A stimulus-activated conductance in isolated taste epithelial membranes

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ABSTRACT Membrane vesicles isolated from the cutaneous taste epithelium of the catfish were incorporated into phospholipid bilayers on the tips of patch pipettes. Voltage-dependent conductances were observed in ~50% of the bilayers and single-channel currents having conductances from 8 to >250 pS were recorded. In 40% of the bilayers displaying no voltage-dependent conductances, micromolar concentrations of L-arginine, a potent stimulus for one class of catfish amino acid taste receptors, activated a nonselective cation conductance. The L-arginine-gated conductance was concentration-dependent, showing half-maximal activation in response to ~15 μM L-arginine. L-Arginine-activated channels had unitary conductances of 40–50 pS and reversed between −6 and +18 mV with pseudointracellular solution in the pipette and Ringer in the bath. L-Alanine, a potent stimulus for the other major class of catfish amino acid taste receptors, did not alter bilayer conductance. D-Arginine, which is a relatively ineffective taste stimulus for catfish but a good cross-adapter of the L-arginine-induced neural response, had no effect on bilayer conductance at concentrations below 200 μM. However, increasing concentrations of D-arginine from 1 to 100 μM progressively suppressed the L-arginine-activated conductance, suggesting that D-arginine competed for the L-arginine receptor, but did not activate the associated cation channel. This interpretation is consonant with recent biochemical binding studies in this system. These results suggest that L-arginine taste receptor proteins in the catfish are part of or closely coupled to cation-selective channels which are opened by L-arginine binding.

INTRODUCTION

Gustatory transduction is initiated by the interaction of taste stimuli with sites on the apical membranes of specialized epithelial receptor cells in the taste buds. Recent experiments in several laboratories indicate that fundamentally different mechanisms mediate the depolarizing receptor potentials elicited in taste cells by some classes of chemical stimuli (for reviews see references 1–4). A variety of studies indicate that taste responses to sodium salts are initiated, at least in part, by direct influx of stimulus cations through voltage-insensitive, amiloride-blockable channels in the apical (receptive) membranes of the receptor cells (5–9). Sour taste, which is produced by acids, appears to result from a transient proton block of a resting conductance to potassium (10, 11) mediated by voltage-dependent potassium channels localized in the apical membranes of the taste cells (12, 13). Although receptor-coupled second-messenger pathways have been implicated in taste cell responses to sugars (14, 15) and the bitter-tasting compound, denatonium (16), the most convincing evidence for specific taste receptor proteins is in the cutaneous taste system of the channel catfish (*Ictalurus punctatus*), where two independent classes of amino acid taste receptors have been identified on the basis of radioligand binding studies (e.g., 17–19) and neural cross-adaptation experiments (20–23). One class of receptors binds L-alanine and other short-chain neutral amino acids (L-ALA receptor), whereas the other binds the basic amino acid L-arginine (L-ARG receptor). These experiments have been possible both because of the high density of taste buds in the skin of the fish, which provides sufficient taste cell membranes for binding studies, and because of the relatively high affinities of these sites for their ligands (K_{Dapp} 10^{-6}–10^{-8} M).

Recent experiments have demonstrated not only that GTP-binding regulatory proteins are present in membrane fractions derived from the catfish taste epithelium (24), but that a potent taste stimulus (L-alanine) for one class of receptors stimulates the production of both cAMP and inositol 1,4,5-trisphosphate ($InsP_3$) (25). L-Arginine, which acts at the other, independent class of receptor sites, does not stimulate either second messenger pathway. These results suggest that activation of the L-ALA taste receptor is coupled to the modulation of one or more second messenger pathways which may, in turn, modulate membrane conductance either directly or through protein-kinase-mediated phosphorylation (26). We report here that planar bilayers, into which purified membrane vesicles isolated from the catfish taste epithelium have been

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incorporated, display reversible increases in conductance in the presence of micromolar concentrations of L-arginine, but not L-alanine. This conductance is mediated by nonselective cation channels which appear to be directly opened by L-arginine. Preliminary reports of these studies have been presented (27, 28).

