monally regulated proteins by modulating its own stability or modulating GR-mediated ubiquitination and subsequent degradation of other proteins. In fact, glucocorticoid hormone treatment has been shown to stimulate protein catabolism associated with muscle wasting (*22*). Because glucocorticoid hormone treatment results in a predominance of the transformed, monomeric GR capable of binding tRNA (as documented in our study), this GR may then participate in the ubiquitin pathway.

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Brain Barrier Tissues: End Organs for Atriopeptins

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Little is known about the pathophysiology of cerebral edema and other disturbances of water balance that involve the barrier tissues at the interface of blood and brain. The present experiments show that these barrier tissues contain receptors and second messenger systems for atriopeptins, recently identified cardiac peptides involved in peripheral water regulation. They also show that atriopeptins can alter the rate of cerebrospinal fluid production. Because the blood-brain and blood–cerebrospinal fluid barriers are involved in normal water movements in the central nervous system, these studies suggest that brain barrier tissues may be important end organs for the atriopeptins and that atriopeptins could have therapeutic application to disorders of water balance in the central nervous system. An isolated, purified population of atriopeptin receptor cells, obtained from choroid epithelium, was used in these experiments. This cell population may provide a valuable model system for investigating the intracellular biochemical mechanisms through which atriopeptins exert their actions.

BECAUSE THE BRAIN IS ENCASED within a rigid skull and lacks a true lymphatic drainage system, it is critically vulnerable to damage from edema. However, when compared with peripheral tissues, relatively little is known about intracranial regulation of water and electrolytes. Extracellular fluid movement into and out of the brain occurs primarily at the blood-brain barrier (capillary endothelium), blood–cerebrospinal fluid (CSF) barrier (choroid plexus epithelium), and CSF outflow system (dural sinus–arachnoid villi) (*1*). Recently, it became apparent that atriopeptins, released from atrial cardiocytes, may be key hormones for regulating fluid volume in the periphery (*2*). Because the endothelial cells of the blood-brain barrier and the epithelial cells of the blood-CSF barrier potentially have access to circulating atriopeptins, we investigated the possibility that brain barrier tissues might be end organs for atriopeptins. Our biochemical and physiological studies, detailed below, support this possibility (*3*). We also describe a cell population that may be useful for studying the mechanisms of action of atriopeptin receptors both in the brain and periphery.

Evidence from peripheral tissues suggests that atriopeptin receptor occupancy is associated with the intracellular production of guanosine 3′,5′-monophosphate (cyclic GMP) (*4*). Therefore, to determine whether atriopeptin receptors might be present in brain barrier tissues, we first attempted to establish whether atriopeptin-activated guanylate cyclase activity could be demonstrated in washed membrane fractions prepared from these tissues. The presence of such activity would indicate that these peptides not only bind to the membrane but that they are capable of exerting a biochemical alteration of membrane activity.

Figure 1A shows that rat atrial natriuretic peptide (rANP) 1–28 (Ser-Leu-Arg-Arg-atriopeptin III) was a potent activator of guanylate cyclase activity in purified rabbit cerebral microvessels [maximum velocity of enzyme activity (V_{max}), 215% of control; activation constant (K_a), 0.5 n*M*]. From other studies, it is known that such microvessels consist of a high percentage of endothelial cell–containing cerebral capillaries (*5*). A similar degree of activation of guanylate cyclase by rANP was observed in microvessels prepared from pig brain.

Figure 1B (middle curve) shows that rANP also stimulated enzyme activity in membrane fractions prepared from whole rabbit choroid plexus obtained from lateral, third, and fourth ventricles. In five separate experiments, the K_a for activation of the enzyme in the choroid (9.8 ± 5.1 n*M*; SEM) was similar to that (K_a = 5 ± 2 n*M*; SEM, n = 4) which we observed in rabbit kidney, a tissue known to be rich in atriopeptin receptors (*4, 6*), and somewhat greater than that which we observed for atriopeptin stimulation in the rabbit cerebellum (Fig 1A), a tissue known to be enriched (relative to other brain areas) in cyclic GMP (*7*). The V_{max} for stimulation of basal activity in the choroid plexus averaged 58% ± 16% (SEM, n = 5).

