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Stimulation of Olfactory Receptors Alters Regulation of $[Ca_i]$ in Olfactory Neurons of the Catfish (*Ictalurus punctatus*)

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Summary. Intracellular calcium was measured in single olfactory neurons from the channel catfish (*Ictalurus punctatus*) using the fluorescent Ca^{2+} indicator fura 2. In 5% of the cells, olfactory stimuli (amino acids) elicited an influx of calcium through the plasma membrane which led to a rapid transient increase in intracellular calcium concentration. Amino acids did not induce release of calcium from internal stores in these cells. Some cells responded specifically to one stimulus (L-alanine, L-arginine, L-norleucine and L-glutamate) while one cell responded to all stimuli. An increase in intracellular calcium could also be elicited in 50% of the cells by direct G-protein stimulation using aluminum fluoride. Because the fraction of cells which respond to direct G-protein stimulation is substantially larger than the fraction of cells responding to amino acids, we tested for possible damage of receptor proteins due to exposure of the olfactory neurons to papain during cell isolation. We find that pretreatment with papain does not alter specific binding of L-alanine and L-arginine to olfactory receptor sites in isolated olfactory cilia. The results are discussed in terms of their relevance to olfactory transduction.

Key Words olfactory transduction · olfactory neurons · calcium · fura-2

Introduction

A role for intracellular calcium in olfactory transduction in vertebrates was first suggested by studies of Huque and Bruch (1986) who showed, using isolated cilia from the olfactory epithelium of the channel catfish *Ictalurus punctatus,* that odor stimuli induce rapid G-protein-mediated production of inositol-1,4,5-trisphosphate (IP_3). This observation is not confined to aquatic organisms because in studies employing stopped-flow techniques Boekhoff et al. (1990) have measured a rapid (within 50–100 msec) increase in IP_3 levels in rat olfactory cilia following exposure to those odor stimuli which do not produce significant adenylate cyclase stimulation. Because IP_3 is well known to release calcium from intracellular stores and to trigger influx of calcium through the plasma membrane (Putney et al., 1988), these observations suggest that odors may trigger an increase in intraneuronal calcium.

Because of the heterogeneous nature of the cell population in the olfactory epithelium, responses to olfactory stimuli are best studied in morphologically identified receptor cells. The calcium sensitive dye fura-2 (Grynkiewicz, Poenie & Tsien, 1985) can be used in conjunction with epifluorescence microscopy to measure intracellular calcium semiquantitatively in single cells (Cobbold & Rink, 1987). The technique has the advantage of being noninvasive, facilitating its use in small cells (such as olfactory neurons) which are not amenable to ion-selective microelectrode studies.

In earlier work we established that fura-2 could be used to monitor changes in intracellular calcium reliably in isolated catfish olfactory neurons (Restrepo & Teeter, 1990). In the present manuscript we show that intracellular calcium increases abruptly and reversibly after exposure of isolated catfish olfactory neurons to olfactory stimuli (L-amino acids) and that this increase in calcium is caused by influx of extracellular calcium through the plasma membrane. Olfactory neurons were also found to respond to direct G-protein stimulation (with aluminum fluoride), suggesting that G-proteins play a role in triggering the increase in $[Ca_i]$. A preliminary account of this work has appeared elsewhere (Restrepo et al., 1990).

Materials and Methods

Materials

Papain, Sigma type III (EC 3.4.22.2), DTT, poly-D-lysine (mol wt: 30,000–70,000), leupeptin and all buffers and salts were obtained from Sigma (St. Louis, MO). Gentamicin was obtained from

GIBCO (Grand Island, NY). Ionomycin was procured from Calbiochem (La Jolla, CA). Fura-2/AM, fura-2 free acid and pluronic F127 were obtained from Molecular Probes (Eugene, OR). Nimodipine (Bay E 9736) was obtained from the Miles Institute for Preclinical Pharmacology (West Haven, CT).

Isolation of Olfactory Neurons

Cells were isolated by brief treatment with 30 U/ml of activated papain in divalent cation free Ringer's as described previously (Restrepo & Teeter, 1990). Cell viability assessed with Trypan blue averaged 95%. Olfactory neurons were easily discriminated from respiratory, sustentacular and basal cells on the basis of their bottle shape, the presence of the olfactory knob and cilia and of axonal fragments (Cancalon, 1978; Restrepo & Teeter, 1990). Neurons gradually lost their characteristic morphology 1 to 4 hr following isolation. Only morphologically identifiable neurons were used for the calcium measurements presented in this manuscript.

Measurement of Intracellular Calcium in Single Cells

Cells, attached to a poly-D-lysine coated coverslip, were loaded with fura-2 by incubation for 1 hr in fish Ringer's (containing in mM: 110 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, titrated to pH 7.6 with NaOH) supplemented with 5 mM D-glucose, 5 μM fura-2/AM and 80 μg/ml pluronic F127. Previous studies indicate that the olfactory neurons hydrolyze fura-2/AM to fura-2 free acid, leaving no detectable traces of intermediate products of hydrolysis (Restrepo & Teeter, 1990).