MATERIALS AND METHODS

Solutions

Solutions were prepared from reagent-grade salts and filtered immediately before use (0.2 μM Acrodisc filters, Gelman Sciences, Inc., Ann Arbor, MI). Most experiments were performed with "catfish" Ringer containing (millimolar): 110 NaCl, 2.5 KCl, 1.6 $MgCl_2$, 1.0 $CaCl_2$, 5 Hepes, pH 7.4, in the bath and a pseudointracellular solution containing (millimolar): 12.5 NaCl, 85 KCl, 1.6 $MgCl_2$, 0.25 $CaCl_2$, 0.5 EGTA, 5 Hepes, pH 7.4, in the recording pipette. A few experiments were performed with these solutions reversed or with various concentrations of $CaCl_2$, $BaCl_2$, KCl, or NaCl and 10 mM Hepes in both the bath and the pipette. Azolectin (Sigma type II-S from soybeans) was obtained from the Sigma Chemical Co., St. Louis, MO, and stored frozen at −70°C. Lipid was prepared fresh for each experiment by dissolving azolectin in analytical grade hexane (Nanograde, Mallinckrodt Inc., St. Louis, MO), at a concentration of 30 mg/ml. Later experiments were performed using bilayers formed from mixtures of palmitoyloleoyl phosphatidylethanolamine (POPE) and phosphatidylserine (PS) (7:3) obtained from Avanti Polar Lipids, Inc., Birmingham, AL. These lipids were dissolved in hexane at 10 mg/ml. Amino acid solutions were prepared from 10 mM stock solutions and filtered before use. Solution changes and application of amino acid stimuli were accomplished using a gravity-flow perfusion system. In early experiments, either the pipette was moved from one chamber to another, each containing a different solution or concentration, or aliquots of stimuli were sequentially added to the recording chamber to provide an ascending series of concentrations. All experiments were performed at room temperature.

Membrane vesicles and bilayers

A sedimentable fraction (P2), which is enriched in amino acid binding activity and marker enzymes for plasma membranes, was prepared from homogenates of catfish taste epithelium by differential centrifugation as described previously (17–19). A nucleotidase-enriched fraction of plasma membranes (QBO) was prepared from fraction P2 by a modification of the procedure described by Cagan and Boyle (29), involving centrifugation of P2 on a 30% (wt/wt) sucrose cushion at 36,000 *g* for 20 min. Samples of QBO were washed to remove residual sucrose and sonicated briefly (30 s). All procedures were carried out at 4°C. Fraction QBO was prepared fresh for each experiment because preliminary studies indicated that stimulus-activated conductances were less frequently encountered using previously frozen membrane vesicles than with fresh material. Subsequently, we found that QBO could be stored for up to 3 d at 4°C with only gradual decline in the frequency with which L-Arg-activated channels were encountered.

Membrane vesicles were incorporated into high resistance bilayers on the tips of patch pipettes using minor modifications of the procedure initially described by Coronado and Latorre (30). A lipid monolayer was formed at the surface of a small (300-μl) plastic chamber containing membrane vesicles in Ringer (50–100 μg protein), by carefully adding 30–50 μl of azolectin or 10 μl of POPE:PS (7:3). After allowing at least 10 min for the solvent to evaporate, high-resistance bilayers were formed on a pipette by inserting it into the chamber under positive pressure, releasing the pressure, withdrawing the pipette, then reinserting it. Bilayers with resistances of 5–150 GΩ were routinely obtained. Vesicles were also incorporated by adding membranes to the bath after a bilayer had been formed at the pipette tip in the absence of vesicles. The presence of voltage-dependent and ligand-activated conductances was detected by measuring the currents produced by voltage pulses or ramps in the absence and presence of amino acid taste stimuli.

