Linoleic Acid Induces Calcium Signaling, Src Kinase Phosphorylation, and Neurotransmitter Release in Mouse CD36-positive Gustatory Cells*

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We have recently demonstrated that the cells expressing CD36, localized apically on the taste buds of mouse circumvallate papillae, act as gustatory cells. In the present study we isolated these CD36-positive cells from mouse circumvallate papillae and investigated intracellular signaling events, triggered by a long-chain polyunsaturated fatty acid, i.e. linoleic acid (LA). LA induced increases in free intracellular calcium, [Ca2+]i, by recruiting calcium from endoplasmic reticulum pool via inositol 1,4,5-triphosphate production followed by calcium influx via opening of store-operated calcium (SOC) channels. LA also induced phosphorylation of Src-protein-tyrosine kinases (Src-PTKs), particularly of Fyn59 and Yes62. LA-evoked phosphorylation of Fyn59 and Yes62 was implicated in the activation of SOC channels. Reverse transcription-quantitative PCR revealed that the CD36-positive gustatory cells possessed mRNA of enzymes like tryptophan hydroxylase-1, L-aromatic amino acid decarboxylase, tyrosine hydroxylase, and dopamine β-hydroxylase, involved in the synthesis of monoamine neurotransmitters. Interestingly, the addition of LA to these cells induced the release of 5-hydroxytryptamine and noradrenalin to the extracellular environment. The LA-induced release of these neurotransmitters was curtailed by SOC channel blockers and Src-PTK inhibitors. These results altogether demonstrate that LA binds to mouse CD36-positive gustatory cells, induces Src-PTKs phosphorylation, triggers calcium signaling, and evokes the release of 5-hydroxytryptamine and noradrenalin, which in turn may be implicated in the downstream signaling to the afferent nerve fibers, thus transmitting the output signal from taste buds to the central nervous system.

The tongue contains primarily four types of papillae. Filiforms are involved in the somesthesic perception of foods, whereas fungiforms, foliates, and circumvallates, which contain taste buds, are responsible for the chemosensory perception of basic taste modalities (sweet, bitter, salt, sour, umami). Recent evidences have strongly suggested the existence of an additive oro-sensory system devoted to the dietary fat perception in rodents. Gilbertson et al. (1) have reported that lipids may be sensed by isolated rat fungiform taste receptor cells (TRC)2 via the inhibition of the delayed rectifying K+ channels by polyunsaturated fatty acids. Fukuwatari et al. (2) and Laugerette et al. (3) documented the expression of the receptor-like lipid-binding protein CD36 in rat and mouse taste bud cells, respectively. Kawai and Fushiki (4) demonstrated that the addition of a lipase inhibitor diminished the spontaneous preference for triglycerides. These investigators proposed that the lingual lipase, present in the rodent saliva, might release free fatty acids that would be detected by gustatory cells. The immunolocalization of CD36 in the apical side of few TRC in circumvallate papillae (3) is especially adequate for this function since CD36 is known to exhibit a very high affinity for long-chain fatty acids (5). Consistent with this assumption, we have recently provided the first evidence that CD36-positive gustatory cells play a significant role in dietary lipid perception in the mouse (3). Indeed, the CD36 gene inactivation fully abolished the spontaneous preference for long-chain fatty acids observed in wild-type mice (3). It is noteworthy that this effect on feeding behavior is lipid-specific since sweet preference and bitter aversion are not affected in these transgenic mice (3).

To further explore whether a sixth taste modality devoted to the “fat” functionally exists in rodents, the downstream signaling events triggered by the free fatty acid/CD36 interaction in gustatory cells must be studied. We have for the first time purified the CD36-positive gustatory cells from mouse CVP and demonstrated that linoleic acid induced increases in [Ca2+]i in these cells via CD36 (6). In the present study we have extended these investigations to characterize the mechanisms of action of linoleic acid on calcium signaling/protein phosphorylation, leading to the release of neurotransmitters, which might be implicated in the activation of afferent nerve fibers.

