# $p^1,p^4$-Diadenosine 5′-tetraphosphate induces the uptake of arginine and citrulline by a pore on the plasma membrane of bovine aortic endothelial cells

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Received 23 October 2000; accepted 23 October 2000

First published online 2 November 2000

Edited by Jacques Hanoune

**Abstract We have previously demonstrated that $p^1,p^4$-diadenosine 5′-tetraphosphate ($Ap_4A$) induces the release of nitric oxide (NO) and modulates the uptake of extracellular L-arginine (L-Arg) and L-citrulline (L-Cit) by bovine aortic endothelial cells (BAEC) [Hilderman, R.H. and Christensen, E.F. (1998) FEBS Lett. 427, 320–324 and Hilderman, R.H., Casey, T.E. and Pojoga, L.H. (2000) Arch. Biochem. Biophys. 375, 124–130]. In this communication we report that extracellular $Ap_4A$ enhances the uptake of L-Arg and L-Cit through a pore on the plasma membrane of BAEC that is selective for these two amino acids. We also demonstrate that $Ap_2A$, which induces NO release, enhances L-Arg uptake while $Ap_5A$, a vasoconstrictor, does not enhance the uptake of L-Arg. The potential physiological significance of the uptake of these two amino acids in relation to NO synthesis is discussed.** 

*Key words:* $Ap_4A$; Pore; L-Arg uptake; Bovine endothelial cell

## 1. Introduction

Adenosine (5′) oligophospho-(5′) adenosine dinucleotides are a novel class of extracellular signal molecules that are stored in platelets and are released into the vascular system following physiological stress [3–5]. Thadenine dinucleotides contain two adenosine moieties linked via their 5′ positions by a chain of phosphates ($Ap_xA$; $x = 2–7$). $p^1,p^4$-Diadenosine 5′-tetraphosphate ($Ap_4A$) is one of the most abundant and best characterized adenine dinucleotides. Extracellular $Ap_4A$ has been shown to modulate blood vessel tone [6–8], induce the release of nitric oxide (NO) from endothelial cells [1], prime respiratory burst and regulate apoptosis in neutrophils [9], inhibit platelet aggregation [10], activate glycogen phosphorylase in hepatocytes [11], elicit smooth-muscle contractions in the vas deferens [12] and urinary bladder [13], and, in other tissues, promote catecholamine release [14].

Many of the biological effects of extracellular $Ap_4A$ are mediated by the interaction of this dinucleotide with cell surface receptors. We have demonstrated that extracellular $Ap_4A$ induces the release of NO in endothelial cells by interacting with a receptor that is specific for some but not all of the α,ω-adenine dinucleotides (termed $P_4$ purinoceptor) [1,15]. In addition, we have shown that extracellular $Ap_4A$ interacts with a second receptor system on endothelial cells to enhance the uptake of L-arginine (L-Arg) and L-citrulline (L-Cit) [2]. Other investigators have demonstrated that bradykinin and ATP stimulate an increase of L-Arg uptake and NO release [16]. Since NO is synthesized by the enzyme nitric oxide synthase (NOS) through the conversion of L-Arg to L-Cit [17], the ability of various ligands to stimulate an increase in both L-Arg uptake and NO release suggests that the intracellular concentration of L-Arg may be rate-limiting for NO production. However, the intracellular concentration of L-Arg has been shown to be significantly higher than the $EC_{50}$ value of NOS for L-Arg [18–20]. These observations suggest that NOS may be saturated with L-Arg and that the supply of extracellular L-Arg is important for NO generation. This discrepancy would be resolved if L-Arg and NOS are compartmentalized in endothelial cells. Other investigators have demonstrated that a major proportion of NOS is particulate [21,22], and may be localized at the plasma membrane [23], which would provide a close link between an arginine transporter and NOS. Thus it is important to characterize L-Arg transport in endothelial cells. As a step in this direction we have demonstrated L-Arg is transported into bovine aortic endothelial cells (BAEC) by at least two different transporter systems [24]. One transporter system is protein synthesis dependent and L-Arg transported by this system is incorporated into proteins. The second transporter system is protein synthesis independent and uptake occurs by facilitated diffusion. However, $Ap_4A$ does not enhance the uptake of L-Arg through either of these transporter systems. In this communication we report that $Ap_4A$ enhances the uptake of L-Arg and L-Cit but not L-Lys, L-Leu or L-Asp through a pore on the plasma membrane of BAEC.

