured to obtain a background measurement (including autofluorescence). This measurement was subtracted from subsequent measurements of cells loaded with fura 2-AM. Cells were then loaded with fura 2-AM (9 \(\mu\)M for MMC and 3 \(\mu\)M for rat MC) and incubated for 45 min at 37°C. The concentrations of fura 2-AM were based on preliminary experimental determinations of optimal fluorescence. The cells were rinsed three times and incubated in the appropriate PBS for 45 min. The incubation time was based on timed studies revealing 45 min as the shortest time period in which the maximal effect was obtained. The cells were placed in fresh solution immediately before insertion into a holding chamber maintained at 37°C. An inverted Nikon microscope with a \(\times 40\) oil immersion lens was used. A field of cells was illuminated with two different wavelengths, using a

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PTI Deltascan. A chopper rapidly alternated between the 350- and 380-nm wavelengths so that almost simultaneous excitation with dual wavelengths was achieved. The emitted fluorescence was fed through a photomultiplier tube via an ultraviolet dichroic cube. The Deltascan was connected to a computer that calculated the wavelength ratios, which correlate with [Ca\(^{2+}\)]. Because in preliminary studies L-Arg did not affect baseline levels of [Ca\(^{2+}\)], we used AVP as a means of stimulating [Ca\(^{2+}\)]. AVP is known to raise [Ca\(^{2+}\)], as well as cause MC contraction (5, 2). Cells were then measured at baseline and after injection of AVP. The experiments were carried out over ~10 min. During this time, the baseline had been noted to be stable when there were no additions.

Background measurements of fluorescence were obtained in the presence and absence of L-Arg, D-Arg, L-Val, L-Orn, and guanidine at 350 and 380 nm. There was no effect on fluorescence or wavelength spectra of these amino acids compared with control solutions.

We expressed the results of [Ca\(^{2+}\)] as the ratio of the peak-to-baseline measurement at 350 nm to that at 380 nm, after injection of AVP (10 \(^{-6}\) M in MMC and 10 \(^{-7}\) M in rat MC). Fura 2 fluorescence peaks at a wavelength of 350 nm when it is bound to Ca\(^{2+}\), and the free form of fura 2 has its fluorescence peak at 380 nm. The simultaneous measurement of fluorescence at these two different wavelengths allows the use of the ratio of the two. This, in turn, eliminates several variables such as cell thickness, fura 2 concentration, and changes in optics that would otherwise make it difficult to compare control with experimental groups. Although there are various methods of calibration by which a calculated concentration can be obtained, each has its own shortcomings. We felt that a more accurate presentation of the results was to express changes in [Ca\(^{2+}\)] as a change in the fluorescence ratio. Although there are fluctuations in the ratios of control values, as is expected in any biological system, we are interested in relative differences between experimental groups and controls and not in absolute numbers.

The concentration of the various amino acids used was 1 mM. This concentration was chosen on the basis of prior information. Serum levels of amino acids vary between 0.1 and 0.7 mM, depending on the individual amino acid (8). Intracellular concentrations can reach levels as high as 5 mM (8). In previous in vitro studies by another author, concentrations between 1 and 5 mM were used (6). In our studies, we found that concentrations in excess of 1 mM (as high as 2 mM) had no further effectiveness. We therefore elected to use 1 mM concentrations throughout the study.

**Solutions and Reagents**

The control solution contained (in mM) 25 Na Hepes, 114 NaCl, 5 KCl, 0.8 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 1.5 CaCl, and 5.5 glucose. All solutions were adjusted to pH 7.4. L- and D-Arg, L- and D-Orn, spermine, spermidine, guanidine hydrochloride, N\(^{\text{nitro-l-arginine methyl ester (L-NAME)}}\), and AVP were obtained from Sigma Chemical (St. Louis, MO). Fura 2-AM was obtained from Molecular Probes (Eugene, OR). N\(^{\text{monomethyl-l-arginine (L-NMMA)}}\) from Calbiochem (La Jolla, CA), L-Val and RPMI 1640 from ICN (Costa Mesa, CA), DMEM from Life Technologies (Gaithersburg, MD), and FCS from Gemini Bio-products (Calabassas, CA).

**Statistics**

Two types of statistical analyses were used. When we dealt directly with the comparison of a control solution and a modified solution in samples originating from the same subculture, we used Student’s paired t-test. When more than one modified solution was used in a given experiment, repeated measures ANOVA was used. If the calculated F revealed statistical significance, the difference between two groups was evaluated by determining the q value using the Newman-Keuls test. In certain sets of experiments involving more than one modified solution, the results did not fit a normal distribution. In these experiments, repeated measures ANOVA on ranks was used. Again, the Newman-Keuls test was used to evaluate specific differences among groups.