However, we found no stimulation of guanylate cyclase activity in membrane fractions prepared from rabbit or pig cerebral cortex (Fig. 1A). In membranes prepared from the ventral portion of pig or rabbit superior sagittal dural sinus, which contains

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arachnoid villi, rANP caused a small stimulation of guanylate cyclase but only at high concentrations (K_a > 1000 n*M*). There was also a very small (15%) degree of enzyme stimulation in the pia arachnoid, a preparation consisting of both pia arachnoid membrane and small extraparenchymal cerebral arterioles and veins.

Stimulation by rANP was selective for guanylate cyclase and did not activate adenylate cyclase in the same brain barrier preparations, although adenylate cyclase was stimulated by isoproterenol (Fig. 1C). As shown in Fig. 1C, high concentrations of rANP caused a small inhibition of adenylate cyclase. Stimulation of guanylate cyclase in barrier tissues was also selective for the intact 28–amino acid peptide. Thus, in choroid plexus, we found that rANP fragment 1–11 was about 10% to 15% as active as the intact peptide and that rANP fragment 13–28 caused almost no stimulation of guanylate cyclase, a pattern of activity nearly identical to that which we found in rabbit kidney. For rANP 1–28, the range of K_a values (0.5 to 10 n*M*) that we observed for guanylate cyclase activation in cerebral microvessels and choroid plexus was similar to the range of binding affinities (0.1 to 2 n*M*) reported in other tissues for radiolabeled atriopeptins (*6*).

Choroid plexus is quite vascular, consisting of capillary and arteriolar loops covered by a single layer of secretory epithelium. Because cerebral microvessels contained rANP-stimulated guanylate cyclase activity, it was possible that the activity observed in the intact choroid was due to atriopeptin receptors present on choroid vascular components. Therefore, in order to localize rANP-stimulated guanylate cyclase activity in the choroid plexus, we isolated highly enriched suspensions of choroid epithelial cells from intact choroid by methods described earlier (*8*) (Fig. 1). The identity of the cells was confirmed through immunohistochemical studies showing the presence (>95%) of morphologically typical epithelial cells strongly stained with a polyclonal antibody to the peripheral form of Na- and K-activated adenosine triphosphatase (Na, K-ATPase) (*9*), which only faintly labels endothelial and stromal cells, and with antibody to the protein, DARPP-32, which solely labels epithelial cells (Fig. 2, a to c) (*10*).

Figure 1B shows that the activity of rANP-stimulated guanylate cyclase in membranes prepared from epithelial cells was much greater than that in membranes prepared from vascular cell–enriched choroid remaining after most epithelial cells were removed. Thus, surprisingly, it is the secretory epithelium itself, and not the vascular components of the choroid, that contains a high concentration of rANP receptors.

When isolated as described above, the choroid epithelium is a nearly homogeneous population of cells that can be identified by cell markers and sustained in short-term culture (*11*). The existence of atriopeptin receptors in such a defined cellular population suggests that these cells might be extremely valuable for studying the receptor and post-receptor mechanisms associated