Cells were imaged under epifluorescence microscopy in a Nikon Diaphot microscope equipped with high UV transmission optics and were excited at alternating 340-nm (calcium-sensitive) and 360-nm (calcium-insensitive) wavelengths using a Photon Technologies Deltascan illumination system (Photon Technology International, South Brunswick, NJ). Because 360 nm is the isosbestic point for fura-2 (the point at which a change in calcium does not produce a change in fluorescence emission), monitoring of fluorescence emission at 360-nm excitation facilities detection of dye leakage, bleaching or of cell movement away from the region monitored by the photomultiplier tube (PMT). Fluorescence emitted from a single cell was collected using a rectangular emission aperture. Emitted light was filtered with a wide bandpass filter with cutoffs at 470 and 580 nm using a low-noise type R1527 Hamamatsu photomultiplier tube (Hamamatsu Photonics, Toyota Village, Japan). A detailed description of the setup is given in Restrepo and Teeter (1990). Cells were continuously perfused with solutions at room temperature. Perfusion rates were such that 90% exchange of solutions occurred within 5 to 30 sec depending on the position of the cell with respect to the intake port.

Intracellular calcium concentration ($[Ca_i]$) was calculated from the fluorescence ratio R (fluorescence emitted with excitation at 340 nm divided by fluorescence with excitation at 360 nm, after background subtraction) using the equation (Grynkiewicz et al., 1985):

$$[Ca_i] = K_d(F_o/F_s)(R - R_{min})/(R_{max} - R)$$

where R is the fluorescence ratio (F_{340}/F_{360}), R_{min} and R_{max} are the minimum and maximum fluorescence ratios, K_d is the apparent calcium dissociation constant and (F_o/F_s) is the ratio of fluorescence intensities when excited at 360 nm at limiting low (F_o) and high calcium (F_s) concentration (because of the choice of wavelengths F_o/F_s has a value of one). The calcium dissociation constant used for fura-2 was 166 nM (this is the appropriate K_d for the ionic strength of fish Ringer's, *see* Restrepo and Teeter, 1990). Cell autofluorescence was negligible and no appreciable bleaching was detected after continuous excitation for as long as 15 min.

As explained in Results, we employed a combination of 30 μM $AlCl_3$ and 10 mM NaF to test for the involvement of G-proteins in the regulation of intracellular calcium in catfish olfactory neurons. In control experiments we found that, when added directly to solutions containing fura-2, AlF_4^- did not cause any artifactual changes in either the 340- or 360-nm fluorescence emission or in the fluorescence ratio.

Olfactory Cilia Isolation and Treatment with Papain

Olfactory epithelia from 15 fish were deciliated by exposure to a medium with 10 mM calcium (10 mM $CaCl_2$, 50 mM NaACES at pH 7.0 plus 10 μg/ml leupeptin). Cilia were collected and washed once with two 20-min centrifugations at 30,000 $\times$ g as previously described (Boyle et al., 1987). Following isolation, cilia were resuspended in either 50 mM NaACES at pH 7.0 (control) or in media with 2 mM EDTA, 50 mM NaACES at pH 7.6 and 30 U/ml of activated papain (papain treated). Papain was activated by treatment of 0.2 ml of enzyme solution (300–400 U/ml) at pH 7.0 with 1 mM DTT for 10 min (DTT solution was made immediately before treatment). After a 15-min incubation with papain at room temperature, mimicking conditions of the cell isolation procedure (Restrepo & Teeter, 1990), digestion was halted by 1 : 1 dilution with ice-cold media with 2 mM $CaCl_2$, 20 μg/ml leupeptin, 50 mM NaACES at pH 7.0. Control cilia were diluted 1 : 1 with ice-cold 50 mM NaACES. Cilia were sedimented by centrifugation at 30,000 $\times$ g for 20 min, and each pellet was resuspended in 2.2 ml ice-cold 50 mM NaACES using a hand-held homogenizer.

Receptor Binding Measurements

Binding of L-[^{3}H]-alanine and L-[^{3}H]-arginine was assayed following the procedure of Kalinoski, Bruch and Brand (1987). Assays were performed in triplicate (variability between samples was typically less than 10%). Competitive binding curves were obtained by measuring displacement of binding of a trace amount of radioligand (0.03–0.15 μM) by different amounts of nonradioactive amino acid (0.5 to 100 μM). Nonspecific binding was determined in parallel samples by addition of 20 mM nonradioactive ligand (counts in nonspecific binding sample were less than 15% of counts in 0.5-μM sample). Because treatment with papain may lead to digestion of extrinsic ciliary proteins, total ciliary protein was not used as a normalization factor for the amount of binding. Instead, total ciliary lipid phosphate (determined by the method of Chen, Toribara & Warner, 1956) was used to normalize the binding data. In control cilia 1 μg of protein is equivalent to 2 nmols of lipid phosphate.