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EXPERIMENTAL PROCEDURES

Materials—C57BL/6J mice were obtained from Janvier Elevage (Le Genest-st-isle, France). Sulfo-N-succinimidyl-olate (SSO) was a generous gift from IF Glatz (Maastricht, The Netherlands). The culture medium RPMI 1640 and l-glutamate were purchased from Lonza Verviers SPRL (Verviers, Belgium). Fura-2/AM was procured from Invitrogen. All other chemicals including linoleic acid (18:2 n-6), collagenase type-I, and trypsin inhibitor were obtained from Sigma. SKF96365, econazole, and SU6656 were purchased from Calbiochem. Elastase and dispase were purchased from Serlabo (Bonneuil/ Marne, France) and Roche Diagnostics, respectively. Anti-CD36 antibody coupled to phycoerythrin and anti-α-gustducin antibody were procured from Santa Cruz Biotechnology, Inc. Anti-p-Fyn59, anti-p-Lyn53/56, and anti-p-Yes62 antibodies were bought from Tebu-Bio (Paris, France), and anti-phosphotyrosine kinase (p-PTK) antibody was obtained from Chemicon (Dundee, UK). Anti-CD36 antibody was procured from Cell Sciences (Dundee, UK). Anti-gustducin antibody was obtained from Boehringer Ingelheim (Germany). Sulfo-N-succinimidyl carbonate (CSP) and Cy3-conjugated goat antiserum to sheep IgG were procured from Invitrogen. Anti-rat CD36 antibody were procured from Santa Cruz Biotechnology, Inc. Anti-CD36 antibody was procured from Cell Sciences (Dundee, UK). Anti-gustducin antibody. It is noteworthy that the clone UA009 also recognizes mouse CD36 (3). After washing, slides were incubated for 2 h at room temperature with Cy3-conjugated anti-mouse IgG (1:600 dilutions) for CD36 expression or with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:600) for α-gustducin detection. Staining specificity was assessed by treating slides in the absence of primary antibodies. After three washings with PBS, a drop of Aqua Poly-Mount mounting medium was added on the slide for the analysis under fluorescence microscope (Zeiss Axioskop).

Measurement of Ca2+ Signaling—The CD36-negative and CD36-positive gustatory cells, cultured for 24 h, were washed with PBS, pH 7.4. The composition of PBS was as follows: 3.5 mM KH2PO4, 17.02 mM Na2HPO4, 136 mM NaCl. The cells were then incubated with Fura-2/AM (1 μM) for 60 min at 37 °C in loading buffer which contained 110 mM NaCl, 5.4 mM KCl, 25 mM NaHCO3, 0.8 mM MgCl2, 0.4 mM KH2PO4, 20 mM Hepes, 0.33 mM Na2HPO4, 1.2 mM CaCl2, and the pH was adjusted to 7.4.

After loading, the cells (2 × 106/ml) were washed 3 times (600 g × 10 min) and remained suspended in the identical buffer. For experiments in Ca2+−free (0% Ca2+) medium, CaCl2 was replaced by EGTA (2 mM). [Ca2+]i was measured according to Grynkiewicz et al. (7). The fluorescence intensities were measured in the ratio mode in PTI spectrophotometer at 340 and 380 nm (excitation filters) and 510 nm (emission filter). The cells were continuously stirred throughout the experimentation. The test molecules were added into the cuvettes in small volumes with no interruptions in recordings. The intracellular concentrations of free Ca2+, [Ca2+]i, were calculated by using the equation [Ca2+]i = Kd × [(R − Rmin)/(Fmax − F)] × (Sf/Sb), where Kd is the dissociation constant for Fura-2/calcium complex, R is the ratio emission with excitation at 340 nm divided by excitation at 380 nm, Rmin is the ratio in the presence of no Ca2+, Fmax is the ratio of saturating [Ca2+]i, and Sf/Sb is the ratio of 380 nm excitation fluorescence at zero and saturating [Ca2+]i. A value of 224 nM for Kd was added into the calculations. Rmax and Rmin values were obtained by the addition of ionomycin (5 μM) and MnCl2 (2 mM), respectively. The SOC channel blockers (SKF96365 and econazole), CD36 inhibitor (SSO), or protein-tyrosine kinase (PTK) inhibitors (PP2, genistein, and SU6656) were added to the cells for 20 min before the addition of linoleic acid. Measurement of Inositol 1,4,5-Triphosphate (IP3) Production—The CD36-negative and CD36-positive gustatory cells were treated with LA in the presence or absence of SSO for 20 min. The vehicle control received 0.1% ethanol (v/v) for 20 min. After stimulation, cells were washed twice with ice-cold PBS. Cell suspension was then lysed with 0.1% Triton X-100 for 30 min at room temperature before overnight incubation at 4 °C with a 1:100 dilution of anti-rat CD36 antibody (UA009) or a 1:150 dilution of polyclonal anti-mouse α-gustducin antibody. It is noteworthy that the clone UA009 also recognizes mouse CD36 (3). After washing, slides were incubated for 2 h at room temperature with Cy3-conjugated anti-mouse IgG (1:600 dilutions) for CD36 expression or with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:600) for α-gustducin detection. Staining specificity was assessed by treating slides in the absence of primary antibodies. After three washings with PBS, a drop of Aqua Poly-Mount mounting medium was added on the slide for the analysis under fluorescence microscope (Zeiss Axioskop).
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decantation, leaving the bound fractions. The radioactivity is measured with a β-scintillation counter.

Immunoprecipitation and Immunodetection of PTKs—First, we detected the presence of CD36 and Src-related kinases (Src total, Fyn, Yes, and Lyn) in CD36-positive cells by employing the specific antibodies (Fig. 3A).