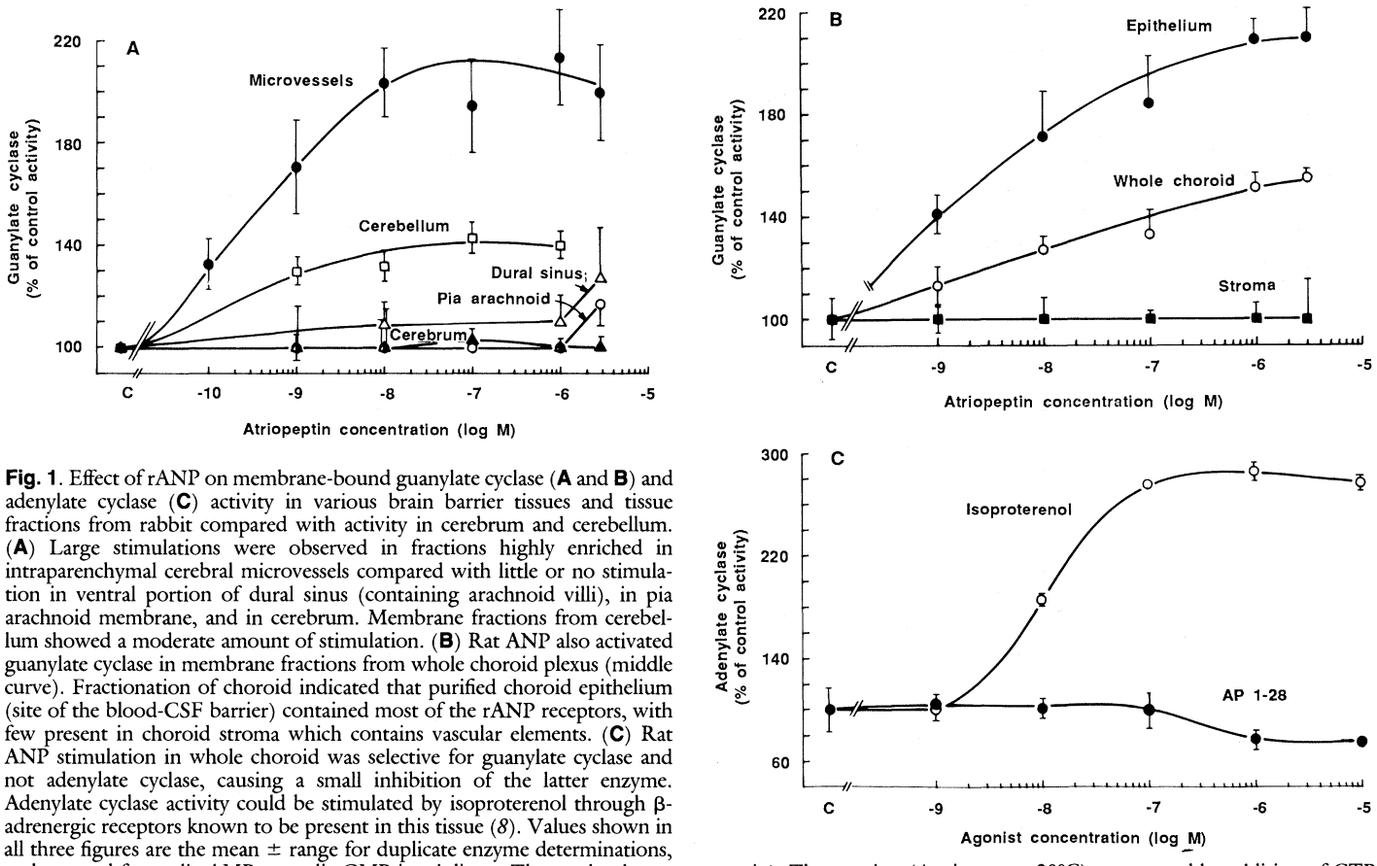

Fig. 1. Effect of rANP on membrane-bound guanylate cyclase (**A** and **B**) and adenylate cyclase (**C**) activity in various brain barrier tissues and tissue fractions from rabbit compared with activity in cerebrum and cerebellum. (**A**) Large stimulations were observed in fractions highly enriched in intraparenchymal cerebral microvessels compared with little or no stimulation in ventral portion of dural sinus (containing arachnoid villi), in pia arachnoid membrane, and in cerebrum. Membrane fractions from cerebellum showed a moderate amount of stimulation. (**B**) Rat ANP also activated guanylate cyclase in membrane fractions from whole choroid plexus (middle curve). Fractionation of choroid indicated that purified choroid epithelium (site of the blood-CSF barrier) contained most of the rANP receptors, with few present in choroid stroma which contains vascular elements. (**C**) Rat ANP stimulation in whole choroid was selective for guanylate cyclase and not adenylate cyclase, causing a small inhibition of the latter enzyme. Adenylate cyclase activity could be stimulated by isoproterenol through β-adrenergic receptors known to be present in this tissue (*8*). Values shown in all three figures are the mean ± range for duplicate enzyme determinations, each assayed for cyclic AMP or cyclic GMP in triplicate. The results shown are typical of those seen in other experiments. Guanylate cyclase was measured as the rate of cyclic GMP formation by modifications of the technique of Waldman *et al.* (*4*). Briefly, tissues were homogenized (10 mg wet weight per milliliter) in 50 m*M* tris-HCl, *p*H 8.0, 1 m*M* EDTA, 1 m*M* dithiothreitol, and 250 m*M* sucrose and centrifuged at 100,000*g* to obtain a P1 pellet. Reaction tubes contained (in 0.3 ml), 50 m*M* tris, *p*H 7.6, 6 m*M* $MnCl_2$, 0.5 m*M* 3-isobutyl-1-methylxanthine (IBMX), 10 m*M* theophylline, 3 m*M* GTP, hormone (in 0.03 ml containing 2.5 m*M* ascorbic acid and 0.1% bovine serum albumin), and 0.06 ml of P1 fraction (40 to 60 μg of protein). The reaction (4 minutes at 30°C) was started by addition of GTP and terminated by addition of 0.3 ml of 150 m*M* sodium acetate (*p*H 4.0) and boiling for 3 minutes. Cyclic GMP formed was subsequently measured by radioimmunoassay. Under these conditions, guanylate cyclase activity was linear with time and tissue concentration. Adenylate cyclase activity (C) in P1 fractions was measured as previously described (*8*). Choroid epithelial cells (B) were isolated by previously described techniques (*8, 11*), but with a lower concentration (0.025%) of trypsin and a rotating tissue tumbler which allowed cleaner fractionation of epithelial cells from erythrocytes and vascular components.