Electrophysiological recordings

Patch pipettes were pulled from Corning borosilicate glass capillaries (Garner Glass Co., Claremont, CA) using a horizontal puller (P-80/PC, Sutter Instruments, San Rafael, CA). The heater current was adjusted to produce pipettes with bubble numbers of 2.5–4.5 (31). Tip resistances measured in intracellular solution were 3–15 MΩ. Whole bilayer currents, as well as single-channel currents, were recorded using a LIST EPC7 patch amplifier (Medical Systems Corp., Greenvale, NY) in the voltage-clamp mode. Current signals were amplified and low-pass filtered (eight-pole Bessel filter; Frequency Devices, Inc., Haverhill, MA) between 500 and 3,000 Hz, depending upon the experiment. In early experiments, currents were displayed on a storage oscilloscope and a Brush 2400S chart recorder (Gould Inc., Cleveland, OH) for data analysis by hand. In later experiments, currents were digitized (2–10 kHz) and stored on the fixed disk of a 286 computer running pCLAMP software (Axon Instruments, Burlingame, CA). Voltage ramps and pulses were generated with a D/A converter under computer control.

RESULTS AND DISCUSSION

Before incorporation of membrane vesicles, lipid bilayers on the tips of the patch pipettes displayed only capacitative transients in response to voltage pulses. Bilayers to which taste epithelial membrane vesicles had been fused or bilayers formed from interfacial monolayers at the surface of solutions containing membrane vesicles frequently displayed an increase in conductance to voltage ramps from −80 to +80 mV (35 of 70 bilayers). Single-channel recordings from these bilayers displayed a variety of single-channel currents, ranging in conductance from 8 to >250 pS.

In 15 of the 35 "silent" bilayers, which displayed no currents in response to voltage ramps from −60 to +60 mV, concentrations of L-arginine >0.5–1 μM elicited reversible increases in bilayer conductance. Current voltage curves for a bilayer before addition of *Control*, after addition of, and after removal of *Wash* 50 μM L-arginine are presented in Fig. 1 *A*. With pseudointracellular solution in the pipette and Ringer in the bath, the L-arginine-activated currents reversed between −18 and +21 mV (+5 mV in Fig. 1 *A*) with a mean value of +10.3 ± 3.2 mV, $n = 10$, suggesting that the channels were relatively nonselective between Na^+ and K^+. L-Arginine also elicited an increase in conductance in bilayers displaying voltage-dependent conductances (4 of 12 attempts) (Figs. 1 *B* and 3 *B*). Current-voltage curves from a POPE/PS