Second, we detected LA-induced phosphorylation at tyrosine residues by employing anti-phosphorylated PTK antibodies. Hence, the cells were incubated with or without SSO (50 μM) for 20 min then stimulated or not with LA (20 μM) as described in the legends of Fig. 3, B and C. After stimulation, cells were lysed for 1 h at 4 °C with 800 μl of ice-cold lysis buffer A (20 mM HEPES, pH 7.4, 2 mM EDTA, 125 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 0.5 mg/ml benzamidine, 1% Nonidet P-40) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2.5 μg/ml pepstatin, 5 μg/ml leupeptin). The lysates were centrifuged (13,500 g × 15 min at 4 °C), and supernatants were subjected to Western blotting. To ensure equal loading of proteins, immunodetection of β-actin was performed in the same assays.

To detect the phosphorylation of Src-related kinases, we first immunoprecipitated the treated-CD36-positive or CD36-negative gustatory cells with anti-phospho-PTK and then probed them with antibodies against Fyn, Yes, and Lyn (Fig. 3D). Briefly, 1 mg of protein was immunoprecipitated with 1 μg of anti-PTK antibody and 25 μl of A/G-Sepharose beads. Phosphotyrosine protein-containing immunoprecipitates were washed three times with buffer A and diluted with Laemmli sample buffer. For Western blotting, denatured proteins (20 μg) were separated on SDS-polyacrylamide/bisacrylamide (8%) gel and transferred onto the PVDF membrane. After saturation for 1 h in the TBS/Tween 20 (10 mM Tris base, 0.15 M NaCl, 0.05% Tween 20) supplemented with delipidated milk 1% (v/v), the PVDF membrane was incubated with primary antibodies for 2 h at room temperature. After washing 4 times in the TBS/Tween 20, the PVDF membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antimouse antibody (1:1000) in TBS/Tween 20 supplemented with 1% delipidated milk (v/v). The PVDF

![Figure 1](https://example.com/figure1.png)

**FIGURE 1. Characterization of purified CD36-positive gustatory cells and effects of linoleic acid on the increases in [Ca\(^{2+}\)](i), and production of inositol trisphosphate.** The CD36-positive and CD36-negative gustatory cells, isolated from CVP of C57BL/6J mice as described under "Experimental Procedures," were used for immunocytochemical detection of CD36 and α-gustducin. Cytospin-prepared slides were fixed and blocked with fetal calf serum before incubation with anti-rat CD36 antibody (A) and a polyclonal anti-mouse α-gustducin antibody (B). Slides were analyzed under fluorescent microscope (Zeiss Axioskop). The CD36-positive gustatory cells (2 × 10⁴/assay) were also loaded with the fluorescent probe, Fura-2/AM, as described under "Experimental Procedures." Experiments were performed on CD36-positive (C) or CD36-negative (D) cells in the presence (100% Ca\(^{2+}\)) or absence (0% Ca\(^{2+}\)) of Ca\(^{2+}\) in the extracellular medium. The arrowheads indicate the time when the linoleic acid at 20 μM was added into the cuvette without interruptions in the recording. Upward deflection indicates increases in [Ca\(^{2+}\)](i), in these cells. The figure shows the single traces of observations which were reproduced several times independently. E, the cells, treated with LA, containing or not SSO at 50 μM for 20 min were assayed for production of IP₃. IP₃ production was measured by [³H] Biotrak Assay. Values are the means ± S.D., n = 6 (*, p < 0.001). $ shows insignificant results as compared with control CD36-negative cells. £ shows insignificant results as compared with LA-treated CD36-negative cells.

### Table 1

| Gene          | Primer sequences                                      |
|---------------|-------------------------------------------------------|
| TPH-1         | Forward, 5′-AAGGGGAAAATCGGCTGGAC-3′; Reverse, 5′-CAATAATGGAGACCTGGCCCG-3′ |
| AADC          | Forward, 5′-ACCCTACCTCTTGCAATCCG-3′; Reverse, 5′-GCACTGTTGTTCCTAATCC-3′ |
| TH            | Forward, 5′-AACCTGTTTTTTGGAGACCTGC-3′; Reverse, 5′-GCACTGTTGTTCCTAATCC-3′ |
| DBH           | Forward, 5′-GAGAGCCCCTTCCCCTACC-3′; Reverse, 5′-TGAGCTGCGAATGAGATCC-3′ |
| VMAT-2        | Forward, 5′-GCGGGGAAATCGTGCGTAC-3′; Reverse, 5′-CAATAGTGATGACCTGGCCGT-3′ |

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### Sequences of the primers used to study the expression of mRNA of different genes

T Bian, Tryptophan hydroxylase-1; AADC, L-α-aminoo acid decarboxylase; TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; VMAT-2, vesicular monoamine transporter-2.
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membrane was washed 3 times in 0.05% TBS/Tween 20 and once in TBS. The phosphorylated bands were revealed with the horseradish peroxidase substrate (Super Signal Substrate, Western blotting kit from Pierce). To ensure the specificity of the detection of phosphorylated Src-related kinases, immunodetection of unphosphorylated Src, Fyn, Yes, and Lyn was performed in each sample (Fig. 3D).

