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Opioid Receptor Function Is Regulated by Post-endocytic Peptide Processing*

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Background: Endothelin-converting enzyme-2 (ECE2) localizes to early endosomes and processes neuropeptides at non-classical sites at acidic pH.
Results: Inhibiting ECE2 activity impairs recycling and resensitization of δ opioid receptors.
Conclusion: ECE2 regulates δ opioid receptor function by endocytic processing of opioid peptide substrates.
Significance: Understanding the involvement of ECE2 in opioid receptor function could open novel avenues for developing pharmacotherapeutics to treat pain.

Most neuroendocrine peptides are generated in the secretory compartment by proteolysis of the precursors at classical cleavage sites consisting of basic residues by well studied endopeptidases belonging to the subtilisin superfamily. In contrast, a subset of bioactive peptides is generated by processing at non-classical cleavage sites that do not contain basic residues. Neither the peptidases responsible for non-classical cleavages nor the compartment involved in such processing has been well established. Members of the endothelin-converting enzyme (ECE) family are considered good candidate enzymes because they exhibit functional properties that are consistent with such a role. In this study we have explored a role for ECE2 in endocytic processing of δ opioid peptides and its effect on modulating δ opioid receptor function by using selective inhibitors of ECE2 that we had identified previously by homology modeling and virtual screening of a library of small molecules. We found that agonist treatment led to intracellular co-localization of ECE2 with δ opioid receptors. Furthermore, selective inhibitors of ECE2 and reagents that increase the pH of the acidic compartment impaired receptor recycling by protecting the endocytosed peptide from degradation. This, in turn, led to a substantial decrease in surface receptor signaling. Finally, we showed that treatment of primary neurons with the ECE2 inhibitor during recycling led to increased intracellular co-localization of the receptors and ECE2, which in turn led to decreased receptor recycling and signaling by the surface receptors. Together, these results support a role for differential modulation of opioid receptor signaling by post-endocytic processing of peptide agonists by ECE2.

Most neuroendocrine peptides are synthesized from precursor proteins; post-translational processing of these precursors is a key step in the production of biologically active peptides. The generation of active peptides from inactive precursors requires limited processing, and in most cases this processing occurs at classical cleavage sites consisting of dibasic residues (1). In general, members of the family of prohormone convertases, belonging to the subtilisin superfamily, endoproteolytically cleave at the dibasic sites, and the C-terminally extended basic residues are then removed by carboxypeptidase E (1–4). The resulting peptides may undergo additional modifications required for biological activity (1, 5).

A subset of neuropeptides generated from non-classical cleavages has been identified using mass spectrometry (1). In the case of peptides that are processed at aliphatic/aromatic residues, members of the endothelin-converting enzyme (ECE)[4] family belonging to the neprilysin superfamily have been implicated in the processing of bioactive peptides at these sites (6). However, the majority of these proteases exhibit near neutral pH optima and cellular and subcellular localizations that are not consistent with a role for these enzymes in neuroendocrine peptide processing (6). ECE2 was discovered as a novel member of the endothelin-converting enzyme-1 (ECE1) gene family (7); the two gene products share only 59% amino acid identity (8). Interestingly, unlike ECE1, ECE2 is optimally active at acidic pH (9), suggesting that it could function in an acidic intracellular compartment such as endosomes. Consistent with this finding, ECE2 has been localized to early and late endosomes (10). The acidic pH optimum and subcellular localization make ECE2 an ideal candidate for the intracellular processing of neuroendocrine peptides including opioid peptides (11–14).

To directly test the ability of ECE2 to process peptides at non-classical sites, we used recombinant purified ECE2 and examined its ability to process a number of opioid peptides as well as other neuropeptides at acidic or neutral pH (13). We found that ECE2 exhibits activity only at acidic pH and is able to process opioid peptides selectively at specific sites (13, 14).

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[4] The abbreviations used are: ECE, endothelin-converting enzyme; CSID, ChemSpider identification number; Delt II, deltorphin II; IBMX, 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione; δOR, δ opioid receptor; ANOVA, analysis of variance.

However, the evaluation of the role of ECE2 in biological systems would be facilitated by reagents that specifically block the enzyme activity, leading us to undertake studies to identify a specific ECE2 inhibitor by virtual docking of a small molecule library to a homology model of ECE2 generated based on the crystal structure of neprilysin (12). The ability of the small molecules identified from this screen to block the activity of neprylysin or ECE2 was tested using a quenched fluorescent substrate (12). This led to the identification of S136492 as a putative ECE2-selective inhibitor (12). This inhibitor was found to block the enzymatic activity of ECE2 but not of neprilysin (12). Furthermore, this inhibitor did not inhibit the activity of ECE1 at either pH 7.4 or 5.5. In this study using S136492 and the δ opioid receptor (δOR) as a model, we directly examined a role for ECE2 in the processing of opioid peptide agonists in the recycling compartment and its implications for δOR function. We found that inhibition of ECE2 activity, but not the activity of other metallopeptidases, impairs receptor recycling as well as peptide agonist degradation. An acidic intracellular compartment is required for both of these processes. This is seen in heterologous cells and in primary neurons. We also found that ECE2 activity plays a role in receptor resensitization, as inhibition of ECE2 activity leads to decreases in signaling mediated by deltorphin II (Delt II; a highly selective peptide agonist) or BAM22 (an endogenous peptide agonist). Together these results suggest that ECE2 is involved in modulating the function of the δOR system by processing endogenous opioid peptides internalized with the receptors.