## 2. Materials and methods

### 2.1. Materials

L-[2,3,4-$^3$H]Arg, L-[4,5-$^3$H]Lys, L-[3,4,5-$^3$H]Leu, L-[2,3-$^3$H]Asp and L-[ureido-$^{14}$C]Cit were purchased from New England Nuclear. Cycloheximide, $Ap_2A$, $Ap_3A$, $Ap_4A$ and $Ap_5A$ were purchased from Sigma. All other reagents were of analytical reagent grade or better.

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0014-5793/00/$20.00 
PII: S0014-5793(00)02187-6

*2.2. Cell culture*

BAEC ($1.0\times10^5$ cells) were seeded in 35-mm gelatin-coated petri dishes and grown as previously described [2]. All experiments were performed with passage 10 cells.

*2.3. Measurement of L-Arg uptake at 4°C*

To ensure that we were measuring L-Arg uptake via a pore and not by a transporter system, all L-[$^3$H]Arg uptake experiments were performed at 4°C. All experiments were performed using cells in early growth phase ($2.5–5\times10^4$ cells/cm$^2$) in 35-mm gelatin-coated petri dishes. Endothelial cells were washed three times with Krebs–Henseleit buffer (10 mM HEPES (pH 7.4), 120 mM NaCl, 4.6 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM $NaH_2PO_4$, 0.7 mM $Na_2HPO_4$, 10 mM glucose), incubated in the presence or absence of 25 μM $Ap_4A$ for 30 min at 37°C in 0.8 ml of the same buffer and then incubated for 20 min at 4°C. Uptake studies were performed at 4°C in a final volume of 1.0 ml and were initiated by the addition of 200 μM L-[$^3$H]Arg (6000–6500 cpm/nmol). The samples were incubated for 30 min at 4°C with shaking and then washed three times with ice-cold Krebs–Henseleit buffer containing 10 mM L-Arg. After washing with Krebs–Henseleit buffer, the samples were solubilized with 0.1 M NaOH and the alkaline solution was counted in a Beckman LS-3133P liquid scintillation counter as described [2]. Blanks were obtained by adding L-[$^3$H]Arg to the cells and immediately washed with ice-cold Krebs–Henseleit buffer prior to solubilizing with 0.1 M NaOH and counted. All experiments were performed in triplicate and repeated at least eight times.

*2.4. Determination of cell number*

After each experiment the total number of cells in four petri dishes was determined following trypsin digestion as described [24]. Cell densities were determined by counting in a hemocytometer and averaging four plates.

*2.5. Metabolism of L-Arg*

Metabolism studies were performed as described above in Section 2.3 except 1 μM L-[$^3$H]Arg ($1–1.4\times10^6$ cpm/nmol) was used in the experiments. Cells were lysed in 0.1 ml of distilled $H_2O$ and cell extracts were obtained by scraping followed by centrifugation at $10\,000\times g$ for 10 min. Supernatants (50 μl) were spotted onto TLC plates (Whatman silica gel AlSil G/UV). The plates were developed as described [2,24] and autoradiography was performed at −80°C for 14 days.

*2.6. Statistics*

Results are presented as means ± standard deviations. Statistical significance was assessed using the Student's *t*-test. Statistical significance was assumed if $P<0.05$.

## 3. Results

*3.1. Effect of $Ap_4A$ on L-Arg uptake by BAEC at 4°C*

The ability of $Ap_4A$ to enhance the uptake of L-Arg at 4°C is presented in Fig. 1. Variable amounts of $Ap_4A$ were incubated with BAEC for 30 min at 37°C, then incubated for an additional 20 min at 4°C prior to the addition of L-[$^3$H]Arg and incubation for 30 min at 4°C. $Ap_4A$ enhanced, in a dose-dependent manner, the uptake of L-[$^3$H]Arg and reached a plateau at 10 μM (Fig. 1). $Ap_4A$ concentrations up to 500 μM did not significantly increase the amount of L-[$^3$H]Arg taken up by BAEC (data not shown). All subsequent experiments were performed with 25 μM $Ap_4A$.