Abbreviations

ACES: (2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid; cAMP: adenosine 3′5′-monophosphate; DTT: DL-dithiothreitol; EDTA: ethylenediamine tetraacetic acid; EGTA: [ethylenebis

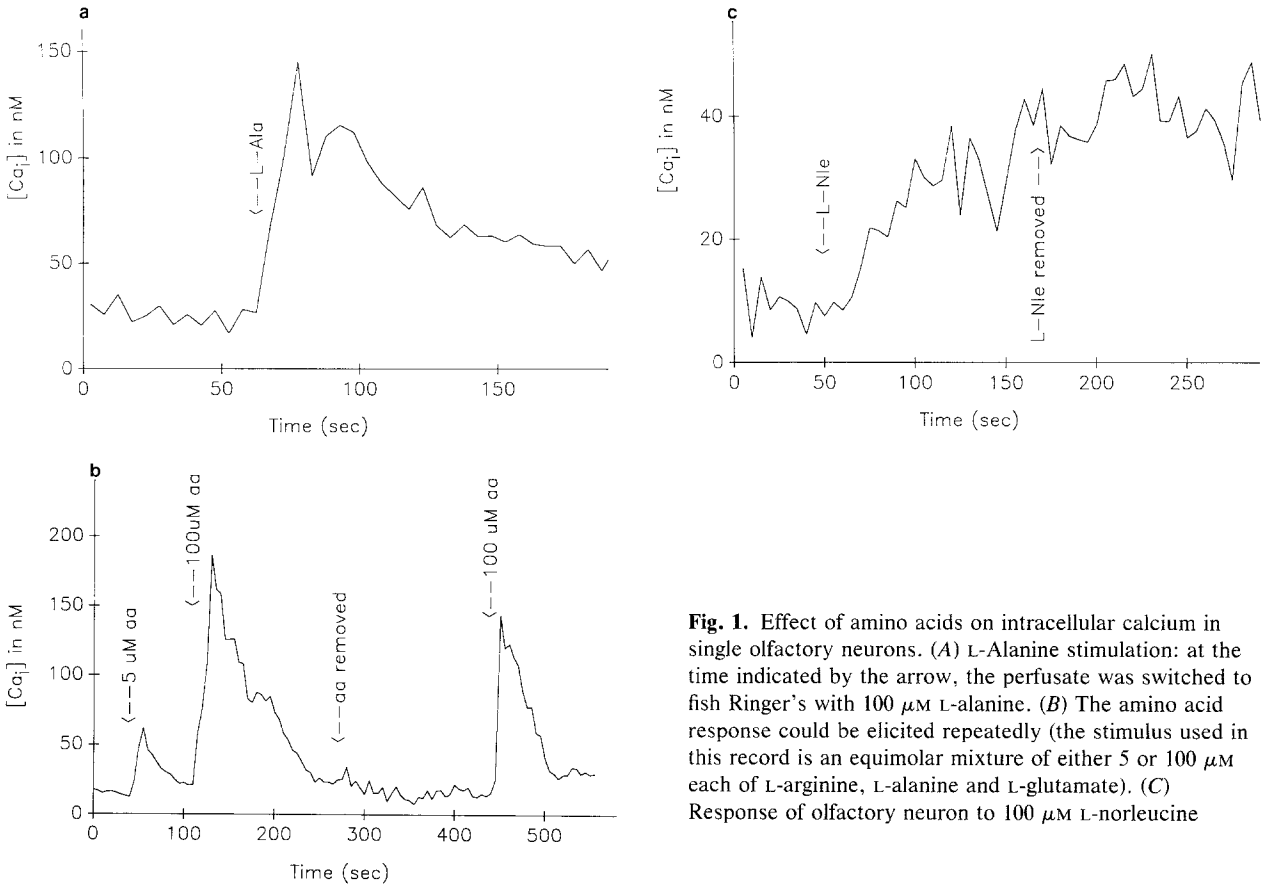

Fig. 1. Effect of amino acids on intracellular calcium in single olfactory neurons. (*A*) L-Alanine stimulation: at the time indicated by the arrow, the perfusate was switched to fish Ringer's with 100 μM L-alanine. (*B*) The amino acid response could be elicited repeatedly (the stimulus used in this record is an equimolar mixture of either 5 or 100 μM each of L-arginine, L-alanine and L-glutamate). (*C*) Response of olfactory neuron to 100 μM L-norleucine

(oxyethylenenitrilo)]tetraacetic acid; fura-2/AM: fura-2 acetoxymethyl ester; HEPES: N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid; IP_3: inositol-1,4,5-trisphosphate.

Results

Amino Acids Elicit Changes in Intracellular Calcium

Electrophysiological cross-adaptation and competitive binding studies indicate that catfish olfactory neurons possess at least four different receptor sites for detection of amino acids (Caprio & Byrd, 1984; Kalinoski et al., 1987; Bruch & Rulli, 1988). These sites can be classified according to the side chain of the amino acids they recognize: short chain neutral (SCN), long chain neutral (LCN), acidic and basic. We have assayed changes in intracellular calcium upon stimulation of morphologically identified olfactory neurons using four amino acids, one from each of these groups (L-alanine (SCN), L-norleucine (LCN), L-arginine (basic) and L-glutamate (acidic)). All stimuli were used at a concentration of 100 μM which is within the physiological response range of the catfish olfactory epithelium (Caprio, 1978). Isolated catfish olfactory neurons kept low resting calcium concentrations in the range of <2 to 100 nM with a median [Ca_i] of 17.6 nM (Restrepo & Teeter, 1990). Of 219 cells tested, 11 cells (5%) responded to stimulation by amino acids with a rapid increase in intracellular calcium (Fig. 1, 177 were tested for stimulation by L-alanine, L-arginine and L-glutamate and 52 were tested for the above amino acids plus L-norleucine). The magnitude of the increase in calcium was widely variable: the smallest increase was 60% over basal and the largest 1400% over basal (mean ± SD from 11 cells was 449 ± 387% over basal).