EXPERIMENTAL PROCEDURES

Materials—Neuro2A and CHO cells were from ATCC. DMEM, F12 medium, penicillin-streptomycin, anti-mouse Alexa-594 and anti-rabbit Alexa-488-conjugated secondary antibodies, Lipofectamine, and 4,6-diamidino-2-phenylindole (DAPI) were from Invitrogen. S136492 (CSID 22902217), SM19712 (PubChem ID 71312044), IBMX, and anti-FLAG M1 mouse monoclonal antibody were from Sigma-Aldrich. Anti-*myc* and anti-HA antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. SNC80, Delt II, cycloheximide, chloroquine, captopril, and BAM22 were from Tocris Bioscience. MS0022129 (22129, ChemBridge catalog No. 5871159, CSID 697993), MS0021474 (21474, ChemBridge catalog No. 5719593, CSID 15358401), 6634449 (CSID 22200660), and 6636797 (CSID 4664999) were from ChemBridge. The HitHunter cAMP HS chemiluminescence detection kit was from DiscoveRx.

Cell Culture and Transfection—CHO cells stably expressing N-terminally FLAG epitope-tagged δOR (F6 cells) were grown in F12 medium containing 10% FBS, streptomycin-penicillin, and 500 μg/ml Geneticin (G418). Neuro2A cells stably expressing N-terminally *myc* epitope-tagged δOR (N2A-δOR) were grown in DMEM containing 10% FBS, streptomycin-penicillin, and 500 μg/ml Geneticin (G418). F6 or N2A-δOR cells were transfected with human HA epitope-tagged ECE2 using Lipofectamine as per the manufacturer's protocol, and colonies with stable expression (F6-ECE2 or N2A-δOR-ECE2 cells) were selected in medium containing 500 μg/ml Geneticin and 250 μg/ml hygromycin B.

Primary Cortical Neurons—Primary cortical neurons were generated from E18 Sprague-Dawley rat pups as described (15).

Enzyme Activity Assays—Recombinant ECE2 (32.5 ng) with a specific activity of 12 pmol/min/μg protein was generated as described previously (12). Secreted soluble recombinant ECE1 (30 ng) with a specific activity of 750 pmol/min/μg protein was generated and purified using a protocol similar to that used for ECE2 (12). Solubilized midbrain membranes (10 μg) from wild-type or ECE2 knock-out mice were prepared as described (16). Enzymatic activity, in the absence or presence of the ECE2 inhibitor S136492 or the ECE1 inhibitor SM19712, was assayed using the synthetic quenched fluorescent substrate McaBk2 (10 μM) at 37 °C with either 0.2 M sodium acetate buffer, pH 5.5, or 50 mM Tris-Cl buffer, pH 7.4, as described previously (12, 16).

Receptor Recycling—Recycling experiments were carried out as described previously (17). Briefly, F6, F6-ECE2, N2A-δOR, and N2A-δOR-ECE2 cells or primary cortical neurons (2×10^5 cells) were seeded into each well of a 24-well polylysine-coated plate. The following day cells were treated either with 100 nM or 1 μM Delt II, SNC80, or leucine-enkephalin or with 100 nM BAM22 for 5, 10, or 30 min to facilitate receptor internalization. The cells were washed to remove the agonist and incubated with medium without the agonist for 5–60 min to facilitate receptor recycling. At the end of the incubation period, cells were chilled to 4 °C and then fixed briefly (3 min) with 4% paraformaldehyde followed by three washes (5 min each) with PBS. Cell surface receptors were determined by ELISA as described below. To calculate percent recycled receptors, the surface level of receptors prior to agonist-mediated internalization (*i.e.* total cell surface receptors) was taken as 100%. Then the percent surface level of receptors following agonist-mediated internalization (taken as $t = 0$) was subtracted from all the time points to obtain the percent recycled receptors. We verified that the cell fixation conditions did not lead to significant cell permeabilization of the primary antibodies by comparing the data from unfixed cells in suspension (to minimize cell loss) (18) with cells fixed at 4 °C for 3 min with 4% paraformaldehyde ("fixed cells," used in most of the studies described herein) or 4% paraformaldehyde containing 0.3% Triton X-100 (to allow permeabilization and detection of intracellular receptors). We found no significant difference in the detection of surface receptors between unfixed and fixed cells. Under conditions of receptor internalization (treatment for 30 min with 1 μM Delt II) 42 ± 2% of the surface receptors were detected in unfixed cells and 39 ± 1% in cells fixed with 4% paraformaldehyde, whereas 66 ± 1% of receptors were detected in cells fixed and permeabilized with 0.3% Triton X-100. Under the conditions of receptor recycling (removal of the drug and recovery in media for 1 h), 72 ± 4% of the surface receptors were detected in unfixed cells and 74 ± 3% in cells fixed with 4% paraformaldehyde, whereas 93 ± 1% of the receptors were detected in cells fixed and permeabilized with 0.3% Triton X-100.