*3.2. Effect of suramin on $Ap_4A$ enhanced uptake of L-Arg by BAEC at 4°C*

Suramin does not inhibit the binding of $Ap_4A$ to the $Ap_4A$ $P_4$ purinoceptor on BAEC but suramin does inhibit the $Ap_4A$ enhanced uptake of L-Arg at 37°C [2,18]. Thus we determined the effect of suramin on $Ap_4A$ enhanced uptake of L-Arg by BAEC at 4°C. As shown in Fig. 2, suramin had no effect on

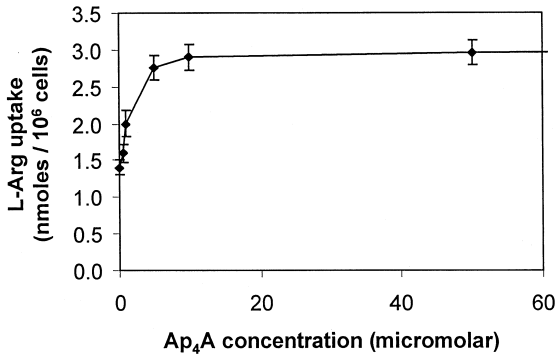


Fig. 1. Effect of $Ap_4A$ on L-Arg uptake by BAEC at 4°C. BAEC were incubated with the indicated concentrations of $Ap_4A$ for 30 min at 37°C followed by incubating the cells for an additional 20 min at 4°C. Uptake studies were then initiated by the addition of 200 μM L-[$^3$H]Arg (6000–6500 cpm/nmol) and the cells were incubated for 30 min at 4°C. After this incubation the cells were washed with ice-cold Krebs–Henseleit buffer, solubilized with 0.1 M NaOH and counted as described [2]. Data are averages of eight different experiments performed in triplicate. Error bars are shown as standard deviations.

L-Arg uptake in absence of $Ap_4A$. In the presence of $Ap_4A$, suramin inhibited 56% of the L-[$^3$H]Arg uptake at 4°C. These data further support the notion that $Ap_4A$ modulates the uptake of L-Arg through the second class of binding sites and not the $Ap_4A$ $P_4$ purinoceptor.

*3.3. Effect of temperature on the metabolism of L-Arg by BAEC*

To determine the effect of temperature on the ability of BAEC to metabolize L-[$^3$H]Arg, $Ap_4A$ was incubated with two sets of BAEC for 30 min at 37°C. Radiolabeled L-Arg was added to the first set of cells and these cells were incubated for an additional 30 min at 37°C. The second set of cells was incubated at 4°C for 20 min prior to the addition of L-[$^3$H]Arg and incubated for 30 min at 4°C. Both sets of cells were then lysed, centrifuged, and spotted on TLC plates, and

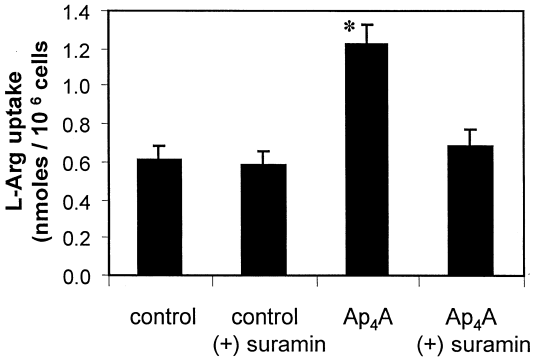


Fig. 2. Effect of suramin on $Ap_4A$ enhanced uptake of L-Arg by BAEC at 4°C. BAEC were incubated in the presence or absence of 500 μM suramin for 15 min at 37°C prior to the addition of 25 μM $Ap_4A$ and the cells were incubated for an additional 30 min at 37°C. The cells were then incubated for an additional 20 min at 4°C and uptake studies were performed at 4°C as described in the legend of Fig. 1. Data are averages of eight different experiments performed in triplicate. Error bars are shown as standard deviations. Significant differences from the control values (amino acid uptake in absence of $Ap_4A$) were determined by Student's *t*-test. *, $P<0.05$ vs. control.