Detection of mRNA of Enzymes Involved in the Synthesis of Neurotransmitters by RT-quantitative PCR and Semiquantitative RT-PCR—Total RNA was extracted from CD36-negative and CD36-positive gustatory cells by using Trisol (Invitrogen) and subjected to DNase treatment using the RNase-free DNase Set (Qiagen). One μg of total RNA was reverse-transcribed with Super script II RNase H-reverse transcriptase using oligo(dT) according to the manufacturer’s instructions (Invitrogen). Real time PCR was performed on the iCycler iQ real time detection system, and amplification was undertaken by using SYBR Green I detection. Oligonucleotide primers were based on the sequences of mice gene in GenBank™ data base (see Table 1). The amplification was carried out in a total volume of 25 μl that contained 12.5 μl of SYBR® Green Supermix buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 3 mM MgCl2, 0.2 mM each dNTPs, 0.63 units of iTaq DNA polymerase, and SYBR®Green 1.0 μM fluorescein) and 12.5 μl (0.3 μM) of each primer and diluted cDNA.

The amplification conditions consisted of an initial denaturation step at 95 °C for 5 min as a “hot start” followed by 40 cycles at 95 °C for 30s and 60 °C for 30s with a single fluorescence detection point at the end of the relevant annealing or extension segment. At the end of the PCR, the temperature was increased from 60 to 95 °C at a rate of 2 °C/min, and the fluorescence was measured every 15 s to construct the melting curve. The standard curves were generated for each gene using serial dilutions of positive control template to establish PCR efficiencies. All determinations were performed at least in duplicate using two dilutions of each assay to achieve reproducibility.

Results were evaluated by iCycler iQ software including standard curves, amplification efficiency (E), and cycle threshold (Ct). Relative quantification of mRNA in different groups was determined as ΔΔCt = ΔCt of gene of interest − ΔCt of β-actin. ΔCt = Ct of CD36-positive or CD36-negative cells − Ct of total cells. Relative quantity (RQ) was calculated as RQ = (1 + E)−ΔΔCt.

For semiquantitative determinations, amplification was performed in the same conditions as described above for 30 cycles followed by a final extension period of 72 °C for 10 min. Reaction products were electrophoresed on a 1.5% agarose gel impregnated with ethidium bromide. The RNA pattern was visualized by UV transillumination.

Determination of Monoamine Neurotransmitters by High Performance Liquid Chromatography (HPLC)—HPLC separation of monoamine neurotransmitters was performed as described elsewhere (8). Cultured CD36-negative and CD36-positive gustatory cells were incubated with LA, as mentioned in the legends, and at the end of incubation supernatant was removed and supplemented with equal volume of solution A (0.2 M perchloric acid, 2.7 mM EDTA, 5.26 mM sodium metabisulfite), then 100 pg of an internal standard (3,4-dihydroxybenzylamine hydrobromide) was added to each sample. Samples were centrifuged for 6 min at 4 °C at 13,000 × g. The supernatants were analyzed by HPLC with a reverse phase column (60 RP Select B, 250 × 4 mm, 5 μm, Merck) with electrochemical detection (Waters, Milford, MA) before or after passing through alumina column, as described previously (9). The potential of the electrochemical detector was set at +0.85 V. The mobile phase consisted of 0.08M sodium phosphate, 0.27 mM EDTA, 3.7 mM octan-1-sulfonic acid, pH 3.5. The biogenic amines were identified and quantified in comparison with commercial standards using the KromaSystem 2000 software (BIO-TEK Instruments, Milano, Italy).

![Figure 2](image-url)

**FIGURE 2. Effects of SOC channel blockers and inhibitors of PTKs on LA-induced increases in \([Ca^{2+}]_i\) in CD36-positive gustatory cells.** The CD36-positive or CD36-negative gustatory cells (2 × 10^4/assay), isolated from CVP of C57BL/6J mice, were loaded with the fluorescent probe, Fura-2/AM, as described under “Experimental Procedures.” A, the cells were added with LA (20 μM) in the presence of SKF96365 (15 μM) or econazole (30 μM). B, the cells were added with LA (20 μM) in the presence of PP2 (10 μM), SU6656 (5 μM), or genistein (30 μM). Values are the means ± S.D., n = 6 (*, p < 0.001).
Measurement of Membrane Potential, $V_{m}$—The probe bisoxonol was used to measure membrane potential in isolated CD36-positive gustatory cells. This probe is chemically unrelated to the cyanines and is not toxic to the cells (10). For these experiments the cells were prepared as described for $[Ca^{2+}]_i$ determinations. After washing, the cells (10^6/1.5 ml) were transferred to the fluorometer cuvettes, and 150 nm of bisoxonol was added. The stock solution of bisoxonol was prepared in DMSO. The cells were allowed to equilibrate with the dye, and after 10 min different test molecules were added. The fluorescent intensities were determined at 540 nm (excitation filter) and 580 nm (emission filter). Upward deflections represent depolarizations.