Single Concentration of Different Inhibitors—Our justification for using a single concentration of different inhibitors in this study is as follows. (i) A 20 μM concentration of the ECE2 inhibitor S136492 blocked 90 ± 4% of ECE2 activity but had no effect on the activity of the closely related ECE1 enzyme (Fig. 1); (ii) a 20 μM concentration of ECE2 inhibitors 22129, 21474,

6634449, and 6636797 was used to compare their effects with that of S136492; (iii) 10 μM phosphoramidon fully inhibited the EC3.4.24.11 family (including neprylisin, ECE, and endopeptidase 24.11, *i.e.* enkephalinase) (12, 19, 20); (iv) 10 μM captopril completely inhibited angiotensin-converting enzyme activity (21); (v) 10 μM thiorphan fully inhibited neprilysin but not endothelin-converting enzyme activity (19, 22); (vi) 10 μM bestatin completely inhibited leucine aminopeptidase activity (23); (vii) 10 μM E64 completely inhibited cysteine protease activity (24); (viii) 10 μM leupeptin fully inhibited serine/cysteine protease activity (as recommended by the manufacturer; Sigma Aldrich); (ix) 10 μM aprotinin fully inhibited serine protease activity (25) (as recommended by the manufacturer, Sigma Aldrich); (x) 100 μM chloroquine increased the intracellular pH at this concentration (26, 27); (xi) 100 nM cycloheximide completely inhibited protein synthesis (28); and (xii) 100 nM bafilomycin, a vacuolar type H^+-ATPase inhibitor, prevented endosomal acidification (28). The relative decrease in cell surface receptors (% inhibition over control) after 60 min of recycling with different inhibitors was calculated by taking the corresponding values in the absence of the inhibitor as 100%.

Enzyme-linked Immunosorbent Assay—ELISA was carried out as described previously (18, 29, 30) to determine cell surface receptor levels following recycling experiments. Briefly, following a brief fixation with paraformaldehyde (as described above) cells were incubated for 1 h at 4 °C with PBS containing 3% BSA followed by a 16-h incubation at 4 °C with a 1:1000 dilution (in PBS containing 1% BSA) of anti-FLAG M1 mouse monoclonal antibody (Sigma-Aldrich) to detect FLAG epitope-tagged δOR, a 1:1000 dilution of anti-*myc* mouse monoclonal antibody (Santa Cruz Biotechnology) to detect *myc*-epitope-tagged δOR, or a 1:500 dilution of rat δOR antibody to detect endogenous δOR. The rat δOR antibody was generated as described previously (29) and was found to be selective, as it showed no specific signal in ELISA, Western blot, or immunocytochemistry assays using membranes from the brains of mice lacking δOR (31). Cells were then washed three times (5 min each wash) with PBS containing 1% BSA and incubated at room temperature with a 1:1000 dilution (in PBS containing 1% BSA) of anti-mouse or anti-rat IgG coupled to horseradish peroxidase (Vector Laboratories). The wells were washed three times with 1% BSA in PBS (5 min, each wash), and color was developed by the addition of the substrate, *o*-phenylenediamine (5 mg/10 ml in 0.15 M citrate buffer, pH 5, containing 15 μl of H_2O_2). Absorbance at 490 nm at different time points was measured with a Bio-Rad ELISA reader.

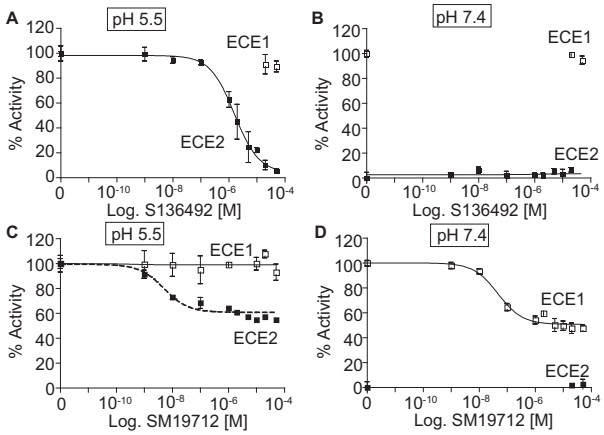
Receptor Endocytosis—In studies investigating the effect of the ECE2 inhibitor on δOR endocytosis, N2A-δOR cells (2×10^5 cells) were seeded into each well of a 24-well polylysine-coated plate. The following day the plate was kept on ice, and cells were incubated at 4 °C for 1 h with a 1:1000 dilution of anti-*myc* antibodies in media to label cell surface δOR. Cells were washed three times and then treated with 100 nM BAM22 for up to 60 min at 37 °C without or with 20 μM S136492. At the end of the incubation period, cells were briefly fixed (3 min) with 4% paraformaldehyde followed by three washes (5 min each) with PBS. Receptors present at the cell surface were determined using a 1:1000 dilution (in PBS containing 1% BSA) of anti-mouse IgG coupled to horseradish peroxidase (Vector Laboratories) followed by color development as described above for ELISA.