**RESULTS**

Evaluation of NO System in MMC: Effects of L-Arg and L-NMMA on AVP-Stimulated [Ca\(^{2+}\)].

In MMC, L-Arg significantly lowered AVP-stimulated [Ca\(^{2+}\)] by 27% (Fig. 1, a representative tracing; control 3.22 ± 0.13 vs. L-Arg 2.34 ± 0.11, n = 43). To determine whether this was due to metabolism of L-Arg to NO, an inhibitor of NO synthase, L-NMMA, was added to the solution containing L-Arg. The inhibition was similar in the presence or absence of 2 mM L-NMMA (Fig. 2; control 4.31 ± 0.74, L-Arg 2.40 ± 0.47, L-NMMA 3.16 ± 0.46, and L-NMMA plus L-Arg 2.60 ± 0.46, n = 5). A second NO synthase inhibitor, L-NAME, was tested to confirm these findings. L-NAME did not reverse the inhibition by L-Arg of AVP-stimulated [Ca\(^{2+}\)] rise. There was no statistical difference between the effects of 1 mM L-Arg alone (1.72 ± 0.39) and of the combination 2 mM L-NAME plus 1 mM L-Arg (1.86 ± 0.26), whereas a comparison of control (2.37 ± 0.32) vs. L-Arg plus L-NAME (1.75 ± 0.55) did reach statistical difference (P < 0.05).

Evaluation of Arginase Pathway in MMC: Effects of L-Arg and L-Orn in Presence and Absence of 20 mM L-Val on AVP-Stimulated [Ca\(^{2+}\)].

L-Arg is metabolized to L-Orn via the arginase pathway. We tested the effect of L-Orn on vasopressin-stimulated MMC [Ca\(^{2+}\)] rise. Like L-Arg, L-Orn inhibited the expected rise, by 24% compared with control (Fig 3; L-Arg 3.52 ± 0.27, L-Orn 2.67 ± 0.14, n = 22). We

![Fig. 1. A representative tracing of fluorescence measured in murine mesangial cells (MMC) as a ratio of measurement at 350 nm wavelength (350/380) to that at wavelength of 380 nm (380), Arrows, peak ratios obtained with addition of Arg-vasopressin (AVP) to cells bathed in control and L-Arg solutions.](http://ajpcell.physiology.org/)
wished to determine whether this was an effect of metabolism of L-Arg to L-Orn or was due to a common charge effect. We therefore tested L-Arg in the presence of L-Val (20 mM), which is a known inhibitor of the arginase pathway (22). L-Arg inhibited hormone-stimulated [Ca\textsuperscript{2+}] rise by 32% in the presence of 20 mM L-Val. This was similar to the effect of L-Orn, which decreased [Ca\textsuperscript{2+}] by 34% (Fig 4; control plus L-Val 3.55 ± 0.41, L-Arg plus L-Val 2.43 ± 0.24, L-Orn plus L-Val 2.36 ± 0.20, n = 11). L-Val by itself, used at a concentration of 20 mM, had no effect on hormone-stimulated [Ca\textsuperscript{2+}] (control 2.84 ± 0.57, Val 3.08 ± 0.42, n = 6). 

Investigation of Possible Charge Influences in MMC

Effects of D-Arg and D-Orn on AVP-stimulated [Ca\textsuperscript{2+}]. D-Arg (1 mM), a nonmetabolized form of Arg, was evaluated to determine whether there is a charge effect of Arg on [Ca\textsuperscript{2+}]. D-Arg and D-Orn inhibited vasopressin-stimulated [Ca\textsuperscript{2+}] rise in MMC by 30% and 24%, respectively (Fig 5; control 3.04 ± 0.30, D-Arg 2.12 ± 0.16, n = 8; control 2.92 ± 0.22, D-Orn 2.21 ± 0.17, n = 8). Evaluation of other cationic amino acids: effects of spermidine and spermine. Both spermine and spermidine inhibited AVP-stimulated [Ca\textsuperscript{2+}] rise by 35% and 42%, respectively (Fig 6; control 4.52 ± 0.59, spermine 2.96 ± 0.33, n = 7; control 4.74 ± 0.65, spermidine 2.77 ± 0.37, n = 6). Evaluation of the functional group of L-Arg: effects of guanidine hydrochloride. Because the effect of L-Arg on MC [Ca\textsuperscript{2+}] appears to be due to its cationic property, we evaluated its functional group, guanidine. We found that, like L-Arg, guanidine (1 mM) inhibits the AVP-stimulated rise in [Ca\textsuperscript{2+}]; the decreases are 34 and 35% by L-Arg and guanidine, respectively (Fig 7; control 3.14 ± 0.84, L-Arg 2.08 ± 0.19, guanidine 2.03 ± 0.12, n = 8).

