The fatty acid oleoylethanolamide (OEA) is a satiety factor that excites peripheral vagal sensory nerves, but the mechanism by which this occurs and the molecular targets of OEA are unclear. In this study the ability of OEA to modulate the capsaicin receptor (TRPV1) was explored. OEA alone did not activate TRPV1 expressed in Xenopus oocytes under control conditions, but produced a differential modulation of agonist-evoked responses. OEA enhanced proton-gated TRPV1 currents, inhibited anandamide-evoked currents and had no effect on capsaicin-evoked responses. Following stimulation of protein kinase C (PKC), OEA alone directly activated TRPV1 channel with an EC_{50} of ~2 μM at room temperature. This effect was due to direct phosphorylation of TRPV1 because no responses to OEA were observed with mutant channels lacking critical PKC phosphorylation sites, S502A/S800A. In sensory neurons, OEA-induced Ca^{2+} rises that were selective for capsaicin-sensitive cells, inhibited by the TRPV1 blocker, capsazepine, and occurred in a PKC-dependent manner. Further, after PKC stimulation, OEA activated TRPV1 channels in cell-free patches suggesting a direct mode of action. Thus, TRPV1 represents a potential target for OEA and may contribute to the excitatory action of OEA on sensory nerves.

The fatty acid oleoylethanolamide (OEA) is a putative, peripheral satiety factor. OEA production and release are stimulated by feeding and inhibited during fasting (1, 2). Moreover, exogenous OEA reduces food consumption in both freely feeding and starved rats. These anorexigenic actions are mediated by stimulation of vagal sensory nerves that in turn stimulate the brainstem and hypothalamus. This hypothesis is supported by the observations that brain administration of OEA is ineffective in reducing food intake, and the effects of OEA are lost by ablation of vagal nerves with neonatal capsaicin treatment (1, 2). However, the mechanism by which OEA excites vagal sensory afferents is unknown. OEA is structurally similar to anandamide (AEA) (Fig. 1A), but unlike AEA has no activity at cannabinoid receptors (3). AEA is an agonist at the capsaicin receptor (TRPV1) (4, 5), and thus, it was of interest to investigate whether OEA might also regulate TRPV1 activity. Although TRPV1 expressed in nociceptive neurons plays an important role in detecting noxious chemical and thermal stimuli (6), there is also significant expression of TRPV1 in visceral sensory neurons (7) and brain (8), suggesting a broader function for this receptor in addition to pain signaling. The results show that OEA differentially modulates agonist-evoked TRPV1 currents. Moreover, OEA directly activates TRPV1 and excites sensory neurons expressing TRPV1, both of which occur in a PKC-dependent manner. Thus, during PKC stimulation the TRPV1 channel may contribute to the sensory nerve actions of OEA.

MATERIALS AND METHODS

Tissues were harvested using protocols approved by the Georgetown University Animal Use and Care Committee. Dorsal root ganglion (ORG) neurons were obtained from P7–18 rats killed by CO_{2} narcosis/decapitation. Oocytes were harvested from adult, female Xenopus laevis anesthetized with tricaine methanesulfonate (0.1 g/l). Frogs were humanely killed following final collection of oocytes.

**Oocyte Electrophysiology—**Defolliculated oocytes were injected with 30–50 ng of rat TRPV1 cRNA. Double electrode voltage clamp was performed using a Warner amplifier (Warner Instruments, OC725C). All the experiments were performed at room temperature of ~22 °C. Oocytes were placed in a perspex chamber and continuously superfused (3–5 ml/min) with Ca^{2+}-free Ringer solution containing (in mM): 100 NaCl, 2.5 KCl, 5 HEPES, 1 Mg^{2+}, and titrated to pH 7.35 with ~5 mM NaOH. For solutions < pH 6.0, HEPES was replaced with 10 mM MES. Ca^{2+}-free conditions were used to minimize VR1 tachyphylaxis and contamination from Ca^{2+}-activated Cl currents. Electrodes were filled with 2 M KCl and had resistances of 0.5–1 MΩ. Oocytes were routinely voltage-clamped at ~60 mV. Voltage ramps consisted of 500 ms pulses from ~60 mV (holding potential) to ~60 mV. Unless otherwise indicated, leak currents measured under control conditions were subtracted from agonist-induced currents. For H^{+} experiments, the test responses were normalized to responses with pH 4 in the same oocyte.