One neuron could be stimulated by all four amino acids. In contrast, five cells responded specifically to one of the amino acids (one cell each for L-alanine, L-arginine, L-norleucine and two for L-glutamate). The other cells were tested with one amino acid alone or with a mixture of amino acids. For cells that responded to L-alanine, L-arginine and L-glutamate, intracellular calcium reached a peak rapidly and then decreased slowly (Fig. 1*A* and *B*). Both phasic and tonic components of the response could be discerned in some of these traces, and the

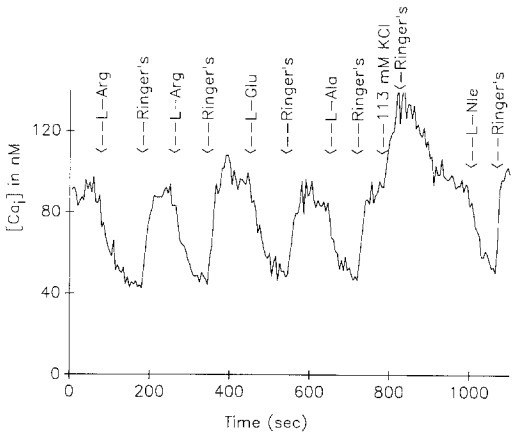

Fig. 2. Decrease in [Ca$_i$] caused by 100 μM L-amino acids in an olfactory neuron. Notice that when extracellular sodium is replaced by potassium, intracellular calcium increases as would be expected because of opening of voltage-dependent calcium channels (Restrepo & Teeter, 1990)

cells responded to repeated application of the stimuli. For cells responding to L-norleucine, intracellular calcium increased slowly and stayed elevated as long as the amino acid was present (Fig. 1*C*). Recovery to basal calcium levels in L-norleucine responsive cells was slow.

In contrast to the transient increase in calcium observed in 11 cells in response to L-amino acids, in one cell, intracellular calcium decreased when the cell was exposed to any of the four amino acids tested and returned to baseline when the amino acid was removed (Fig. 2). This cell had an unusually high basal calcium concentration (92 nM) compared with either the mean of the population (23 ± 19, mean ± SD, n = 140, *see* Restrepo & Teeter, 1990) or with the mean basal calcium of those cells that responded to amino acid stimulation with an elevation of intracellular calcium (18 ± 12 nM, mean ± SD, n = 11). This high calcium concentration was not simply due to damage to the cell membrane since after elevation of [Ca$_i$], induced by replacing extracellular sodium with potassium (Restrepo & Teeter, 1990), return to fish Ringer's led to a rapid recovery of Ca$_i$ towards basal levels (*see* Fig. 2).

The Stimulus-Induced Increase in [Ca$_i$] Was Probably Mediated by Calcium Influx

As shown in Fig. 3*A*, olfactory neurons possess intracellular stores of calcium which, when released by addition of ionomycin in the absence of extracellular calcium, were large enough to increase intracellular calcium substantially over basal [Ca$_i$]. To determine whether the effect of amino acids was mediated

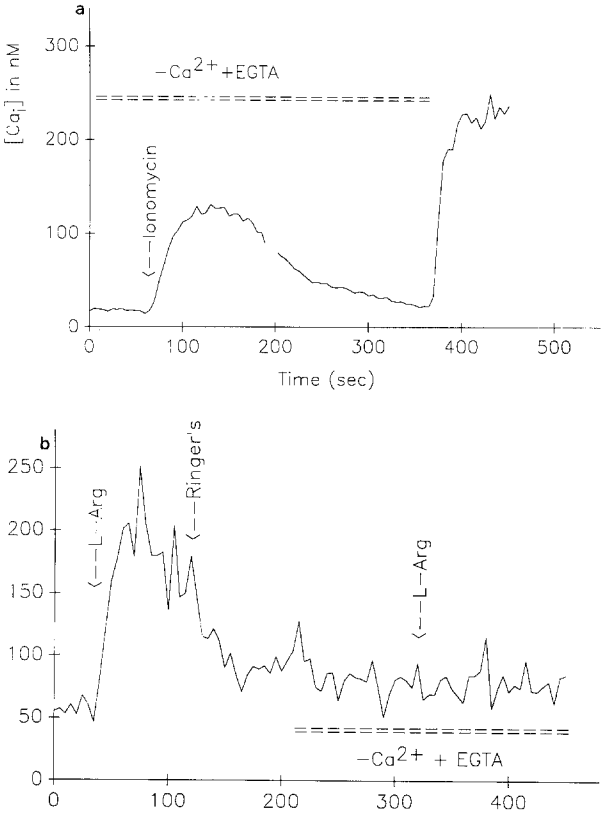

Fig. 3. (*A*) Ionomycin releases calcium from internal stores. When an isolated olfactory neuron was exposed to 1 μM ionomycin in the absence of extracellular calcium, intracellular calcium increased transiently. Extracellular calcium was removed by perfusing with fish Ringer's with no added calcium and 1.5 mM EGTA. The calcium concentration of this solution was below 5 nM (measured with fura-2). (*B*) Removal of extracellular calcium leads to abolishment of the increase of [Ca$_i$] caused by amino acids (one representative trace from a total of four). The stimulus used in this trace is 100 μM L-arginine

by release of calcium from these stores, cells were stimulated in the absence of extracellular calcium (Fig. 3*B*). Removal of extracellular calcium abolished the increase in intracellular calcium in four out of four cells tested, suggesting that this increase elicited by olfactory stimuli was mediated by influx of extracellular calcium across the plasma membrane.