Studies in Rat MC

We then repeated some of our MMC studies using rat MC. Again, L-Arg, L-Arg plus L-NMMA, and D-Arg all inhibited vasopressin-stimulated [Ca\textsuperscript{2+}], rise. (Figs. 8 and 9; control 3.53 ± 0.39, L-Arg 2.66 ± 0.34, L-Arg plus L-NMMA 1.99 ± 0.21, n = 8; control 4.44 ± 0.58, L-Arg 2.54 ± 0.25, n = 8; control 4.37 ± 0.65, D-Arg 2.46 ± 0.25, n = 6). In contrast to the cationic amino acids, the neutral amino acid L-Val had no effect (control 3.12 ± 0.28, L-Val 3.31 ± 0.47).

DISCUSSION

We found that L-Arg does inhibit the rise in [Ca\textsuperscript{2+}] expected with AVP. One possibility is that this decrease in [Ca\textsuperscript{2+}] is due to NO, a metabolite of L-Arg. In a rat mesangial model, NO inhibited angiotensin II-induced contraction (25), which may be a Ca\textsuperscript{2+}-dependent phenomenon. Our study, however, found that although L-Arg decreased MMC and rat MC [Ca\textsuperscript{2+}], the addition of the NO synthase inhibitor L-NMMA did not reverse this effect. This is consistent with an NO-independent mechanism for inhibition of rise in [Ca\textsuperscript{2+}], but does not rule out a separate effect of [Ca\textsuperscript{2+}] on MC relaxation. NO-independent MC relaxation was reported in another study: in a Wistar rat model, somatostatin was found to inhibit angiotensin II-stimulated MC contrac-
The inhibitory effect of somatostatin was not reversed by L-NMMA, suggesting that this somatostatin effect was also not dependent on NO.

In addition to an effect on MC contraction, this inhibition of [Ca\(^{2+}\)]\(_i\) rise may actually represent a negative feedback mechanism of L-Arg on NO. Although under certain conditions L-Arg serves as a substrate for NO synthesis (18), on the basis of our findings, L-Arg could potentially inhibit NO formation. In particular, L-Arg may affect constitutively activated NO production, which is dependent on an increase in [Ca\(^{2+}\)]\(_i\). In Chinese hamster ovary cells, endothelin-stimulated [Ca\(^{2+}\)]\(_i\) rise was inhibited by NO (10), suggesting a possible negative feedback mechanism of NO on itself. In our study, however, the feedback of L-Arg on NO would be through [Ca\(^{2+}\)]\(_i\), but not through NO itself.

Because the production of NO did not mediate the inhibition of [Ca\(^{2+}\)]\(_i\) rise, we investigated other possibilities. The metabolism of L-Arg to L-Orn also does not appear to cause the decrease in [Ca\(^{2+}\)]\(_i\). Although L-Orn does inhibit [Ca\(^{2+}\)]\(_i\) rise, the effect of L-Arg was unchanged in the presence of 20 mM L-Val, a potent inhibitor of the arginase pathway (22), compared with L-Arg alone. If the effect of L-Arg were due to its metabolism to L-Orn, the inhibition produced by L-Arg (but not by L-Orn) would have been reversed by L-Val.

Most likely, we are seeing a charge effect of these cationic amino acids. Both the D and L forms of Arg and Orn inhibit the rise in [Ca\(^{2+}\)]\(_i\), to the same degree. In addition, other cationic amino acids, L-spermine and L-spermidine, inhibit [Ca\(^{2+}\)]\(_i\) rise. To confirm the importance of the effect of L-Arg on [Ca\(^{2+}\)]\(_i\), we demonstrated that the functional cationic group of L-Arg, guanidine, also inhibits [Ca\(^{2+}\)]\(_i\) rise. L-Val, a branched-chain amino acid, had no effect on vasopressin-stimulated [Ca\(^{2+}\)]\(_i\), thereby ruling out a nonspecific amino acid effect. The exact mechanism by which L-Arg exerts this structural effect is unknown. However, because L-Arg also inhibits endothelin-stimulated rise in [Ca\(^{2+}\)]\(_i\) (Balsam, unpublished data), it would appear that L-Arg acts on [Ca\(^{2+}\)]\(_i\) through a common pathway to both hormones, possibly the phosphatidylinositol pathway. It is therefore unlikely that the structural effect of the various amino acids is at the receptor site, as AVP and endothelin act through different receptors.