Degradation of [^{3}H]Delt II by ECE2—In one set of experiments, purified ECE2 (32.5 ng) was incubated for 30 min at 37 °C with 10 nM [^{3}H]Delt II in 0.2 M sodium acetate buffer, pH 5.5, without or with 20 μM S136492, and the processing of [^{3}H]Delt II was monitored by fractionation on thin-layer chromatography using *n*-butanol:acetic acid:water (3:1:1 by volume). ~3-mm fractions were cut, and the radioactivity in each fraction was measured using a scintillation counter.

In another set of experiments, the processing of [^{3}H]Delt II in F6-ECE2 cells was examined. 2×10^5 cells/well were seeded into a 24-well polylysine-coated plate. On the following day the cells were incubated with 10 nM [^{3}H]Delt II for 30 min at 37 °C without or with either 20 μM S136492 or 100 μM chloroquine. The cells were chilled to 4 °C, and the total radioactivity (representing surface plus intracellular radiolabeled ligand), radioactivity in an acid wash (representing surface radiolabeled ligand), and radioactivity remaining in the cells (representing intracellular radiolabeled ligand) were determined as described previously (17). Briefly, cells were washed three times in ice-cold 50 mM Tris-Cl, pH 7.5, and the total bound radioactivity was measured by scintillation counting. A parallel set of wells was then subjected to two washes in ice-cold 0.2 M sodium acetate buffer, pH 4.8, containing 500 mM sodium chloride (acid wash), and the radioactivity in the acid wash and in the cells was measured by scintillation counting. A parallel set of acid-washed cells was lysed and subjected to thin-layer chromatography as described above.

Immunocytochemistry and Confocal Microscopy—Immunocytochemical staining and confocal microscopy were carried out as described previously with some modifications (32). Briefly, F6-ECE2 or primary cortical neurons were first plated on polylysine-coated coverslips in 12-well dishes (Corning Inc., Corning, NY). Cells were treated with either serum-free medium containing vehicle or 1 μM Delt II for 30 min at 37 °C prior to fixation. Cells were fixed using 4% paraformaldehyde in PBS. Following fixation, cells were rinsed repeatedly with PBS and then permeabilized with 0.1% Triton X-100 in PBS and incubated with 4% BSA in PBS for 1 h. Following this initial blocking, cells were incubated with primary antibodies directed against FLAG (Sigma-Aldrich) or HA epitopes (Santa Cruz Biotechnology) (1: 500 in 4% BSA in PBS), endogenous δOR (1:1000 in 4% BSA in PBS), or ECE2 (1:500 in 4% BSA in PBS) for 1 h. Cells were then rinsed repeatedly with PBS and incubated with anti-mouse Alexa-594- or anti-rabbit Alexa-488-conjugated secondary antibodies (1:1000 in 4% BSA in PBS) (Invitrogen) for 1 h. Cells were rinsed thoroughly with PBS and fixed to microscope slides using ProLong Gold anti-fade reagent containing DAPI (Invitrogen). Imaging was carried out using a Leica TCS SP5 DM confocal fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany), and image analysis was performed using ImageJ software.

Determination of cAMP Levels—cAMP levels were determined as described previously (33–35) with minor modifications. Briefly, F6-ECE2, N2A-δOR, or N2A-δOR-ECE2 cells or



E

Inhibitor	IC_{50} (μM)			
	ECE2		ECE1	
	pH 5.5	pH 7.4	pH 5.5	pH 7.4
S136492	1.6 ± 0.1	n.a.	>100	>100
SM19712	0.005 ± 0.001	n.a.	>100	1.1 ± 0.2

FIGURE 1. **Dose-response studies showing the selectivity of the inhibitor S136492 for ECE2.** *A* and *B*, the activity of purified ECE1 or ECE2 was assayed in the absence or presence of S136492 (0–50 μM) at pH 5.5 (*A*) or pH 7.4 (*B*) using the synthetic quenched fluorescent substrate McaBk2 as described under "Experimental Procedures." *C* and *D*, the activity of purified ECE1 or ECE2 was assayed in the absence or presence of SM19712 (0–50 μM) at pH 5.5 (*C*) or pH 7.4 (*D*) using the synthetic quenched fluorescent substrate McaBk2 as described under "Experimental Procedures." *E*, IC_{50} for inhibition of purified ECE1 or ECE2 activity by S136492 and SM19712 (*A*–*D*). Enzyme activity in the absence of the inhibitor is taken as 100%. Data represent mean ± S.E., $n = 3$ independent experiments in triplicate.