Statistical Analysis—Statistical analysis of data was carried out using Statistica (Version 4.1, Statsoft, Paris, France). The significance of the differences between mean values was determined by analysis of variance one way followed by a least significant difference test. For all the tests the significance level chosen was $p < 0.05$.

RESULTS

Linoleic Acid Induces Increases in $[Ca^{2+}]_i$ in CD36-positive Gustatory Cells—To explore whether LA triggers a calcium response via CD36 receptor, we purified the CD36-positive gustatory cells which expressed CD36 (Fig. 1A) and $\alpha$-gustducin, a G-protein considered as a specific marker of TRC (Fig. 1B). We observed that LA induced a rapid increase in $[Ca^{2+}]_i$, which was followed by a stationary phase both in calcium-free (0% Ca^{2+}) and calcium-enriched (100% Ca^{2+}) buffers (Fig. 1C). LA-induced increases in [Ca^{2+}]_i were more important in 100% Ca^{2+} buffer than those in 0% calcium buffer. We also measured the production of IP_3, known to recruit calcium from endoplasmic reticulum (ER) pool. Fig. 1E shows that LA induced 4-fold increases in IP_3 production in comparison to unstimulated cells. To shed light on whether LA induces the production of IP_3 via binding to CD36, we employed sulfo-N-succinimidyl derivative of oleate (SSO) which is known to inhibit the binding of fatty acids to CD36 (11). We observed that SSO curtailed LA-induced increases in IP_3 in these cells. As far as the CD36-negative cells are concerned, we observed that these cells, in response to LA, exhibited a weak increase in [Ca^{2+}]_i in 100% Ca^{2+} buffer without influencing the same in 0% Ca^{2+} buffer (Fig. 1D). Furthermore, in CD36-negative cells, LA failed to induce IP_3 production, and SSO exerted no effect on it (Fig. 1E).
It is important to mention that SSO treatment exerted no cytotoxic effect on either of the cell populations as assessed by trypan blue exclusion assay (not shown).

**LA Stimulates Capacitative Calcium Influx via a PTK-dependent Mechanism in CD36-positive Gustatory Cells**—Because LA-induced increases in [Ca^{2+}]_i are more important in 100% calcium buffer than those in 0% Ca^{2+} buffer, it is possible that LA activates a capacitative calcium entry due to calcium recruitment from internal stores in CD36-positive gustatory cells. To support this hypothesis we conducted experiments in 100% calcium buffer in the presence of SKF96365 and econazole, the inhibitors of store-operated calcium (SOC) channels. Fig. 2A shows that the addition of either SKF96365 or econazole significantly diminished the LA-induced increases in [Ca^{2+}]_i in CD36-positive gustatory cells. These two SOC channel blockers exerted no effect on CD36-negative gustatory cells (Fig. 2A).

Because PTKs have been shown to modulate the activation of SOC channels (12), we investigated the involvement of PTKs in the LA-induced SOC influx. We used the inhibitors of PTKs: PP2 (known to inhibit the PTK activity of Src in a non-competitive manner against ATP), genistein (a soybean isoflavone, known to selectively inhibit PTK), and SU6656 (a selective inhibitor of Fyn, Yes, and Lyn). We observed that in CD36-positive gustatory cells, these inhibitors significantly abolished the increases in [Ca^{2+}]_i induced by LA; however, these agents exerted no effects in CD36-negative cells (Fig. 2B).

**LA Induces the Phosphorylation of Src, Fyn, and Yes in CD36-positive Gustatory Cells**—We observed that mouse lingual CD36-positive gustatory cells expressed abundantly CD36, Src, and Src-PTK-like Fyn and Yes. The CD36-negative cells also expressed these Src-PTK but to a lesser extent than CD36-positive cells. The two cell populations do not express the Lyn isoform of Src-PTK (Fig. 3A). Fig. 3B shows that LA induced the phosphorylation of PTKs, which was well apparent at 5 min and started declining at 15 min of incubation. Moreover, the LA-induced phosphorylation of PTKs was completely abolished by SSO in these cells (Fig. 3C). In CD36-negative cells, LA failed to induce PTK phosphorylation, and SSO exerted no effect on the same (Fig. 3C). Furthermore, we immunoprecipitated the cell lysates with total anti-phospho-PTKs and detected different isoforms of Src-PTKs. We noticed that LA induced the phosphorylation of both Fyn59 and Yes62, but not of Lyn56/53, at 5 and 15 min of incubation (Fig. 3D).