**DRG Electrophysiology—**Single-channel patch clamp recordings were performed using an EPC8 amplifier (HEKA). The bath solution contained (in mM): 140 Na gluconate, 10 NaCl, 1 MgCl_{2}, 1 EGTA, 10 HEPES, pH 7.3; and the pipette solution contained (in mM): 140 Na gluconate, 10 NaCl, 1 MgCl_{2}, 1 EGTA, 10 HEPES, pH 7.3. The current signal was sampled at 3 kHz and digitized at 5 kHz. Currents were further filtered for display purposes. Open probability was evaluated from continuous data stretches of at least 20 s.

**Ca^{2+}-imaging—**DRG neurons were loaded with 1 μM Fluo4-AM (Molecular Probes, Eugene, OR) for 20 min. Neurons were washed for a further 10–20 min prior to recording. The dye was excited at 488 ± 15 nm, and emitted fluorescence was filtered with a 535 ± 25 nm bandpass filter, captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and read into a computer. Analysis was performed offline using Simple PCI software (Compix Inc., Cranberry, PA). Drugs were applied using pressure injection pipettes (1–3 μm diameter) positioned at a distance of ~50–100 μm from the neuron of interest. Pressure alone (~1 psi) did not elicit any Ca^{2+} responses.

**Chemicals—**Capsaicin, capsaicinamide, and phorbol 12,13-dibutyrate (PDBu) were obtained from Sigma. OEA and AEA were obtained from Tocris Cookson (Ellisville, MO). Bisindolylmaleimide 1 (BIM) was from ICN (Aurora, Ohio). Drugs were prepared as stock solutions in MeSO (BIM, 2 mM) or ethanol (10 mM for PDBu and 100 mM for all other drugs) and diluted into physiological solution prior to experiments. OEA solutions were made fresh before every experiment because we found that bioactivity of these solutions decreased markedly with time (half-life of ~30 min in standard buffer). Data are given as mean ± S.E., and statistical significance was evaluated using Student’s t test.
RESULTS

The effect of OEA on capsaicin receptors was studied by applying the fatty acid to cloned rat TRPV1 expressed in *Xenopus* oocytes. OEA alone (1–80 μM) produced no detectable currents at membrane potentials range from −60 to +60 mV (Figs. 1B and 2B). In contrast, OEA markedly enhanced TRPV1 currents activated by acidic (pH 4) solution (Fig. 1B). The potentiation of H⁺-evoked currents by OEA (10 μM) was rapidly reversed upon washout. C, summary of effects of OEA on agonist-evoked currents. OEA (20 μM) enhanced H⁺-gated currents (pH 6–4, n = 12, p < 0.01), reduced currents activated by AEA (14 μM, n = 4, p < 0.05) and had no effect on currents activated by capsaicin (100 nM, n = 5). D, dose response relationship for H⁺ activation (n = 3 for each point) with or without 20 μM OEA. OEA increased the maximal response from 1.1 ± 0.1 to 3.7 ± 0.4, without significantly changing the EC₅₀ (4.6 ± 0.03 and 4.4 ± 0.16 for control and OEA, respectively).