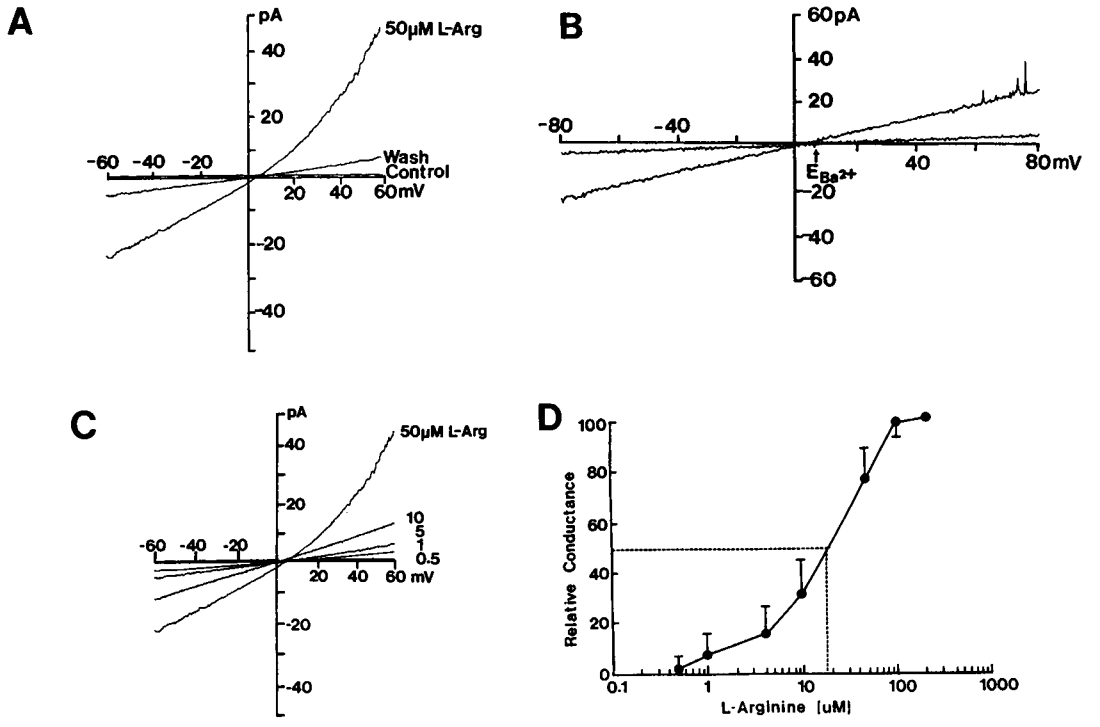

FIGURE 1 Conductance changes elicited by L-arginine in phospholipid bilayers into which purified membrane vesicles isolated from the taste epithelium of the catfish had been incorporated. (*A*) Current-voltage relationships in a bilayer before addition of (*Control*), after addition of, and after removal of (*Wash*) 50 μM L-arginine. Original bilayer resistance was 40 GΩ. Ringer in the bath, pseudointracellular solution in the pipette. (*B*) Ba^{2+} currents in a POPE/PS bilayer before and after addition of 100 μM L-arginine. The pipette contained 55 mM $BaCl_2$, 10 mM Hepes, and the bath contained 73.3 mM $BaCl_2$, 10 mM Hepes. (*C*) Currents elicited by −60 to +60 mV voltage ramps with increasing concentrations of L-arginine. Increasing concentrations of L-arginine were successively added to the Ringer solution in the bath. Pipette contained pseudointracellular solution. Same bilayer as in *A*. (*D*) Concentration-response curve for L-arginine. The relative conductance is the peak current at +60 mV for each concentration of L-arginine as a percentage of the maximum current at +60 mV elicited by 200 μM L-arginine. Mean ± 1 SD for three bilayers. Solutions as in *C*.

bilayer, with 55 mM $BaCl_2$, 10 mM Hepes in the pipette and 73.3 mM $BaCl_2$, 10 mM Hepes in the bath, obtained before and after addition of 100 μM L-arginine, are shown in Fig. 1 *B*. Similar results were obtained with $CaCl_2$ (not shown) and indicate that the L-arginine-activated channels were permeable to divalent as well as monovalent cations. The L-arginine-activated current in Fig. 1 *B* reversed near the equilibrium potential for Ba^{2+}, indicating that under these conditions the permeability to anions was negligible. The permeability ratio of Ba^{2+} relative to Na^+, calculated from reversal potentials in four experiments, was 0.12, indicating that the channel is about eight times more permeable to Na^+ than Ba^{2+}. These data also support the conclusion that the channels are not permeable to Cl^-. The permeability ratio of Ba^{2+} to Ca^{2+} (two experiments) was ~0.1, whereas that for Ca^{2+} to Na^+ (three experiments) was close to 1. These results indicate that although the channels are permeable to Ba^{2+}, they are much more permeable to Na^+, Ca^{2+}, and K^+. Under the ionic conditions in the layer of mucus that covers the apical surfaces of the taste cells (~15 mM Na^+ and 10–1,000 μM Ca^{2+}, depending on the water; Restrepo, D. and J. H. Teeter, unpublished observations) >90% of the inward current through the L-arginine-activated channel would be carried by Na^+.

The L-arginine gated conductance was concentration dependent, activating between 0.5 and 1 μM and saturating at concentrations between 100 and 150 μM (Fig. 1, *C* and *D*). The concentration of L-arginine that produced half-maximal increase in the conductance of these bilayers was ~15 μM (Fig. 1 *D*). The current-voltage relationship in the presence of L-arginine was often curvilinear, particularly at higher concentrations of L-arginine (Fig. 1, *A* and *C*). This apparent voltage dependence of the L-arginine-activated conductance suggests that membrane potential can influence either the gating of the channel or the affinity of the binding sites for L-arginine. The qualitative difference between the macroscopic and single-channel current voltage curves (compare Figs. 1 *C* and 2 *B*) supports the latter alternative.