primary cortical neurons (2×10^5 cells) were seeded into each well of a 24-well polylysine-coated plate. Cells were incubated with 1 μM Delt II or 100 nM BAM22 for 30 min at 37 °C in the absence or presence of 10 μM forskolin and 100 μM IBMX. The cells were washed and incubated without or with 20 μM ECE2 inhibitor (S136492), 100 μM chloroquine, 10 μM captopril, or 100 nM cycloheximide for 60 min, and the levels of cAMP in response to a second pulse (5-min treatment) of Delt II or BAM22 were determined using the HitHunter cAMP HS chemiluminescence detection kit from DiscoveRx (36). This kit measures cAMP in cells using an enzyme fragment complementation technology in which cAMP from cells and cAMP labeled with a β-galactosidase fragment compete for binding to an antibody to cAMP. The unbound labeled cAMP binds to a complementary β-galactosidase fragment to form the active enzyme; its activity can be measured following the addition of a chemiluminescent substrate. Thus the amount of signal obtained is directly proportional to the amount of cAMP present in the cells.

For experiments examining receptor desensitization, cAMP was measured in cells incubated with either 1 μM Delt II or 100 nM BAM22 along with 10 μM forskolin and 100 μM IBMX in the absence or presence of 20 μM ECE2 inhibitors, 100 μM chloroquine, or 100 nM cycloheximide for 5 to 120 min at 37 °C.

Statistical Analysis—GraphPad Prism 4.0 was used for data analysis, and statistical significance was determined by *t* test or one-way ANOVA.

RESULTS

Characterization of S136492 as an ECE2-selective Inhibitor—In a previous study we identified S136492 as a putative ECE2-selective inhibitor using a combination of homology modeling, *in silico* screening of a small molecule library, and enzymatic analysis of the predicted hits (12). This inhibitor was found to block the activity of ECE2 but not of neprilysin (12). To examine the selectivity of S136492 for ECE2, we tested whether it could also block the activity of ECE1, an enzyme that exhibits ~59% homology to ECE2. To this end, we carried out enzymatic assays at pH 5.5 and pH 7.4 using purified enzymes and the synthetic quenched fluorescent substrate McaBk2 in the absence or presence of S136492 (0–50 μM). We found that purified ECE2 exhibited activity at pH 5.5 (Fig. 1, *A* and *B*); no activity was detectable at pH 7.4, and the activity at pH 5.5 was dose-dependently inhibited by S136492 (IC_{50} 1.6 ± 0.1 μM) (Fig. 1*E*). Moreover, S136492 did not block the activity of ECE1 at either pH 5.5 and 7.4, even at 50 μM concentration (Fig. 1, *A* and *B*). Therefore we selected a 20 μM concentration that was found to block 90 ± 4% of ECE2 activity and had no effect on ECE1 activity for the rest of our studies (Fig. 1). We also examined the selectivity of SM19712, which has been used as an ECE1 inhibitor (28, 37–43). We found that SM19712 partially blocked the activity of ECE2 at pH 5.5 (IC_{50} 5.1 ± 0.2 nM) (Fig. 1, *C*–*E*). Together, these results suggest that S136492 is selective for ECE2. We confirmed the selectivity of S136492 for ECE2 using tissues from wild-type and ECE2 knock-out mice. We found that only the enzyme activity at pH 5.5, and not at 7.4, in solubilized midbrain preparations from wild-type mice was completely inhibited by 20 μM S136492 (Fig. 2). This inhibitor had no effect on the enzymatic activity at either pH level in ECE2 knock-out midbrain membranes (Fig. 2), although we did detect enzyme activity a low pH in these mice, suggesting a compensatory up-regulation of related enzymes (albeit not sensitive to S136492) in the absence of ECE2. Taken together, these results indicate that S136492 is an ECE2-selective inhibitor.

Co-localization of ECE2 and δ Opioid Receptors—In a previous study we showed that purified ECE2 selectively processes some opioid peptides (13) and exhibits intracellular localization (14) and optimal activity at acidic pH (9, 13). This led us to postulate that ECE2 may play a role in opioid peptide processing in an endocytic compartment. This would require intracellular co-localization of ECE2 with opioid receptors. Therefore, we used cells expressing FLAG-tagged δOR and HA-tagged ECE2 (F6-ECE2 cells) to examine whether ECE2 and δOR co-localize in intracellular compartments. We detected the presence of δOR and ECE2 both at the cell surface and in intracel-