Notably, L-NMMA itself decreased AVP-stimulated [Ca\(^{2+}\)]\(_i\), but the results did not reach statistical significance compared with control. The [Ca\(^{2+}\)]\(_i\), of cells bathed in L-Arg plus L-NMMA was identical to that of cells bathed with L-Arg alone and statistically lower than that of cells bathed in control solution or in L-NMMA alone. This is consistent with the inhibitory effect being due to L-Arg, since the addition of L-NMMA did not bring the [Ca\(^{2+}\)]\(_i\) back even to the level of L-NMMA alone. The decrease in [Ca\(^{2+}\)]\(_i\) by L-NMMA is most likely due to the structural similarity of L-NMMA and L-Arg.

Work by other investigators supports our findings that cationic compounds influence [Ca\(^{2+}\)]\(_i\). It has been shown that the aminoglycosides, which are organic polycations, preferentially bind to anionic phospholipids, especially phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P\(_2\)] (12). Binding of the cationic aminoglycosides to anionic PtdIns(4,5)P\(_2\) is probably responsible for the disruption of the phosphatidylinositol cascade by these agents. A similar process may be occurring with the cationic amino acids, whose inhibition of
[Ca^{2+}]_i rise may be mediated by binding and disruption of the Ptdlns(4,5)P_2 pathway. Inhibiting the metabolism of Ptdlns(4,5)P_2 decreases the formation of d-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which, in turn, decreases the release of Ca^{2+} from intracellular stores (4). Preliminary data in our laboratory demonstrated that AVP-stimulated [Ca^{2+}], was inhibited by dantrolene (3), an inhibitor of Ca^{2+} release from intracellular stores. Dantrolene is believed to work through an inhibition of Ins(1,4,5)P_3 (16). When cells were bathed in both L-Arg and dantrolene, there was no additive inhibition. This suggests that L-Arg may work in a manner similar to that of dantrolene, namely via an Ins(1,4,5)P_3-mediated mechanism. However, direct measurements of Ins(1,4,5)P_3 and other metabolites of the Ptdlns(4,5)P_2 pathway are necessary to definitively determine whether L-Arg affects [Ca^{2+}]_i through an Ins(1,4,5)P_3-mediated mechanism.

Because the peak MC [Ca^{2+}]_i response to AVP is dependent both on release from intracellular Ca^{2+} stores and on extracellular Ca^{2+} (5, 14), mechanisms other than that of Ins(1,4,5)P_3 may be playing a role. There may be additional effects of L-Arg on other intracellular pathways or on Ca^{2+} fluxes into or out of the cell. In a canine cardiac model, polycytons were found to affect Ca^{2+}-Mg^{2+}-ATPase on the sarcoplasmic reticulum membrane (30). However, because we are using whole cells and a different cell type and species, other factors and/or membranes may be important, such as mitochondrial or plasma cell membranes.

Further studies are needed to characterize the exact mechanism by which L-Arg affects MC [Ca^{2+}]_i. Several issues should be addressed. First, it would be interesting to expand on the effect that L-Arg has on endothelin-stimulated [Ca^{2+}]_i, as well as to explore its action on other agonists. Another question involves the potential effect of L-Arg on Ins(1,4,5)P_3. Can Ins(1,4,5)P_3 directly stimulate these cells to release Ca^{2+} from the sarcoplasmic reticulum? If so, would L-Arg affect this release of Ca^{2+}? A further issue entails the possible action of L-Arg on Cl^- channels. AVP depolarizes MC by activating Cl^- channels, which, in turn, modulate [Ca^{2+}]_i (14). Does L-Arg affect AVP-stimulated Cl^- conductance?

In addition to the mechanism by which L-Arg and AVP interact, the significance of inhibition by L-Arg of AVP-stimulated [Ca^{2+}]_i must be more clearly delineated. Whether this inhibition represents modulation of MC tension and/or a feedback mechanism on NO production remains to be elucidated.

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