**mRNA Expression of the Enzymes Involved in the Synthesis of Monoamine Neurotransmitters in CD36-positive Gustatory Cells**—By employing RT-PCR, we have assessed the expression of mRNA of the enzymes involved in the synthesis of 5-HT and catecholamines. We have measured tryptophan hydroxylase-1 mRNA as the tph1 gene that encodes for the synthesis of tryptophan hydroxylase in peripheral tissues (13). Tryptophan hydroxylase-1 converts l-tryptophan to l-5-hydroxytryptophan. We have also detected l-aromatic amino acid decarboxylase that converts 1-5 hydroxytryptophan to 5-HT. 1-Aromatic
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Amino acid decarboxylase has been detected both in neuronal and non-neuronal cells (14). We have also determined the mRNA of the enzymes involved in the biosynthesis of catecholamines, like tyrosine hydroxylase that converts tyrosine to 3,4-dihydroxyphenylalanine and dopamine β-hydroxylase that converts dopamine to NA. We evaluated the expression of vesicular monoamine transporter-2, implicated in the vesicular storage and subsequent release of neurotransmitters.

We observed that CD36-positive gustatory cells expressed a high level of tryptophan hydroxylase-1 and 1-aromatic amino acid decarboxylase (AADC) mRNA (Fig. 4, A and B). However, tyrosine hydroxylase mRNAs are less abundantly expressed in purified CD36-positive gustatory cells (Fig. 4, A and B). Dopamine β-hydroxylase mRNAs were identically present in unpurified (total), CD36-positive, and CD36-negative cells (Fig. 4B). Vesicular monoamine transporter-2 mRNA is highly expressed in CD36-positive gustatory cells (Fig. 4, A and B). It is also noteworthy that the unpurified total cells also express in relatively small quantities the aforementioned mRNAs (Fig. 4B).

Production and Release of 5-HT and NA in CD36-positive Gustatory Cells—At first we standardized the HPLC detection of biogenic neurotransmitters. Fig. 5A shows the HPLC profile of a chromatogram of the supernatant of LA-treated CD36-positive gustatory cells, indicating the presence of NA and 5-HT in these cells. Because dopamine was barely detectable and the NA peak was not very-well apparent, we improved the detection efficiency of biogenic amines by passing the supernatants through the alumina column and then onto HPLC. Fig. 5B shows a clear isolation of NA though dopamine was barely detectable in these cells. CD36-negative cells failed to produce NA (Fig. 5D) and synthesized a detectable quantity of serotonin (Fig. 5C), which remained lower than that in CD36-positive cells (Fig. 5A).

Fig. 6 shows that, in CD36-positive gustatory cells, LA induced 6-fold increases in the release of 5-HT and NA compared with unstimulated cells. To shed light on whether the binding of LA to CD36 is implicated in the release of 5-HT and NA, we employed SSO. We observed that SSO significantly curtailed the LA-induced release of neurotransmitters into the extracellular environment (Fig. 6, A and B). Interestingly, LA failed to induce the release of serotonin and NA in CD36-negative cells, and SSO exerted no effect on the same (Fig. 6, A and B).

We further employed SOC channel blockers and PTK inhibitors to probe the implication of SOC channels and PTK phosphorylation in the release of 5-HT and NA. Fig. 7 shows that SKF96365 and econazole significantly diminished the LA-induced release of two neurotransmitters into the extracellular environment from CD36-positive cells without influencing the same from CD36-negative cells (Fig. 7, A–D). Similarly, genistein, PP2, and SU6656 inhibited the release of 5-HT and NA from CD36-positive cells without influencing the same from CD36-negative cells (Fig. 7, A–D).

We also employed ionomycin to demonstrate that an increase in $\left[Ca^{2+}\right]_{i}$ is mandatory for the release of neurotransmitters. Hence, we observed that this ionophore increased $\left[Ca^{2+}\right]_{i}$ in both types of cells (Fig. 8, A and B); however, this agent induced the release of 5-HT and NA only from CD36-positive cells but not from CD36-negative cells (Fig. 8, C and D).

LA Induces Depolarization in CD36-positive Gustatory Cells—To elucidate whether LA induces plasma membrane activity, we measured the alterations in the plasma membrane potential ($V_m$) in these cells by using bisoxonol. We observed that LA induced the depolarization in CD36-positive cells (Fig. 9A). As a control, we used KCl, and as expected, this agent also induced plasma membrane depolarization in these cells (Fig. 9B). Such responses are specific to CD36-positive cells as the CD36-negative cells exhibited little depolarization (data not shown).

DISCUSSION

We have recently isolated the CD36-positive gustatory cells to a high degree of purity from mouse lingual CVP. These purified cells, being CD36- and α-gustducin-positive, have been found to abundantly express CD36 mRNA (6), consistent with our previous immunohistochemical data showing co-expression of CD36 and α-gustducin in circumvallate papillae (3). Recently, we have shown that LA induced a significant increase...
In [Ca^{2+}], (6); however, we did not elucidate whether this fatty acid recruited calcium from an intracellular or extracellular environment.