In principle it could also have been possible that removal of extracellular calcium led to quick depletion of intracellular stores distinct from the calcium stores that were released by addition of ionomycin in calcium-free medium (Fig. 3*A*). In this case, the lack of an increase in [Ca$_i$] upon stimulation with amino acids in the absence of extracellular calcium (Fig. 3*B*) could be explained by the quick depletion of calcium from these internal stores triggered by the decrease in extracellular free calcium. However,

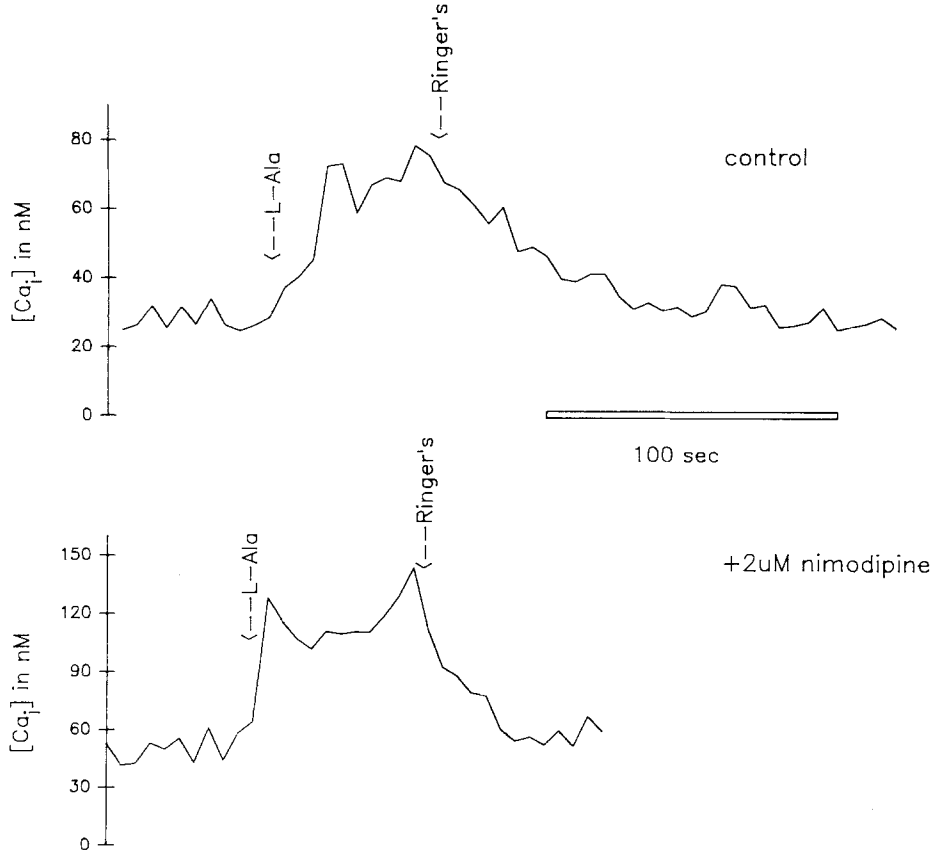

Fig. 4. Addition of 2 μM nimodipine did not prevent the amino acid-induced increase in intracellular calcium. The stimulus used was 100 μM L-alanine in Ringer's

removal of extracellular calcium (by addition of EGTA as in Fig. 3*A*) did not lead to a noticeable change in resting [Ca_i] (the ratio of [Ca_i] during the 40 sec following addition of calcium-free medium to the level of calcium for the 40 sec immediately before addition of calcium-free medium was 0.94 ± 0.04 (mean ± SEM, n = 5). Thus, to trigger the release of the hypothetical quick-release internal calcium stores, these stores must have been able to sense a decrease in extracellular calcium *directly* (because there was no change in cytoplasmic calcium). In addition, a quick depletion of internal stores would have been expected to elicit a transient change in intracellular calcium contrary to the very slow decline in [Ca_i] levels which was observed and which only became apparent after prolonged (7–15 min) incubation in calcium-free medium. The only way to avoid such a transient change in [Ca_i] levels upon release of calcium from the hypothetical quick-release stores would have been if the release of calcium from internal stores were offset by a transient increase in calcium efflux across the plasma membrane. Because of these reasons, we believe that it is unlikely that quick-release internal stores for calcium were involved in the response of olfactory neurons to L-amino acids shown in Figs. 1 and 3.

One pathway that could possibly mediate this calcium influx would be entry of calcium through voltage-dependent calcium channels (Restrepo & Teeter, 1990). However, as shown in Fig. 4, amino acids elicited the elevation in [Ca_i] in the presence of the L-type calcium channel inhibitor nimodipine, which inhibits depolarization-induced increases of intracellular calcium in catfish olfactory neurons (Restrepo & Teeter, 1990). This indicates that the increase in intracellular calcium elicited by odors was mediated by entry of calcium through a nimodipine-insensitive calcium pathway.