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**FIG. 1.** OEA modulates agonist-evoked TRPV1 currents. A, chemical structures of AEA (an endogenous cannabinoid and TRPV1 agonist) and oleoylethanolamide. B, oocytes expressing TRPV1 were voltage clamped at −60 mV. OEA (20 μM) alone evoked no current but enhanced the magnitude of currents evoked by pH 4 solution. The potentiation of H⁺-evoked current by OEA (10 μM) was rapidly reversed upon washout. C, summary of effects of OEA on agonist-evoked currents. OEA (20 μM) enhanced H⁺-gated currents (pH 6–4, n = 12, p < 0.01), reduced currents activated by AEA (14 μM, n = 4, p < 0.05) and had no effect on currents activated by capsaicin (100 nM, n = 5). D, dose response relationship for H⁺ activation (n = 3 for each point) with or without 20 μM OEA. OEA increased the maximal response from 1.1 ± 0.1 to 3.7 ± 0.4, without significantly changing the EC₅₀ (4.65 ± 0.03 and 4.45 ± 0.16 for control and OEA, respectively).

**FIG. 2.** OEA activates TRPV1 directly after PKC stimulation. A, OEA (3 and 10 μM) evoked inward currents in oocytes after treatment with the PKC activator PDBu (500 nM, 3 mins). B, current-voltage relationship for OEA evoked responses before and after PDBu treatment. Following PDBu treatment OEA activated an outwardly rectified current. Note that PDBu treatment alone partly activated TRPV1 receptors. C, the dose-response relationship for activation by OEA (after PDBu treatment). The holding potential was −60 mV. EC₅₀ was 2.0 ± 0.3 μM, and the Hill slope was 1.
with the phorbol ester, PDBu, used to stimulate PKC, OEA (10 μM) alone was capable of activating current (Fig. 2A). OEA-induced current reversed at −5 to −10 mV (the reversal potential for capsaicin-evoked current), exhibited outward rectification characteristic of TRPV1 (Fig. 2B), and could be blocked with the TRPV1 inhibitor, capsazepine (10 μM, n = 4). Further, OEA produced no current in uninjected oocytes. Thus, these results indicate that the current activated by OEA is via
TRPV1 channels. The dose response relationship for OEA following PKC stimulation shows that OEA produced half-maximal activation at $10^{-6}$ M concentrations at room temperature (Fig. 2C). This value is comparable with the EC$_{50}$ for AEA, under similar conditions (4, 5).

To test whether PKC signaled through direct phosphorylation of the TRPV1 channel or via accessory proteins, these experiments were repeated with oocytes expressing TRPV1 channels lacking the serine residues (S502A and S800A), identified as critical for PKC regulation (11). These mutant channels retained sensitivity to $10^{-6}$ M and capsaicin but were completely unresponsive to OEA after phorbol ester treatment (Fig. 3, A–C). In summary, $10^{-6}$ M OEA evoked little detectable current ($<5$ nA) at $-60$ mV in these mutant receptors ($n=8$) compared with $145 \pm 27$ nA for wild type TRPV1 ($n=9$). Thus, phosphorylation of the TRPV1 channel by PKC is a critical prerequisite for direct activation by OEA. In addition, the potentiation of $10^{-6}$ M-evoked currents by OEA was smaller in mutant compared with wild-type receptors (Fig. 3D). These data show that PKC regulates both the direct effects of OEA and the effects of OEA on agonist-induced responses.

To assess whether OEA could activate endogenous TRPV1 channels, Ca$^{2+}$ imaging of cultured adult sensory neurons was performed, using the Ca$^{2+}$ permeability of TRPV1 as a marker of channel activity. DRG neurons were loaded with the Ca$^{2+}$-sensitive dye, Fluo4, and treated sequentially with OEA (10 $\mu$M) and capsaicin (2 $\mu$M) to confirm expression of TRPV1. Fig. 4, A and B, shows the results from representative capsaicin-sensitive neurons conducted with or without PDBu pretreatment (500 nM, 3–5 min). In the absence of PDBu, OEA did not produce a Ca$^{2+}$ rise. In contrast, after treatment with PDBu, OEA produced a clear Ca$^{2+}$ elevation although somewhat smaller than a saturating capsaicin concentration. Note that normalized Ca$^{2+}$ rises in response to capsaicin were smaller after PDBu treatment because PKC activation alone evokes a small Ca$^{2+}$ rise via TRPV1 (9). In summary, responses to OEA after PDBu were observed in 28 of 38 neurons with a mean increase of $-1.6$-fold ($n=38$) compared with $-4.4$-fold for 2 $\mu$M capsaicin (Fig. 4C). No responses to OEA were seen in capsa-
Application of capsaicin to the intracellular face of cell-free recordings were made from cell-free patches of DRG neurons. via activation of TRPV1, and as with the cloned TRPV1, re-}