Single-channel recordings from bilayers displaying an increased conductance in the presence of L-arginine revealed bursts of current fluctuations resembling single-channel currents (Fig. 2 *A*). Both individual openings and bursts of openings lasting several seconds were observed. Single L-arginine-activated channels were encountered only infrequently. More typically, from 3 to 10 channels were present in a bilayer, with one or more of the channels being continuously open in the presence of L-arginine. The records in Fig. 2 *A* show three L-arginine-gated channels, each with a unitary conductance of 45 pS, one of which was continuously open. Additional small conductance channels or subconductance states (*arrows*) were also present. This apparent clustering of L-arginine-activated channels made it difficult to obtain reliable open and closed time histograms. In the few cases where single channels were present (Fig. 2 *B*), analysis indicated that the frequency of channel opening increased with increasing concentrations of L-arginine.

The L-arginine-activated channels appeared to desensitize with prolonged exposure to L-arginine, although the rate varied considerably from one bilayer to another. Some displayed nearly complete desensitization within 3–4 s, while others showed no clear change in conductance for tens of seconds. However, nearly all bilayers showed some desensitization after a 2-min exposure to 50 μM L-arginine. The L-arginine-activated single-channel currents had slope conductances of 40–50 pS and reversed between -6 and $+18$ mV ($+7.4 \pm 3.9$ mV, $n = 8$) with Ringer in the bath and pseudointracellular solution in the pipette.

L-Alanine, which is a potent stimulus (threshold 10^{-7}–10^{-8} M) for the other major class of amino acid taste receptors in the catfish, had no effect on bilayer conductance (seven experiments) at concentrations up to 100 μM (Fig. 3 *A*). This is consistent with the conclusion that L-alanine and L-arginine act at independent sites, which is based on the observations that L-arginine and L-alanine do not significantly cross-adapt in electrophysiological recordings from the taste nerve (19), nor do they compete for the same binding sites in radioligand binding studies (18, 19).

D-Arginine, which is a relatively poor taste stimulus, but a good cross-adapter of L-arginine-induced activity in neural recordings (19), had no effect on bilayer conductance at concentrations below 200 μM (shown for 100 μM in Fig. 3 *A*). However, at concentrations between 200 and 500 μM, D-arginine did elicit small increases in conductance in two bilayers (not shown). Although D-arginine did not activate channels, at least at moderate concentra-