Response of Olfactory Neurons to Addition of Aluminum Fluoride

Huque and Bruch (1986) and Boekhoff et al. (1990) found that direct G-protein stimulation with guanine nucleotides led to an increase in the rate of formation of inositol-1,4,5-trisphosphate. This suggests involvement of regulatory G-proteins in the stimulation of IP_3 formation by odor stimuli, which is presumably followed by an increase in [Ca_i]. To test the involvement of G-proteins in the regulation of intracellular calcium, we assayed changes in [Ca_i]

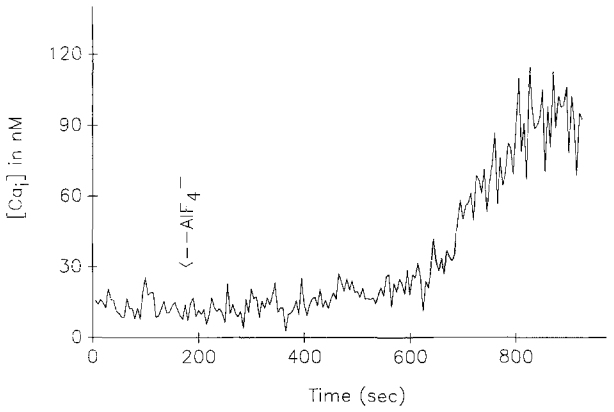

Fig. 5. Response of a single olfactory neuron to addition of 10 mM NaF plus 30 μM $AlCl_3$. Notice the lag between the time of addition of AlF_4^- and the start of the increase in intracellular calcium

following stimulation with aluminum fluoride (a combination of 10 mM NaF and 30 μM $AlCl_3$), which is known to lead to activation of G-proteins in intact cells (Blackmore & Exton, 1986; Dubyak, Cowen & Meuller, 1988; Marc, Leiber & Harbon, 1988). Only neurons unresponsive to addition of all four L-amino acids tested were used. Seven out of a total of thirteen cells tested responded to AlF_4^- with an abrupt increase in intracellular calcium (Fig. 5). This increase in calcium was preceded by a lag period of variable duration (0–10 min) and was not readily reversible. Also, there was a large variation in the magnitude of the increase (14 to 140 nM). These observations are consistent with studies which have determined that the action of AlF_4^- on intracellular calcium in other cells is preceded by a lag of variable duration and that the increases in calcium are not reversible.

As in the case of the amino acid-induced increase in intracellular calcium, [Ca_i] did not increase upon addition of AlF_4^- in the absence of extracellular calcium, indicating that the increase in calcium is due to influx through the plasma membrane. Of eight cells tested none responded to AlF_4^- in the absence of extracellular calcium which is significantly different from the response in the presence of calcium ($P < 0.05$, chi-squared test). In addition, no changes in [Ca_i] were recorded in neurons incubated in fish Ringer's for the same length of time of the incubation with AlF_4^-, indicating that the increase in Ca^{2+} is not a nonspecific effect (no increases were found when calcium was recorded continuously for over 10 min in 12 cells; this is significantly different from the response in the presence of AlF_4^- at the $P < 0.02$ level using a chi-squared test).

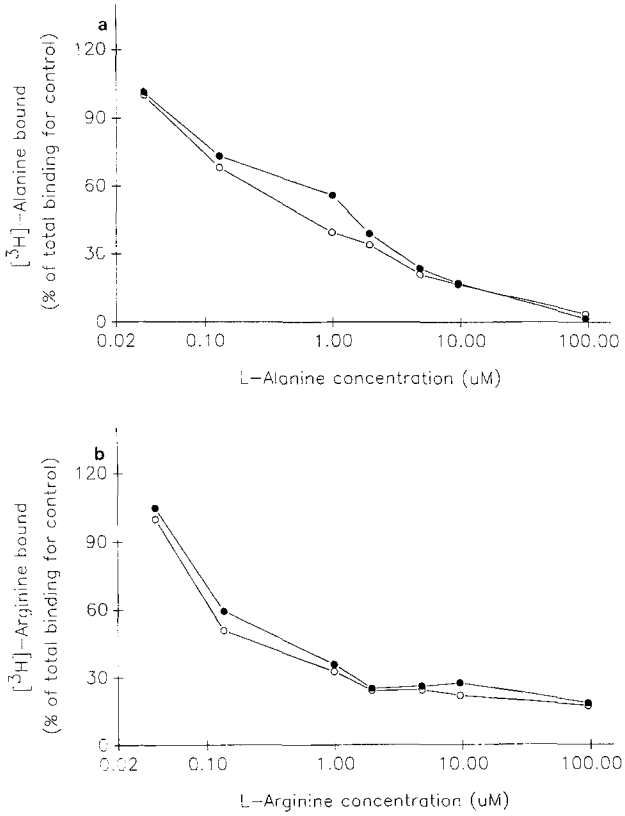

Fig. 6. Effect of papain pretreatment on specific binding of tritiated amino acids to isolated olfactory cilia. (*A*) L-alanine binding. (*B*) L-arginine binding. Open circles: control; closed circles: papain treated. Each graph is from one representative experiment from a total of six which display similar results (three with alanine and three with arginine)

Papain Treatment Did Not Affect Binding of L-Alanine and L-Arginine to Olfactory Receptors

The fact that 50% of the cells responded to direct G-protein stimulation with aluminum fluoride indicated that the cellular machinery necessary to trigger the calcium influx was not damaged by the cell isolation procedure in these cells. Why then did only 5% of the cells respond to olfactory stimuli? One possibility is that the treatment with papain during cell isolation could lead to damage of the olfactory receptor proteins which are exposed to the extracellular medium. To explore this possibility, we assayed binding to isolated olfactory cilia of L-alanine and L-arginine which bind to distinct receptor sites in catfish (Kalinoski et al., 1987; Bruch & Rulli, 1988). Figure 6 shows that the specific binding of these two amino acids to olfactory membranes is not affected by pretreatment with 30 U/ml of activated papain in a wide range of ligand concentrations (0.5 to 100 μM).