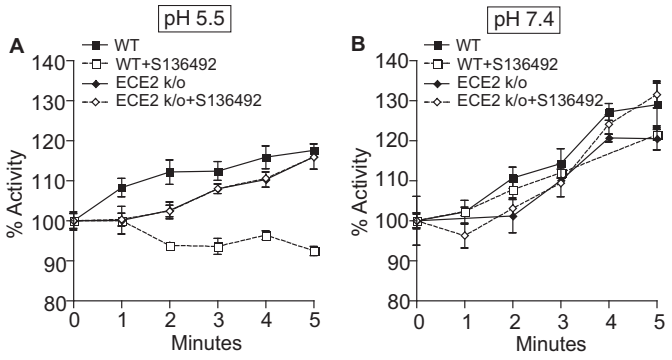


FIGURE 2. **The inhibitor S136492 blocks ECE2 activity in membranes from wild-type but not ECE2 knock-out mice.** The activity of solubilized midbrain preparations from WT and ECE2 knock-out (*ECE2 k/o*) mice was assayed using the synthetic quenched fluorescent substrate McaBk2 in the absence or presence of S136492 (20 μM) at pH 5.5 (*A*) or pH 7.4 (*B*) as described under "Experimental Procedures." The relative fluorescence units at time 0 are taken as 100% activity. Data represent mean ± S.E. from three animals/group in triplicate.

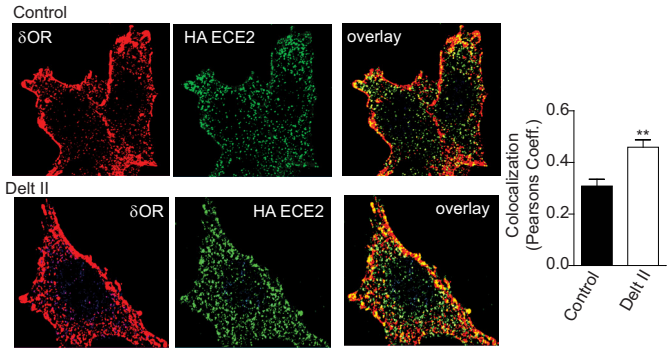


FIGURE 3. **Co-localization of ECE2 and δ opioid receptors.** Cells expressing FLAG epitope-tagged δOR and HA-epitope tagged ECE2 were treated with vehicle or 1 μM Delt II for 30 min, and the level of co-localization was examined by staining cells with antibodies to the epitope tags as described under "Experimental Procedures." Pearson's coefficient analysis shows a significant increase in co-localization of δOR and ECE2 when cells are treated with 1 μM Delt II for 30 min *versus* untreated cells. Each cohort represents $n = 10$ fields (10–15 cells/field); **, $p < 0.01$; *t* test.

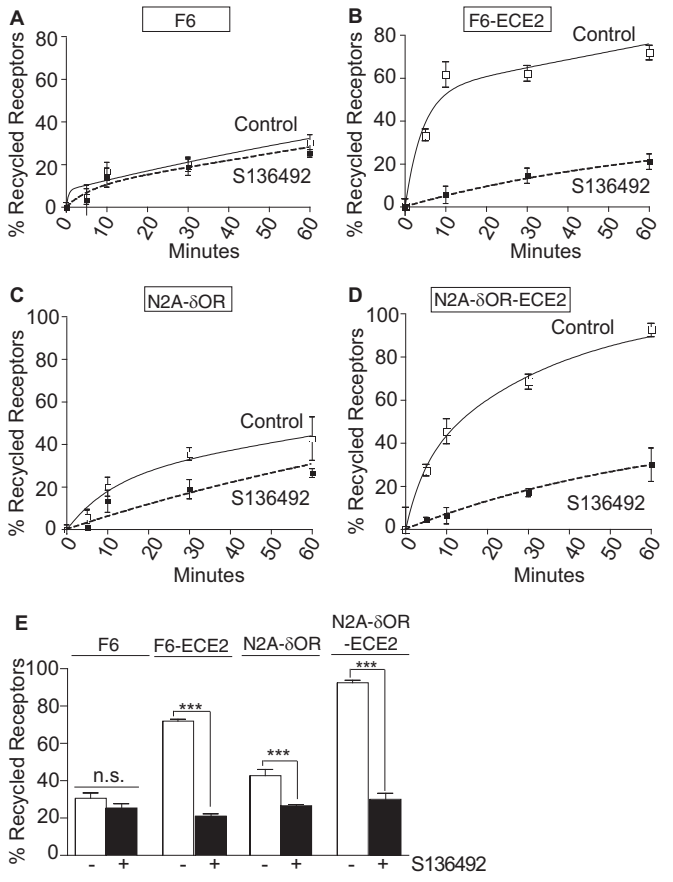


FIGURE 4. **ECE2 activity regulates δ opioid receptor recycling.** F6 (*A* and *E*), F6-ECE2 (*B* and *E*), N2A-δOR (*C* and *E*), or N2A-δOR-ECE2 (*D* and *E*) cells were treated with 1 μM Delt II for 30 min to induce δOR internalization ($t = 0$). The cells were washed and incubated without (*Control*) or with 20 μM ECE2 inhibitor (S136492) for the indicated time periods, and ELISA with antibodies to the epitope tag on the receptor was used to monitor the reappearance of cell surface receptors as described under "Experimental Procedures." The percent recycled receptors was calculated by subtracting the percentage of surface receptors at $t = 0$ (30 min internalization) from all time points. *E*, represents % δOR recycled at 60 min in the absence or presence of S136492. The data represent mean ± S.E. of 5–6 independent experiments in triplicate. ***, $p < 0.001$; *n.s.*, not significant; *t* test.