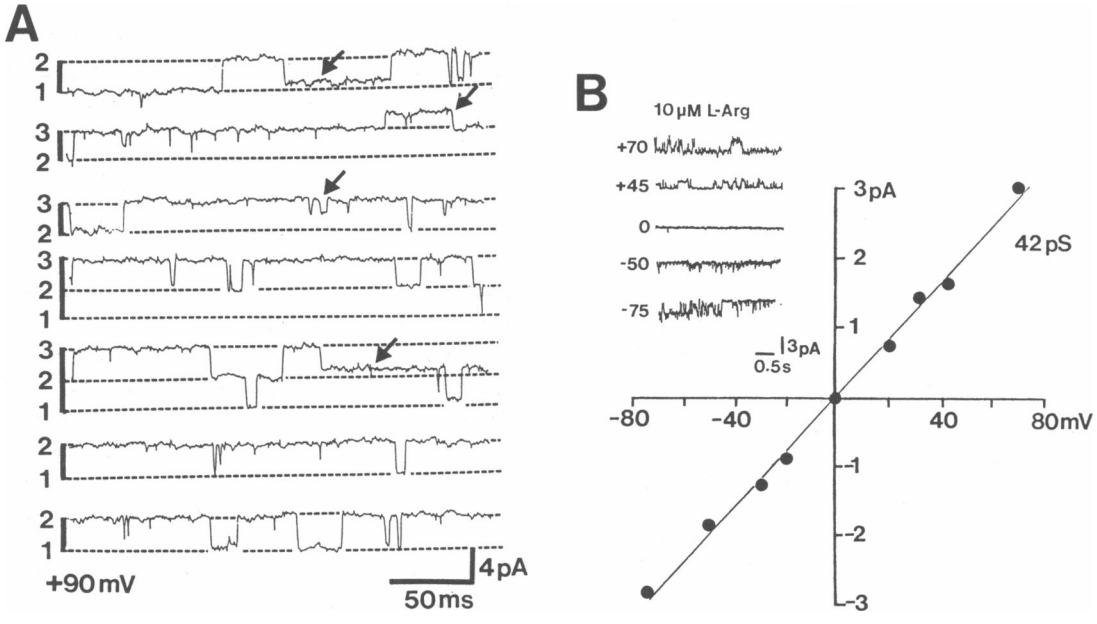

FIGURE 2 (*A*) Single-channel current fluctuations at +90 mV in the presence of 10 μM L-arginine. Currents from at least three L-arginine-activated channels, each with an amplitude of 4 pA, were present. One of these channels was open during the entire record. Channel openings correspond to cation flux from the pipette to the bath and are displayed as upward deflections. Smaller amplitude currents which may represent a separate class of channel or a subconductance of the L-arginine gated channels, were also present (*arrows*). Ringer in bath, pseudointracellular solution in pipette. (*B*) Steady-state current-voltage relationship for the L-arginine-activated channel displayed in the inset. The slope conductance was 42 pS and the current reversed at 0 mV. Solutions as in *A*.

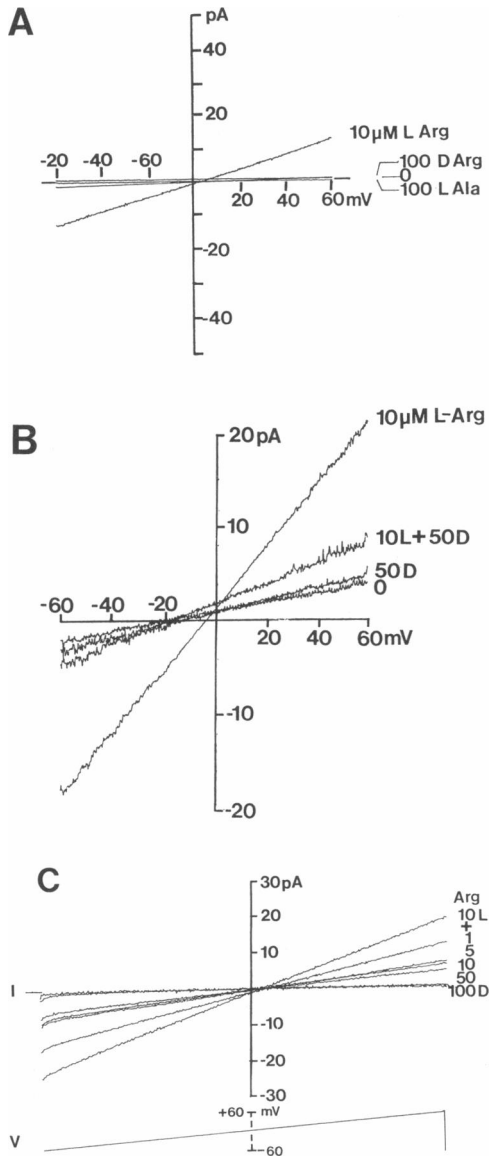

FIGURE 3 Current-voltage relationships for bilayers in the presence of various concentrations of L-arginine and D-arginine. (*A*) Addition of 10 μM L-arginine elicited an increase in bilayer conductance, whereas neither 100 μM L-alanine nor 100 μM D-arginine had any effect on conductance. (*B*) Addition of 50 μM D-arginine, which alone had little effect on bilayer conductance to the bath suppressed the conductance elicited by 10 μM L-arginine by ~70%. (*C*) The conductance elicited by 10 μM L-arginine was progressively reduced by increasing the concentration of D-arginine from 0 to 100 μM. The conductance was completely suppressed by 100 μM D-arginine. The maximum decrease in concentration of L-arginine resulting from dilution was 20%. Ringer in the bath, pseudointracellular solution in the pipette.