with atriopeptin action. Such information would be potentially applicable to the action of atriopeptins, not only in brain barrier tissues, but in peripheral end organs as well.

Therefore, to determine whether isolated, intact epithelial cells maintain their responsiveness to atriopeptins, we isolated purified secretory epithelial cells and maintained them in tissue culture medium in the absence of any hormones. After 3 hours, rANP was added either alone or in presence of phosphodiesterase (PDE) inhibitors. After the cells were incubated for 5 minutes, they were killed and their cyclic GMP content was determined. The isolated epithelial cells showed more than an eightfold increase in cyclic GMP content when incubated with rANP alone (Fig. 2D). Basal cyclic GMP content was increased by the PDE inhibitors, and the combination of rANP and PDE inhibitors caused more than 30-fold increase in cyclic GMP content. (Similar but somewhat larger increases were seen after 15 minutes of incubation.) These marked increases in cyclic GMP provide further evidence that the choroid epithelium is an atriopeptin end organ.

The presence of atriopeptin receptors in choroid epithelium suggested that atriopeptins might affect the secretory function of these cells, which produce CSF. To test this possibility, we next carried out experiments in living rabbits, examining the effects of rANP on CSF production measured by ventricular-cisternal perfusion (Fig. 3). Drug was given intraventricularly, either by bolus injection or by continuous addition (during a 10-minute period) to the CSF perfusion system. Out of 14 rabbits in which CSF production could be adequately assessed throughout the experiment, 13 showed a decrease in the rate of CSF production. In one animal, there was no change in CSF secretion rate, and in no case was there an increase in CSF production. Figure 3 shows a positive response to a bolus injection of 30 pmol of rANP into the lateral ventricle. During a period of 100 minutes, secretion rate dropped by 70%. In the 13 positive responses, the mean decrease observed was 35.3% ± 6.9% (SEM) from a starting basal CSF secretion of 8.3 ± 0.8 μl/min. In other experiments, we found that intravenous injection of similar doses of rANP caused no change in peripheral blood pressure, suggesting that the decreased CSF production rate observed was not due to a systemic effect on vascular perfusion pressure.