the plates were developed and analyzed by autoradiography. As shown in lane 2 of Fig. 3, L-[$^3$H]Arg taken up by BAEC at 37°C migrates as three components: L-Arg, material remaining at the origin and a component migrating with an $R_f$ value of 0.8. We have previously demonstrated that the material at the origin is L-[$^3$H]Arg incorporated into proteins and the material with an $R_f$ value of 0.8 co-migrates with L-argininosuccinate standards [24]. Lane 3 of Fig. 3 demonstrates that L-[$^3$H]Arg taken up by BAEC at 4°C migrates as L-Arg and L-argininosuccinate with a small amount of material remaining at the origin. These data demonstrate that BAEC converts a small amount of L-Arg to L-argininosuccinate at 4°C. To determine whether eNOS converts L-Arg to L-argininosuccinate at 4°C, BAEC were incubated with $Ap_4A$ and diphenyleneiodonium chloride (an irreversible inhibitor of eNOS [25]) for 30 min at 37°C. Then these cells were subsequently incubated at 4°C for 20 min prior to the addition of L-[$^3$H]Arg and incubated for an additional 30 min at 4°C. As shown in lane 4 of Fig. 3, diphenyleneiodonium chloride inhibits the ability of BAEC to convert L-Arg to L-argininosuccinate. These data support the notion that L-Arg is converted to L-argininosuccinate at 4°C by eNOS.

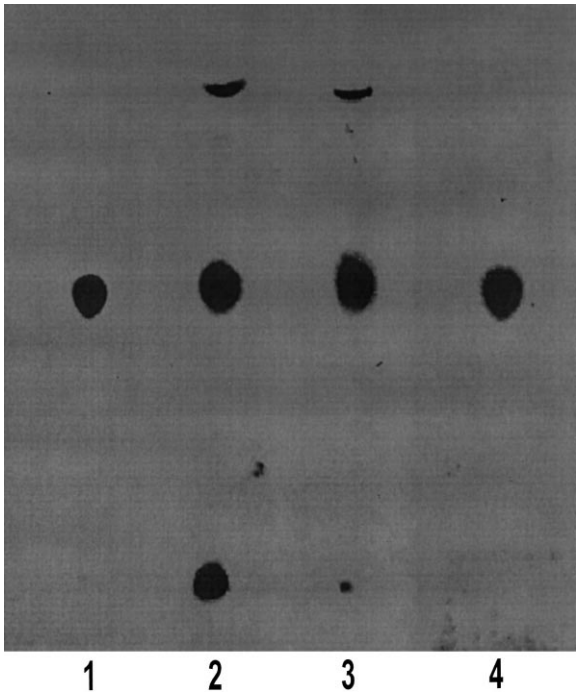


Fig. 3. Effect of temperature on the metabolism of L-Arg by BAEC. BAEC were treated as described below prior to being analyzed by TLC and autoradiography as described [2,24]. Lane 1, 1 μM L-[$^3$H]Arg ($1–4\times10^6$ cpm/nmol) incubated in Krebs–Henseleit buffer for 30 min at 37°C prior to TLC. Lane 2, BAEC were incubated with 25 μM $Ap_4A$ for 30 min at 37°C. Uptake studies were initiated by the addition of 1 μM L-[$^3$H]Arg ($1–4\times10^6$ cpm/nmol) and the cells were incubated for 30 min at 37°C prior to TLC. Lane 3, BAEC were incubated with 25 μM $Ap_4A$ for 30 min at 37°C prior to incubating at 4°C for 20 min. Uptake studies were initiated by the addition of 1 μM L-[$^3$H]Arg ($1–4\times10^6$ cpm/nmol) and the cells were incubated for 30 min at 4°C prior to TLC. Lane 4, BAEC were incubated with 25 μM $Ap_4A$ and 600 μM DPI for 30 min at 37°C prior to incubating at 4°C for 20 min. Uptake studies were initiated by the addition of 1 μM L-[$^3$H]Arg ($1–4\times10^6$ cpm/nmol) and the cells were incubated for 30 min at 4°C prior to TLC.