lular compartments using antibodies to the epitope tags and fluorescently labeled secondary antibodies (Fig. 3). Treatment with the δOR agonist Delt II (1 μM) increased the extent of co-localization of ECE2 and δOR in intracellular compartments (Fig. 3). These results suggest that the receptor-ligand complex can localize to ECE2-containing early endosomes, which may play a role in receptor trafficking.

ECE2 Inhibitor Blocks the Recycling of δ opioid Receptors—To examine the involvement of ECE2 in modulating δOR recycling, we expressed ECE2 in CHO cells (with no detectable ECE activity (9)) and examined the effect of ECE2 expression on the rate and extent of recycling. Receptor recycling was examined by treating cells with the selective peptidic δOR agonist Delt II to induce receptor internalization, removing the agonist to facilitate recycling, and monitoring the reappearance of cell surface δOR by ELISA using antibodies to the epitope tag on the receptor. We found that the presence of ECE2 substantially increased the rate and extent of δOR recycling compared with cells lacking ECE2 (Fig. 4, *A*, *B*, and *E*). Next we examined the effect of ECE2 inhibition on recycling by incubating cells with 20 μM S136492 during the recycling phase. We found that the ECE2 inhibitor S136492 prevented δOR recycling (Fig. 4, *A*, *B*, and *E*, and Table 1). It is interesting to note that the extent of recycling in the presence of the inhibitor is similar to that seen in cells not expressing ECE-2. Additionally, S136492 inhibited δOR recycling irrespective of the level of receptor endocytosis produced by various periods of agonist treatment (5, 10, or 30 min) (Table 1). Together, these results suggest a critical role for ECE2 in regulating δOR recycling.

Next we examined a role for endogenous ECE2 in δOR recycling using Neuro2A neuroblastoma cells; these cells have been used previously to study opioid receptor activation events (44). N2A cells contain a small but detectable level of ECE2. We also generated cell lines that express higher levels of ECE2 (named N2A-δOR-ECE2) to examine the effect of increased enzyme levels on the extent of recycling. We found that increasing the levels of ECE2 led to an increase in the extent of δOR recycling (Fig. 4, *C–E*). Moreover, both in N2A-δOR and N2A-δOR-ECE2 cells, the ECE2 inhibitor S136492 substantially inhibits δOR recycling (Fig. 4 and Table 1); the extent of inhibition in N2A-δOR-ECE2 cells was higher than seen in N2A-δOR cells

TABLE 1

Inhibition of deltorphin II-mediated recycling by S136492, chloroquine, and cycloheximide

F6-ECE2, N2A-δOR, or N2A-δOR-ECE2 cells (2×10^5 cells/well) seeded into each well of a 24-well polylysine-coated plate were treated with 1 μM Delt II for the indicated time periods (to induce δOR internalization), washed, and incubated without (control) or with 20 μM S136492, 100 μM chloroquine, or 100 nM cycloheximide for 60 min; cell surface receptors were monitored by ELISA using antibodies to the epitope tags on the receptor as described under "Experimental Procedures." The data represent mean ± S.E. of 3 independent experiments in triplicate. The relative decrease of cell surface receptors (% inhibition over control) after 60 min of recycling with the inhibitors was calculated by taking corresponding values in the absence of the inhibitor as 100%. **, $p < 0.01$, one-way ANOVA.

Cell line	Delt II-mediated endocytosis	Inhibitor	% Inhibition of recycling over control
	min		
F6-ECE2	5	S136492	46 ± 2**
	10	S136492	36 ± 1**
	30	S136492	71 ± 1**
	30	Chloroquine	62 ± 2**
	30	Cycloheximide	<1
N2A-δOR	30	S136492	38 ± 8**
	30	Chloroquine	61 ± 3**
	30	Cycloheximide	<1
N2A-δOR-ECE2	30	S136492	68 ± 4**
	30	Chloroquine	78 ± 2**
	30	Cycloheximide	<1

TABLE 2

Inhibition of deltorphin II-mediated recycling by different endopeptidase inhibitors

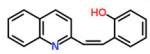
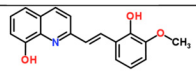
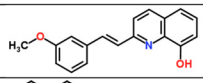
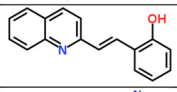
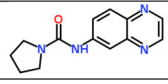
F6-ECE2 cells (2×10^5 cells/well) seeded into each well of a 24-well polylysine-coated plate were treated with 1 μM Delt II for 30 min (to induce δOR internalization), washed, and incubated without or with either 20 μM ECE2 inhibitors (S136492) or 10 μM phosphoramidon, thiorphan, captopril, bestatin, E64, leupeptin, or aprotinin for 60 min; cell surface receptors were monitored by ELISA using antibodies to the epitope tag on the receptor as described under "Experimental Procedures." The data represent mean ± S.E. of 3 independent experiments in triplicate. The relative decrease of cell surface receptors (% inhibition over control) after 60 min of recycling with the inhibitors was calculated by taking corresponding values in the absence of the inhibitor as 100%. **, $p < 0.01$, one-way ANOVA.