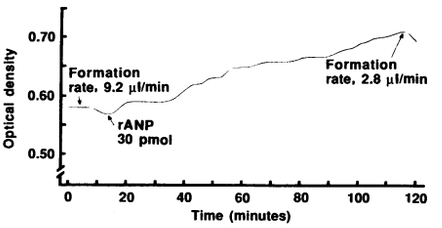

Fig. 3. Inhibitory effect of intraventricular rANP on CSF production rate (increase in dye absorbance) in rabbit with chronically implanted lateral ventricular and cisternal catheters. Ventricular-cisternal perfusion of Blue dextran was carried out by a closed system (*14*) with active pumping both into and out of the catheters at a rate of 30 μl/min. Output dye concentration was monitored continuously with an in-line photocell; and a separate, contralateral ventricular catheter independently measured intracranial pressure. Positive responses (see text) were observed in 13 of 14 experiments, in 3 of which rANP (30 pmol) was given by bolus intraventricular injection and in 10 of which rANP was given by continuous intraventricular infusion at 3 to 30 pmol/min for 10 minutes. Drug was administered in an artificial CSF containing 0.01% rabbit serum albumin (RSA) and 0.25 m*M* ascorbate. In addition, all plastic surfaces were pretreated with 0.1% RSA to reduce peptide loss by adsorption.