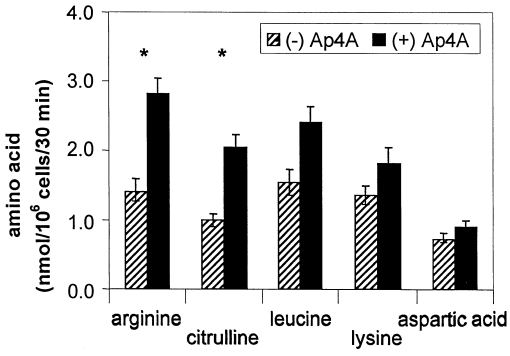


Fig. 4. Specificity of $Ap_4A$ on amino acid uptake by BAEC at 4°C. BAEC were incubated in the presence or absence of 25 μM $Ap_4A$ for 30 min at 37°C followed by the incubation of the cells for an additional 20 min at 4°C. Uptake studies were performed at 4°C as described in the legend of Fig. 1 using 200 μM L-[$^3$H]Arg (6000–6500 cpm/nmol), L-[$^{14}$C]Cit (4500–5000 cpm/nmol), L-[$^3$H]Leu (3000–3500 cpm/nmol), L-[$^3$H]Lys (3000–3500 cpm/nmol) or L-[$^3$H]Asp (7000–7500 cpm/nmol). Data are averages of eight different experiments performed in triplicate. Error bars are shown as standard deviations. Significant differences from the control values (amino acid uptake in absence of $Ap_4A$) were determined by Student's *t*-test. *, $P < 0.05$ vs. control.

### 3.4. Specificity of $Ap_4A$ on amino acid uptake by BAEC at 4°C

The ability of $Ap_4A$ to enhance the uptake of radiolabeled L-Arg, L-Cit, L-Leu, L-Lys and L-Asp at 4°C is shown in Fig. 4. $Ap_4A$ significantly enhanced the uptake of only L-Arg and L-Cit when compared to controls (Fig. 4). $Ap_4A$ enhanced the uptake of L-Arg and L-Cit over controls 1.96- and 2.03-fold, respectively. $Ap_4A$ did not enhance the uptake of the fluorescent dyes ethidium, propidium or lucifer yellow at either 4 or 37°C (data not shown). These data suggest that $Ap_4A$ renders a pore selective for the amino acids involved in NO biosynthesis.

### 3.5. Specificity of α,ω-adenine dinucleotides on L-Arg uptake by BAEC at 4°C

Since $Ap_4A$ and $Ap_2A$ induce the release of NO from BAEC while $Ap_3A$ and $Ap_5A$ do not [1], we determined whether these dinucleotides enhanced the uptake of L-Arg by BAEC at 4°C. As shown in Fig. 5, only $Ap_4A$ and $Ap_2A$ significantly enhanced the uptake of L-Arg when compared to controls. The enhancement of L-Arg over controls for $Ap_4A$ and $Ap_2A$ was 1.96- and 1.81-fold, respectively. In addition, $Ap_6A$, ADP, UTP and benzoyl-ATP (Bz-ATP) did not enhance the uptake of L-Arg at 4°C when compared to controls (data not shown). These data support the notion that

Table 1
Effect of protein synthesis on the $Ap_4A$ enhanced uptake of L-Arg by BAEC at 4°C

| Treatment | L-[$^3$H]Arg uptake (relative percentage) |
|---|---|
| 25 μM $Ap_4A$ | 100 |
| 200 μg cycloheximide and 25 μM $Ap_4A$ | 48 |

BAEC were incubated in the presence or absence of 200 μg of cycloheximide for 15 min at 37°C prior to the addition of 25 μM $Ap_4A$; the cells were then incubated for an additional 30 min at 37°C. The cells were then incubated for 20 min at 4°C and uptake studies were as described in the legend of Fig. 1. Data are averages of eight different experiments performed in triplicate.

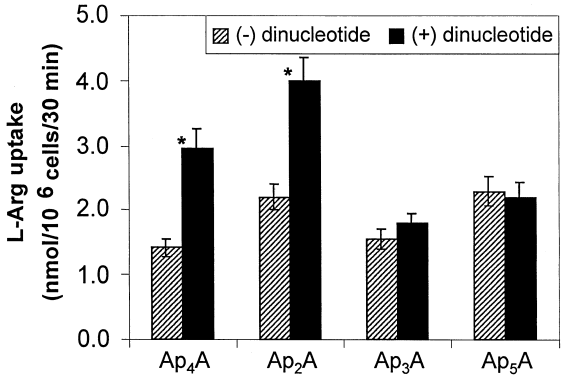


Fig. 5. Specificity of α,ω-adenine dinucleotides on L-Arg uptake by BAEC at 4°C. BAEC were incubated in the presence or absence of 25 μM of the indicated α,ω-adenine dinucleotide for 30 min at 37°C followed by incubation of the cells for an additional 20 min at 4°C; uptake studies were performed at 4°C as described in the legend of Fig. 1. Data are averages of eight different experiments performed in triplicate. Error bars are shown as standard deviations. Significant differences from the control values (amino acid uptake in absence of α,ω-adenine dinucleotide) were determined by Student's *t*-test. $*P < 0.05$ vs. control.

the α,ω-adenine dinucleotides that induce the release of NO from BAEC also enhance the uptake of L-Arg through a pore.