tions, it did suppress the conductance elicited by L-arginine (Fig. 3 *B*). In this example, 50 μM D-arginine had no significant effect on bilayer conductance, whereas 10 μM L-arginine elicited a marked increase in conductance. Exposure of the bilayer to a mixture containing 10 μM L-arginine and 50 μM D-arginine resulted in a conductance that was ~30% of that recorded in the presence of 10 μM L-arginine. Increasing concentrations of D-arginine from 1 to 100 μM, added to the bath in the presence of 10 μM L-arginine, progressively reduced the bilayer conductance until it reached control levels at 100 μM D-arginine (Fig. 3 *C*). This decrease in conductance was not accounted for by desensitization of the L-arginine receptor, which was bathed continuously in 10 μM L-arginine. Although the L-arginine-induced conductance usually displayed some decrease in magnitude with prolonged exposure to L-arginine, the response of the bilayer in Fig. 3 *C* decreased <20% during the 4-min period before the addition of D-arginine. In addition, the maximum dilution of L-arginine resulting from the addition of aliquots of D-arginine was 20% at 100 μM D-arginine. This would not have resulted in dilution of L-arginine to a concentration below the threshold for producing an increased conductance, as was evident in this experiment (Fig. 3 *C*). Direct addition of 100 μM D-arginine, which diluted the L-arginine <5%, completely blocked the conductance induced by 10 μM L-arginine (not shown). These results suggest that D-arginine binds to the L-arginine receptor site but does not activate the conductance increase. They are also consistent with binding studies which indicate that D-arginine competes for the L-arginine binding site (19).

In summary, these results indicate that plasma membranes from the taste epithelium of the catfish contain cation-selective channels that are specifically and reversibly activated (opened) by L-arginine, a potent stimulus for one of the two classes of taste receptors delineated on the basis of radioligand binding and neural cross-adaptation studies. The most potent stimulus for the other class of receptors, L-alanine, had no effect on bilayer conductance, or on the L-arginine-activated channels. The purified plasma membrane vesicles used in the present experiments consisted of membranes from both taste receptor cells and nontaste surface epithelial cells. Consequently, we cannot attribute the L-arginine-activated conductance solely to taste cells. Clearly, if the L-arginine-activated channels are directly involved in taste transduction, they must not only be present in taste cells, but they must be located in the apical membrane. However, the presence of similar channels in nontaste cells would not necessarily mean that they do not play a role in taste reception. Both amiloride-blockable Na^+ channels and proton-block of voltage-gated K^+ channels are "nonspecific" properties of many cells, yet both appear to serve as transduction pathways when they occur in taste receptor cells (7, 9, 12). Conversely, the presence of L-arginine-activated channels in taste cells, although a necessary prerequisite, would not prove their function in taste

reception. However, we would interpret this result as strong evidence for a role for these channels in taste transduction. The close correspondence of the specificity and sensitivity of the observed conductance for L-arginine and its block by D-arginine, with the results of independent radioligand binding studies (17–19) and taste nerve recordings (20–23) in catfish, suggests that this conductance is present in taste receptor cells. In addition, the plasma membrane fraction (QBO) derived from P2 is known to bind amino acid taste stimuli (29; Brand, J. G., unpublished observations) and contains antigenic sites for a monoclonal antibody (32) shown to label primarily cutaneous taste cells of *I. punctatus* (33). Taken together, these results suggest that the L-arginine receptors are part of or closely associated with cation-selective channels, which are directly opened by L-arginine binding. The L-alanine receptors, on the other hand, appear to be coupled to taste cell activation via an independent pathway, probably involving generation of one or more second messengers (25).

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