Discussion

We have shown that a small percentage of isolated catfish olfactory neurons (5%) respond to L-amino acid stimulation with a large transient increase in intracellular calcium mediated by entry of extracellular calcium through the plasma membrane. In addition, direct G-protein stimulation with aluminum fluoride elicits increases in intracellular calcium, also mediated by calcium influx through the plasma membrane, in approximately 50% of the cells. Since catfish olfactory receptors are linked to G-proteins (Bruch & Kalinoski, 1987), this observation suggests that a G-protein-linked second messenger system may mediate the L-amino acid-induced increase in $[Ca_i]$. Because the change in $[Ca_i]$ occurs via calcium influx through the plasma membrane, which could cause cell depolarization, calcium may participate directly in mediating olfactory transduction. In addition, calcium may play a role in either adaptation or in termination of the phasic component of the response because the increase in intracellular calcium concentration caused by exposure to olfactory stimuli could lead to opening of calcium-activated potassium channels (Maue & Dionne, 1987), leading to repolarization of the neuronal plasma membrane and causing a decrease in the action potential firing rate.

Two second messenger systems have been shown to be activated upon stimulation of catfish olfactory cilia with amino acids: cAMP formation (Bruch & Teeter, 1990) and phosphoinositide turnover (Huque & Bruch, 1986). Both second messengers (cAMP and IP_3) could trigger calcium influx. Opening of the olfactory cAMP-gated channel (Nakamura & Gold, 1987; also shown to be present in catfish cilia by Bruch & Teeter, 1990) could cause significant calcium influx because this channel (Suzuki, 1989; Kolesnikov, Zhainazarov & Kosolapov, 1990), like the cGMP-gated channel of vertebrate rods (Yau & Nakatani, 1985), is permeable to calcium. On the other hand, opening of IP_3-gated cation channels on the plasma membrane of the cilia of catfish olfactory neurons (Restrepo et al., 1990) could also mediate the influx of calcium. Regardless of whether one pathway or both are mediating the change in internal calcium, the data in Fig. 3 indicate that calcium is not being released from internal stores. For the IP_3 pathway this implies that either internal calcium stores in olfactory neurons are not releasable by IP_3 or that the formation of IP_3 occurs only in the cilia and that little IP_3 diffuses to the cell body where it could lead to release of calcium from endoplasmic reticulum.

Although the upstroke of the increase in calcium caused by addition of amino acids in Fig. 1*A* and *B* is fast enough that it is probably limited by the perfusion of amino acid onto the cell, the increase in intracellular calcium for the cell in Fig. 1*C* is clearly not limited by perfusion rates. It must be stressed that a slow increase in intracellular calcium does not rule out the role of IP_3 as mediator of the olfactory response because it is the opening of the IP_3-gated channel, causing depolarization, and not the increase in intracellular calcium that must be fast enough to accommodate for the speed of the olfactory response. Even though a plasma membrane channel opens instantaneously, the increase in intracellular ion activity caused by influx of ions through this channel may be slow. This is well illustrated by the increase in calcium triggered by membrane depolarization in isolated olfactory neurons; even though membrane depolarization and opening of voltage-dependent calcium channels happens instantaneously, the increase in $[Ca_i]$ is gradual in a subpopulation of olfactory neurons, presumably because the net number of calcium ions which enter the cell per unit time is small compared to the buffering capacity of the cytosol for calcium (Restrepo & Teeter, 1990).

Figure 1*A* and *B* shows that the increase in calcium triggered by exposure of olfactory neurons to olfactory stimuli is transient. This transient elevation in $[Ca_i]$ could be explained by the opening (and subsequent closing) of cAMP- or IP_3-gated channels due to transient increases in second messenger concentrations (Boekhoff et al., 1990). Opening of the channels would lead to an influx of Ca^{2+} (and Na^+) which would rapidly elevate $[Ca_i]$ and would depolarize the ciliary membrane, resulting in an increase in the rate of firing of action potentials. After this first phase, a decrease in second messenger formation would then lead to closing of the cAMP- and/or IP_3-gated channels, leading to a recovery of $[Ca_i]$ levels towards basal, presumably through the action of the plasma membrane calcium ATPase which displays a robust activity in olfactory cilia membranes (Lo, Bradley & Rhoads, 1990).