### 3.6. *Effect of cycloheximide on the $Ap_4A$ enhanced uptake of L-Arg by BAEC at 4°C*

To determine whether the $Ap_4A$ enhanced uptake of L-Arg requires protein synthesis, we determined the effect of cycloheximide on the uptake of L-[$^3$H]Arg. As shown in Table 1, cycloheximide inhibited 52% of the $Ap_4A$ enhanced uptake of L-Arg by BAEC. Furthermore, the incubation of BAEC in the presence of $Ap_4A$ at 4°C instead of 37°C prior to performing L-[$^3$H]Arg uptake studies also inhibited the $Ap_4A$ enhanced uptake of L-Arg (data not shown). These data support the notion that protein synthesis is required for the selective uptake of L-Arg and L-Cit at 4°C.

## 4. Discussion

The studies in this communication demonstrate that extracellular $Ap_4A$ selectively increases the permeability of BAEC plasma membranes at 4°C to L-Arg and L-Cit but not to L-Lys, L-Leu or L-Asp (Fig. 4). Membrane permeability to fluorescent dyes was also not enhanced by $Ap_4A$. These data suggest that $Ap_4A$ renders a pore selective for the amino acids involved in NO biosynthesis. L-Arg uptake at 4°C was also enhanced by $Ap_2A$ but not by $Ap_3A$ or $Ap_5A$ (Fig. 5). Since $Ap_4A$ and $Ap_2A$, but not $Ap_3A$ or $Ap_5A$, induce NO release above basal levels in BAEC [1], these data further support the notion that extracellular signal molecules that induce NO release also enhance the uptake of extracellular L-Arg. Furthermore, $Ap_4A$ not only enhances the uptake of L-[$^3$H]Arg at 4°C but BAEC also converts L-[$^3$H]Arg to L-[$^3$H]argininosuccinate at 4°C (Fig. 3). These data are consistent with the hypothesis that NOS is compartmentalized on the plasma membrane and that $Ap_4A$ and $Ap_2A$ deliver extracellular L-Arg to NOS via a selective pore.

Extracellular $ATP^{-4}$ has been shown to affect plasma membrane permeability by a non-selective pore ($P_{2Z}$ receptor) [26–28]. $ATP^{-4}$ has been shown to increase the plasma membrane permeability of ions and fluorescent dyes such as of ethidium, propidium or lucifer yellow in various cell types [26–29]. Since $Ap_4A$ does not enhance the uptake of ethidium, propidium or lucifer yellow in BAEC at either 4 or 37°C and $Ap_4A$ selectively enhances the uptake of L-Arg and L-Cit at 4°C, these data are consistent with the notion that $Ap_4A$ does not alter BAEC plasma membrane permeability by interacting with a $P_{2Z}$ receptor. Since Bz-ATP, a $P_{2Z}$ agonist, does not enhance the uptake of L-Arg at 4°C, this further supports the notion that $Ap_4A$ does not alter BAEC plasma membrane permeability by interacting with a $P_{2Z}$. The inability of ADP, UTP and $Ap_6A$ to enhance L-Arg uptake at 4°C is consistent with $P_{2Y1}$ and $P_{2Y2}$ receptors not being involved in L-Arg uptake at 4°C.

Cycloheximide inhibits the $Ap_4A$ enhanced uptake of L-Arg at 4°C (Table 1). These data suggest protein synthesis is required for the $Ap_4A$ enhanced uptake of L-Arg. At the present time it is unclear whether the receptor with which $Ap_4A$ interacts is a ligand-operated channel or whether it is a signaling device that activates other membrane molecules, leading to pore formation. In addition, it is also unclear whether L-Arg uptake at 4°C in the absence of $Ap_4A$ is through the same pore that occurs in the presence of $Ap_4A$. In any event, $Ap_4A$ permeabilization of endothelial cell plasma membranes may be of physiological importance as the mechanism by which extracellular L-Arg is delivered to NOS for the generation of NO.

*Acknowledgements:* This research was supported in part by NSF MCB-9816681, South Carolina Experiment Station Grant SC01630 and the Greenville Hospital System. The authors would like to thank Dr. Jim Zimmerman of Clemson University for critically evaluating the manuscript.

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