AEA currents. In contrast to the data reported here, Smart TRPV1 would also be consistent with the observed inhibition of These data suggest that activation by OEA occurs through a TRPV1 receptors lacking critical serines (Ser-502 and Ser-800). by OEA required phosphorylation of TRPV1 and was absent in alternative pathways for factors that activate or sensitize sensory nerves, including bradykinin and extracellular ATP, use signaling pathways that converge on PKC activation (9, 12). Protein kinase A-mediated phosphorylation provides yet another pathway for phosphorylation of TRPV1 because mutant receptors lacking critical PKC phosphorylation sites were unresponsive to OEA, and a PKC inhibitor blocked the actions of OEA in sensory neurons. PKC also enhanced the synergistic action of OEA and protons. These data confirm the important role for PKC in TRPV1 channel regulation. Many of the signaling factors that activate or sensitize sensory nerves, including bradykinin and extracellular ATP, use signaling pathways that converge on PKC activation (9, 12).

**DISCUSSION**

This study has identified the TRPV1 channel as a potential sensory nerve target of OEA. OEA regulated the activity of both cloned and endogenous TRPV1 channels. At rest, OEA did not directly gate the TRPV1 channel but instead modulated agonist-induced responses; OEA enhanced the effects of protons ~3-fold and inhibited the current activated by AEA by ~50%. Importantly, OEA alone activated TRPV1 channels following stimulation of PKC. This effect was dependent on phosphorylation of TRPV1 because mutant receptors lacking critical PKC phosphorylation sites were unresponsive to OEA, and a PKC inhibitor blocked the actions of OEA in sensory neurons. PKC also enhanced the synergistic action of OEA and protons. These data confirm the important role for PKC in TRPV1 channel regulation. Many of the signaling factors that activate or sensitize sensory nerves, including bradykinin and extracellular ATP, use signaling pathways that converge on PKC activation (9, 12).

Protein kinase A-mediated phosphorylation provides yet another pathway for PKC dependence of activation (18). Thus, it is likely that responses to OEA, especially after PKC activation, would be markedly greater at 37 °C than the responses observed here at 22 °C.

The finding that OEA regulates TRPV1 activity may be relevant to the satiety actions of this lipid. OEA is believed to excite vagal sensory afferents (1, 2), and these neurons highly express TRPV1 (7). Thus, activation of these TRPV1 channels in vagal neurons by OEA may contribute to the excitatory actions of OEA. Further experiments in vagal neurons are needed to confirm this directly. Interestingly, AEA, an endogenous cannabinoid also activates TRPV1, but in contrast to OEA produces a well described hyperphagia believed to be mediated via actions at both central (19) and peripheral cannabinoid receptors (2). OEA, in contrast, lacks cannabinoid activity and appears to act exclusively by exciting peripheral vagal sensory neurons. Indeed, OEA may counteract the hyperphagic effects of AEA (2). In this regard, it may be significant that OEA can inhibit AEA-evoked activation of TRPV1.

The PKC dependence of OEA effects on TRPV1 suggests that OEA might synergize with other signaling molecules to mediate its excitatory action. One attractive candidate is the peripheral satiety factor, cholecystokinin (CCK). CCK like OEA is released during feeding and signals via type A CCK receptors located on vagal nerves (20, 21). This leads to activation of the phospholipase C-PKC pathway. Thus, activation of PKC during CCK signaling could act synergistically with OEA to excite vagal neurons.

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