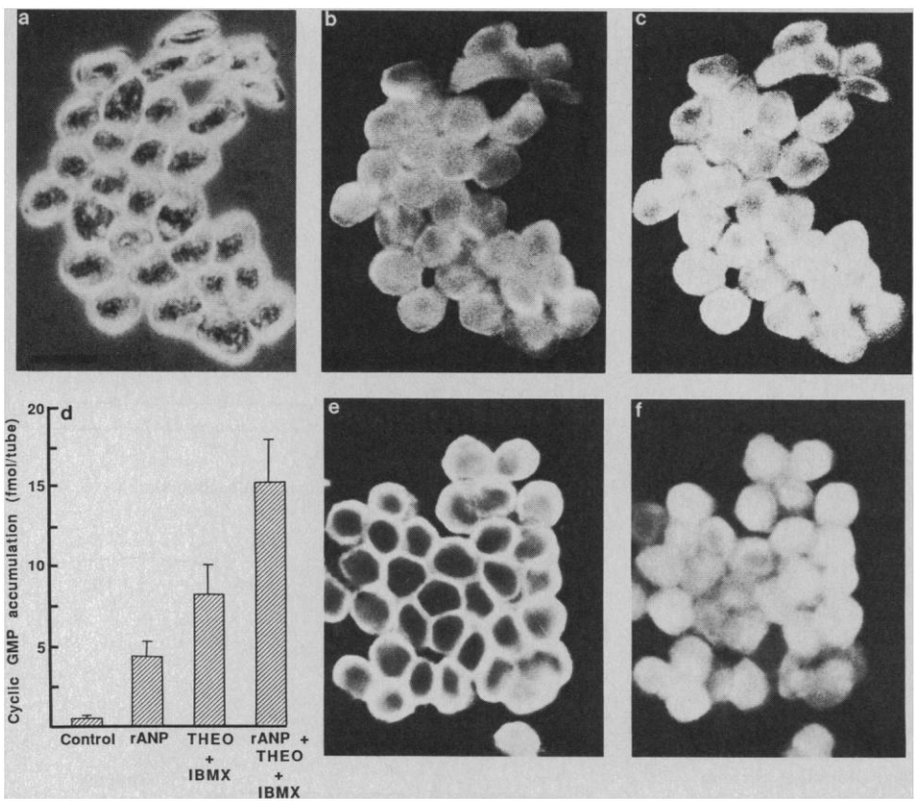

Fig. 2. Appearance and hormone responsiveness of intact isolated and purified choroid epithelial cells. (**a**) Appearance of a small group of epithelial cells after isolation and purification (phase contrast; marker, 20 μm). (**b**) Same cells immunostained with a rabbit polyclonal antibody (1:150 dilution) to the alpha form of Na,K-ATPase (*9*), followed by second antibody (Cappel Laboratories 1:100 dilution). Plasma membrane fluorescence [see also (e)] was characteristic of choroid epithelium (rhodamine optics). (**c**) Cells were also immunostained with mouse monoclonal antibody to DARPP-32 (*10*) followed by second antibody (Cappel, 1:100 dilution). More diffuse staining was found only in epithelium and not in vascular or stromal components of choroid (fluorescein optics). (**d**) Suspension of epithelial cells in artificial CSF at 37°C exposed for 5 minutes to 1 μ*M* rANP showed marked increase in intracellular cyclic GMP content, an effect that was potentiated by the phosphodiesterase inhibitors theophylline (THEO) (10 m*M*) and IBMX (0.5 m*M*). For one experiment, the mean and range are shown for duplicate determinations, each assayed for cyclic GMP content in triplicate. In four separate experiments, the degree of stimulation by rANP alone varied from 290% to 1460%. (**e**) Another group of epithelial cells showing bright plasma membrane immunostaining of Na,K-ATPase. (**f**) Same cells immunostained with antibody to DARPP-32. As shown here (bottom third, center), fewer than 5% of cells were DARPP-negative.

Although it is difficult to rule out the possibility that the observed action of rANP on decreasing CSF production was due to an indirect effect of the intraventricular injection on the central nervous system (CNS), we also observed, in other rabbits, that intravitreal injection of rANP into one eye (a procedure that allows the peptide to have direct access to the aqueous humor–secreting ciliary process) caused a much greater ipsilateral than contralateral decrease in intraocular pressure, a result not likely to be due to a systemic or CNS effect. These intraocular effects of rANP are consistent with results of autoradiographic studies showing that the ocular ciliary process, which is pharmacologically and physiologically similar to the choroid plexus, binds labeled atrial natriuretic factor (*12*).

Taken together, the above physiological and biochemical studies support the possi-

bility that brain barrier tissues may be end organs for atriopeptins, findings that are relevant to CNS water regulation under normal conditions and in pathophysiological situations such as cerebral edema or hydrocephalus. Our findings also suggest that isolated choroid epithelial cells may provide a useful model system for studying the receptor and post-receptor actions of atriopeptins in a defined cell population. For example, characterization of tertiary messengers, such as proteins whose phosphorylation is regulated by cyclic GMP–dependent protein kinase, should provide clues to biochemical mechanisms mediating the physiological effects of atriopeptins.

Note added in proof: In recent autoradiographic studies, McCarthy and Plunkett (*13*) have reported that labeled rANP binds to whole rat choroid plexus with an affinity (7 n*M*) similar to that which we found for activation of guanylate cyclase in rabbit choroid plexus.

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Macrophage Cytotoxicity: Role for L-Arginine Deiminase and Imino Nitrogen Oxidation to Nitrite

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Previous studies have shown that cytotoxic activated macrophages cause inhibition of DNA synthesis, of mitochondrial respiration, and of aconitase activity in tumor target cells. An L-arginine–dependent biochemical pathway synthesizing L-citrulline and nitrite, coupled to an effector mechanism, is now shown to cause this pattern of metabolic inhibition. Murine cytotoxic activated macrophages synthesize L-citrulline and nitrite in the presence of L-arginine but not D-arginine. L-Citrulline and nitrite biosynthesis by cytotoxic activated macrophages is inhibited by N^G-monomethyl-L-arginine, which also inhibits this cytotoxic effector mechanism. This activated macrophage cytotoxic effector system is associated with L-arginine deiminase activity, and the imino nitrogen removed from the guanido group of L-arginine by the deiminase reaction subsequently undergoes oxidation to nitrite. L-Homoarginine, an alternative substrate for this deiminase, is converted to L-homocitrulline with concurrent nitrite synthesis and similar biologic effects.