In the present study we have characterized the molecular mechanism of action of LA in isolated CD36-positive gustatory cells. We have observed that LA induces increases in [Ca^{2+}], even in the absence of calcium in the extracellular medium, suggesting that LA recruits calcium from intracellular stores. We further observed that LA induced the production of IP_3, and SSO significantly diminished this phenomenon in CD36-positive gustatory cells. These observations suggest that LA, by binding to CD36, recruits calcium from ER pool via a PLC-dependent mechanism. Interestingly, LA failed to induce increases in [Ca^{2+}], in CD36-negative cells, and SSO exerted no effect on the same. Our observations on IP_3-evoked increases in [Ca^{2+}], corroborate the study of Bernhardt et al. (15) who have shown the production of IP_3 and increases in [Ca^{2+}], in response to sweeteners in rat CVP. However, it remains to be ascertained whether CD36 is coupled to direct activation of PLC in CD36-positive gustatory cells. Not much is known on the coupling of CD36 to PLC activation; however, Gousset et al. who have shown that mouse α-gustducin-positive TRC express SOC channels.

As far as the regulation of SOC channels is concerned, several reports have suggested a role for PTK phosphorylation (21, 22). It is also noteworthy that CD36 has been shown to be coupled to Src-PTK phosphorylation in several cell types like human platelets, melanoma, and erythroleukemia cells (23). We observed that inhibitors of PTKs and Src-related kinases significantly diminished the LA-induced increases in [Ca^{2+}], in CD36-positive gustatory cells. These PTK/Src-PTK inhibitors exerted no effect on CD36-negative cells. Furthermore, the binding of LA to CD36 seemed to result in the phosphorylation of PTKs as SSO blocked their phosphorylation, evoked by this fatty acid in CD36-positive gustatory cells. However, the intermediary second messengers like Vav, which may link Src-PTK phosphorylation and PLC activation in CD36-positive cells, remain to be shown (17). Besides, TRC have been shown to express PLCβ2 (18).

As per the capacitative model of calcium homeostasis, the depletion of intracellular stores of calcium is followed by calcium influx via the opening of calcium channels to refill the intracellular pools (19). These channels have been termed store operated calcium channels, and their activity is maintained as long as the stores are not refilled (19). To determine whether LA-induced recruitment of calcium from ER pool results in the opening of SOC channels, we employed SOC channel blockers, which as anticipated, decreased significantly the LA-induced calcium response in 100% calcium buffer. The SOC channel blockers exerted no effect in CD36-negative cells. Our results on CD36-positive gustatory cells are in close agreement with Pérez et al. (20),
Increases in $[Ca^{2+}]$, have been shown to be involved in several cell functions, including neurotransmitter release (25). Several neurotransmitter candidates have been proposed for synapses between gustatory TRC and primary sensory afferent fibers, including acetylcholine, NA, 5-HT, amino acids (glutamate and $\gamma$-aminobutyric acid), and peptides (substance P and calcitonin gene-related peptide) (26, 27). Among these candidates it has been postulated that 5-HT may be the major taste bud neurotransmitter (22, 28, 29). To probe whether CD36-positive gustatory cells can synthesize the biogenic neurotransmitters and release them upon stimulation with LA, we conducted further experiments. We observed that CD36-positive gustatory cells expressed the mRNAs of tryptophan hydroxylase-1 and $\alpha$-aromatic amino acid decarboxylase. Our observations corroborate several studies that have reported the presence of $\alpha$-aromatic amino acid decarboxylase (30) and tryptophan hydroxylase-1 (31) in mouse taste buds. Interestingly, tyrosine hydroxylase and dopamine $\beta$-hydroxylase mRNAs were also present to a lesser extent in these cells.

We further observed that the addition of LA to CD36-positive gustatory cells induced a significant release of 5-HT and NA into the extracellular environment. To our knowledge ours is the first study to detect quantitatively 5-HT and NA in mouse TRC. Most of the studies conducted hitherto have detected these neurotransmitters immunocytochemically (32) or with fluorescent biosensor probes (29). LA-induced release of 5-HT and NA is mediated via CD36 as SSO curtailed the same. Interestingly, the release of these two neurotransmitters seem to be mediated by calcium influx as SOC channel blockers abolished the LA-induced release of neurotransmitters. We mentioned here before that the phosphorylation Src-PTKs is implicated in the opening of SOC channels. Consequently, the Src-PTKs inhibitors abolished the LA-induced release of the two monoamines into the extracellular medium. The use of ionomycin further strengthened the implication of calcium in the efflux of neurotransmitters as this agent induced both increases in $[Ca^{2+}]$, and the release of 5-HT and NA in CD36-positive gustatory cells. Our findings corroborate the observations of Huang et al. (29) who have shown by using biosensors that increases in $[Ca^{2+}]$, induce serotonin release in response to taste stimuli. Furthermore, Haskew et al. (33) have reported the release of excitatory amino acids in a Src-PTK-dependent man-