Figure 6 shows that treatment with papain does not affect amino acid binding to olfactory receptors in catfish olfactory cilia. A lack of an effect of papain is not surprising since olfactory cilia proteins are heavily glycosylated and because the amino acid binding sites are believed to be located near sugar side chains of glycoproteins (Kalinoski et al., 1987). This heavy glycosylation may minimize accessibility of the protease to the olfactory receptors. In addition, although it is likely that cellular damage occurs in some cells after dissociation, it appears that this is not the only reason why only a small percentage

of cells respond to amino acids with an increase in $[Ca_i]$ because: (*i*) unresponsive cells keep low calcium concentrations indicating that cells are metabolically viable and that the plasma membrane is not leaky, and (*ii*) many of the cells which do not respond to amino acids respond with an elevation of intracellular calcium to direct G-protein stimulation with aluminum fluoride (Fig. 5) or to membrane potential depolarization (Restrepo & Teeter, 1990). These results suggest that olfactory neurons are functionally heterogeneous in agreement with electrophysiological and immunohistochemical studies which find that olfactory neurons are heterogeneous in their response to odor stimuli and in their ability to be recognized by antibodies (Margolis, 1972, 1988; Getchell & Shepherd, 1978; Akeson, 1988; Frings & Lindemann, 1988; Morgan, 1988; Verhaagen et al., 1988; Firestein & Werblin, 1989).

Several reasons can be envisioned for the absence of a change in intracellular calcium in response to stimulation with L-amino acids in many of the cells. (*i*) Some of the unresponsive cells could respond specifically to chemosensory stimuli other than the four amino acids tested. (*ii*) Different second messenger systems whose stimulation does not cause an increase in $[Ca_i]$ may be activated by odor stimuli in some neurons. (*iii*) The elevation in internal calcium may be spatially localized to the apical region of the cell. Because we measure the average calcium concentration in the whole cell, a localized increase in $[Ca_i]$ could have been missed due to the noise in the fluorescence signal. (*iv*) Some neurons may not express chemosensory receptors because they are at an early stage of cell differentiation (Graziadei & Monti-Graziadei, 1978).

As mentioned above, because of the presumed location of the second messenger-gated channels at the cilia of the olfactory neurons, it is likely that regional increases in $[Ca_i]$ take place upon stimulation with olfactory stimuli. From the data in Fig. 1 it is possible to calculate the smallest portion of the total cellular volume in which a large increase in $[Ca_i]$ could account for the measured increases in measured average $[Ca_i]$ as follows: addition of 100 μM L-amino acids caused an increase in $[Ca_i]$ from approximately 20 to 180 nM in Fig. 1*B*. Using the equation with $(R_{max}/R_{min}) = 0.3$ and $K_d = 166$ nM it is possible to calculate the fluorescence ratios corresponding to the measured $[Ca_i]$ (R/R_{max} at 20 nM = 0.375 and (R/R_{max}) at 180 nM = 0.664). The maximum increase in the fluorescence ratio that can take place in any region of the cell is when R becomes equal to R_{max}. If we assume that the fluorescence ratio in a small fraction (f) of the cytoplasm approached R_{max} while the rest of the cell remained at the basal calcium concentration of 20 nM ($(R/R_{max}) = 0.375$), it is possible to calculate a cytoplasmic fraction (f) of (0.664–0.375)/(1 − 0.375) or 0.46. Thus, a minimum of 46% of the fluorescence inside the cell had to display a large increase in $[Ca_i]$ to account for a 160-nM increase in average measured $[Ca_i]$. A similar calculation with the data in Fig. 1*C*, in which the elevation in $[Ca_i]$ was much smaller, indicates that in this cell 10% of the total fluorescence had to increase to account for the change in average $[Ca_i]$. Thus, because the cilia account for only a few percent of the volume of the neuron, and assuming that fura-2 fluorescence is evenly distributed throughout the cell, it is possible to conclude that the increases in $[Ca_i]$ measured in this study must have occurred over a substantial portion of the cell. It is likely that in some cases $[Ca_i]$ increased, not only in the dendrite, but in the cell body as well. This does not, of course, mean that at early times a localized change could not have taken place in the cilia.

Although the group of cells that responded to amino acids is small (11 cells), there is clear indication that cells respond heterogeneously to amino acids. Some catfish olfactory neurons responded specifically to only one stimulus while one neuron responded to all four stimuli. In addition, one cell responded to stimulation with amino acids with a *decrease* in intracellular calcium. It is tempting to associate this decrease in $[Ca_i]$ with observations in single unit recordings that show that in a small number of responding cells addition of olfactory stimuli leads to a *decrease* in the basal electrical activity of the neuron (O'Connell & Mozell, 1969; Gesteland, 1971; Mathews, 1972; Duchamp et al., 1974; Getchell & Shepherd, 1978; Sicard & Holley, 1984) and with intracellular recordings in lobster olfactory neurons which show that some stimuli lead to plasma membrane hyperpolarization (McClintock & Ache, 1989). This kind of behavior could be exhibited by cells in which basal IP_3 or cAMP levels are high, maintaining either the IP_3- or the cAMP-gated channel open, leading to a low resting membrane potential and to a high basal intracellular calcium concentration. In these cells, if it is postulated that addition of odor stimuli leads to a decrease in second messenger concentration, the IP_3- or cAMP-gated channels would close, leading to membrane potential repolarization and to a decrease in $[Ca_i]$.

In summary, we have determined that exposure of catfish olfactory neurons to olfactory stimuli (L-amino acids) triggers entry of calcium through the plasma membrane, leading to a transient elevation in intracellular calcium. This influx of calcium and the concomitant elevation in $[Ca_i]$ may play an important role in olfactory transduction. In addition, we have shown that optical measurement of intracel-

lular calcium in isolated olfactory neurons is a powerful technique suitable for the study of the functional heterogeneity of the olfactory neuron population.

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