MOUSE PERITONEAL MACROphages activated in vivo by intracellular pathogens such as *Mycobacterium bovis*, strain Bacillus Calmette-Guérin (BCG), or in vitro by lymphokines, are cytotoxic for tumor cells after exposure to a second signal such as lipopolysaccharide (LPS) (*1–4*). Cytotoxic activated macrophages (CAM) cause a slowly developing and reproducible pattern of metabolic inhibition in tumor target cells which, in many cases, is fully reversible. For example, CAM cells inhibit mitochondrial respiration (*5–7*), the citric acid cycle enzyme aconitase (*7*), and DNA synthesis (*8*), while certain other metabolic pathways such as glycolysis remain functional (*5*). This reproducible and selective pattern of metabolic inhibition is, at least in part, due to CAM-induced iron loss from tumor target cells (*7, 9*). Earlier experiments showed that CAM-induced metabolic inhibition in tumor target cells is L-arginine–dependent (*10*). We therefore tested L-arginine and its homolog L-homoarginine, which substitutes for L-arginine as an inducer of CAM-associated cytotoxicity (*10*), as substrates for a CAM-associated deiminase synthesizing L-citrulline and L-homocitrulline, respectively. Arginine deiminase activity has not been described previously in mammalian cells (*11*).

L-Citrulline accumulation in the medium was measured after 24 hours by a colorimetric reaction that detects carbamido compounds (*12*). Urea was removed from samples prior to the assay by incubation with urease (1 unit/ml) at 37°C for 1 hour. The experiments were performed in medium prepared without amino acids except L-glutamine (*13*) and the desired concentration of L-arginine, L-homoarginine, or D-arginine. L-Glutamine does not affect the L-arginine–dependent CAM-effector mechanism (*10*). In the presence of L-arginine but not D-arginine, CAM cultured alone and CAM cocultivated with L10 cells released a molecule containing the carbamido group (L-citrulline) into the medium (Table 1). The quantity of L-citrulline synthesized was proportional to the initial L-arginine concentration. Table 1 also shows a correlation between the L-arginine concentration in the medium, the synthesis of L-citrulline, and the development of inhibition of mitochondrial respiration in L10 target cells as determined by lysis in a glucose-free second incubation medium. These results demonstrate an L-arginine–dependent CAM effector mechanism with deiminase activity that converts L-arginine to L-citrulline. L-Homoarginine is also a substrate for this deiminase and when added to the medium under identical conditions of culture or coculture produced similar results (Table 1).

$$\underset{\text{(L-homoarginine)}}{\text{L-arginine}} + H_2O \xrightarrow{\text{Deiminase}} \underset{\text{(L-homocitrulline)}}{\text{L-citrulline}} + NH_3$$

$$NH_3 + 1\text{-}1/2\ O_2 \xrightarrow{\text{Oxidase}} NO_2^- + H_2O + H^+$$

Fig. 1. Proposed pathway for L-citrulline and L-homocitrulline synthesis from L-arginine and L-homoarginine with subsequent oxidation of imino nitrogen to nitrite.

Arginine deiminase converts L-arginine to L-citrulline and ammonia (Fig. 1). We used an experimental protocol identical to that outlined in Table 2 to measure ammonia in the culture medium. Ammonia was measured by two methods—ion exchange chromatography (Biotronik LC 5001) and an enzymatic assay (*14*) (Aca II clinical analyzer, DuPont). We found no correlation between the ammonia concentration in the

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