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**FIGURE 8.** Effects of ionomycin on the increases in $[Ca^{2+}]$, and release of monoamine neurotransmitters in CD36-positive gustatory cells. The CD36-positive (A) and CD36-negative (B) gustatory cells (2 x 10$^6$/assay), isolated from CVP of C57BL/6J mice, were also loaded with the fluorescent probe, Fura-2/AM, as described under “Experimental Procedures.” Experiments were performed in the presence of 100% Ca$^{2+}$ buffer. The arrows indicate the time when the ionomycin at 50 nM was added into the cuvette without interruptions in the recording. Upward deflection indicates increases in $[Ca^{2+}]$, in these cells. The CD36-positive (C) and CD36-negative (D) gustatory cells (2 x 10$^6$/assay), isolated from CVP of C57BL/6J mice, were incubated for 5 min with or without (control) ionomycin (50 nM). The supernatant was saved, and biogenic amines (C and D) were extracted and determined by HPLC coupled to electrochemical detection as described under “Experimental Procedures.” Values are the means ± S.D., n = 6 (*, p < 0.001). NS, not significant.

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**FIGURE 9.** Effects of LA and KCl on membrane potential ($V_m$) in CD36-positive gustatory cells. The CD36-positive gustatory cells (2 x 10$^6$/assay) were loaded with the fluorescent probe, bisoxonol, as described under “Experimental Procedures.” The arrows indicate the time when the test molecules, KCl (40 mM) and LA (20 M), were added into the cuvette.

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We further observed that Fyn$^{99}$ and Yes$^{62}$ control the opening of SOC channels in response to LA in CD36-positive gustatory cells. Our observations can be substantiated by the results of Babnigg et al. (24) who have shown that Ca$^{2+}$ influx, after store depletion, is dramatically diminished in fibroblasts, derived from src knockout mice, and SOC influx can be restored to control levels by transfection with src gene.
Linoleic Acid Induces Signaling in Mouse CVP Cells

In primary cultures of astrocytes, Wang (34) has demonstrated that activation of Src kinases is responsible for the release of glutamate in rat cerebrocortical nerve terminals. The aforementioned observations provide for the first time evidence on the coupling of PTKs with the release of 5-HT and NA in lipid gustatory cells.

We would like to recall that we have detected the mRNA of vesicular monoamine transporter-2, which is implicated in sequestering monoamine neurotransmitters into vesicles for storage and subsequent exocytosis. We have also observed that CD36-positive gustatory cells are excitable ones as both LA and KCI induced the depolarization which may also contribute to the exocytosis of neurotransmitters in these cells. It is noteworthy that the depolarization in these cells cannot be attributed to calcium influx via voltage-dependent calcium channels since diltiazem and ω-conotoxin, the respective inhibitors of L-type and N-type calcium channels, failed to curtail the LA-induced calcium influx, and plasma membrane depolarization though these agents diminished the response of KCI on depolarization and calcium influx (results not shown). Hence, it is possible that the transient receptor potential melastatin-5 (TRPM5) channels may be implicated in LA-induced plasma membrane depolarization as Liu and Liman (35) have shown that an initial increase in [Ca$^{2+}$]$^i$ activates TRPM5 channels which allow Na$^+$ influx, responsible for the depolarization of the cell. The TRC have been shown to express the transient receptor potential melastatin-5 channels (36). Recently, Sclafani et al. (37) have demonstrated that transient receptor potential melastatin-5 KO mice showed no preference for fat and carbohydrates, showing the implication of these channels in lipid taste signaling.

Regarding the physiological relevance of the release of these neurotransmitters, we can state that these agents might exert their actions via their receptors present on the neighboring cells. Indeed, 5-HT$_{1b}$ and 5-HT$_{3}$ serotonergic receptors are expressed in rat taste buds; the former is present on TRC, and the latter is found on sensory primary afferent fibers (38). Despite some discrepancy on the presence of NA in papillae, a expressed in rat taste buds; the former is present on TRC, and NA into the extracellular medium, and these neuromediators in turn will activate afferent termination nerve fibers, which will transmit information on the quality and intensity of taste stimuli to the brain. However, it remains to be studied as to which subtype of cells (type I, type II, or type III) the CD36-positive gustatory cells belong. Besides, more detailed studies are required to show whether CD36-positive gustatory cells can establish synaptic contacts onto nerve processes and whether these cells utilize the SNARE protein machinery syntaxin, SNAP-25, and synaptobrevin, as is used by synapses in the central nervous system for Ca$^{2+}$-dependent exocytosis. The investigations in these directions are in progress in our laboratory.

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