Inhibitor	Specificity	% Inhibition over control
S136492	Endothelin-converting enzyme-2	71 ± 1**
Phosphoramidon	EC 3.4.24.11 family	47 ± 1**
Thiorphan	Neprilysin	4 ± 2
Captopril	Angiotensin-converting enzyme	<1
Bestatin	Leucine aminopeptidase	<1
E64	Cysteine protease	22 ± 2**
Leupeptin	Serine/cysteine protease	<1
Aprotinin	Serine protease	<1

TABLE 3

Inhibition of deltorphin II-mediated recycling by different ECE2 inhibitors

F6-ECE2 cells (2×10^5 cells/well) seeded into each well of a 24-well polylysine-coated plate were treated with 1 μM Delt II for 30 min (to induce δOR internalization), washed, and incubated without (control) or with 20 μM ECE2 inhibitors S136492, 6634449, 6636797, 21474, or 22129 for 60 min; cell surface receptors were monitored by ELISA using antibodies to the epitope tag on the receptor as described under "Experimental Procedures." The data represent mean ± S.E. of 3 independent experiments in triplicate. The relative decrease of cell surface receptors (% inhibition over control) after 60 min of recycling with the inhibitors was calculated by taking corresponding values in the absence of the inhibitor as 100%. *, values taken from Ref. 12; **, $p < 0.01$ inhibitor *vs.* absence of inhibitor, one-way ANOVA.

Inhibitor	Structure	IC_{50} (μM)*	% inhibition over control
S136492		4.63	71 ± 1**
6634449		32.9	60 ± 1**
6636797		35.7	67 ± 1**
21474		6.42	66 ± 1**
22129		5.83	27 ± 1**

(Fig. 4). The fact that cells with higher level of ECE2 exhibited higher recycling and that the selective inhibition blocked this process is consistent with a critical role for ECE2 in regulating δOR recycling.

Next, we examined whether inhibitors of other peptidases known to process opioid peptides *in vitro* (45) would also influence the recycling of δOR. For these studies we used compounds that are reported to be classic inhibitors of specific peptidases and selected an optimal, single concentration (10 μM) based on published reports (12, 19–24) (See "Experimental Procedures" for detailed justification). The inhibitors used included phosphoramidon (an EC3.4.24.11 family inhibitor including inhibition of neprylisin, ECE1, and ECE2) (12, 19, 20); thiorphan, an inhibitor of neprilysin but not of ECE activity (19, 22); captopril, an angiotensin-converting enzyme inhibitor (21); bestatin, a leucine aminopeptidase inhibitor (23); E64, a cysteine protease inhibitor (24); leupeptin, a serine/cysteine protease inhibitor; and aprotinin, a serine protease inhibitor (25). We found that only the inhibitors of ECE2 (S136492) or the ECE family (phosphoramidon), but not inhibitors of other peptidases, substantially inhibited receptor recycling (Table 2). To further examine ECE2 involvement in δOR recycling, we examined a panel of ECE2 inhibitors that were identified previously by high throughput screening (12) and found that all of the inhibitors substantially blocked δOR recycling (Table 3). Among these, S136492 had the highest potency for inhibiting ECE2 enzymatic activity (12), further justifying its use for all other studies.

Changes in Intracellular pH Modulate δ opioid Receptor Recycling—Because ECE2 exhibits optimum activity at acidic pH, a condition found in endocytic vesicles, we examined the requirement for an acidic environment for δOR recycling. For this we carried out the recycling studies in the presence of chloroquine, an agent that increases the pH of intracellular organelles (26). We used chloroquine at a concentration of 100 μM based on previous reports (27, 46). As a control, and to rule out the involvement of newly synthesized proteins in modulation of δOR recycling, a protein synthesis inhibitor, cycloheximide, was used at a concentration of 100 nM, which has been reported to block protein synthesis (28). As expected, we found that in cells expressing ECE2 (F6-ECE2, N2A-δOR, and N2A-δOR-ECE2) receptor recycling was significantly and substantially inhibited by chloroquine (Fig. 5 and Table 1). Interestingly, the extent of inhibition of δOR recycling by chloroquine was similar to that of the ECE2 inhibitor (Fig. 5 and Table 1). In addition, we found that the protein synthesis inhibitor cycloheximide had no effect on either the rate or extent of recycling (Fig. 5 and Table 1), suggesting that newly synthesized proteins do not contribute to the increase in cell surface receptors seen during

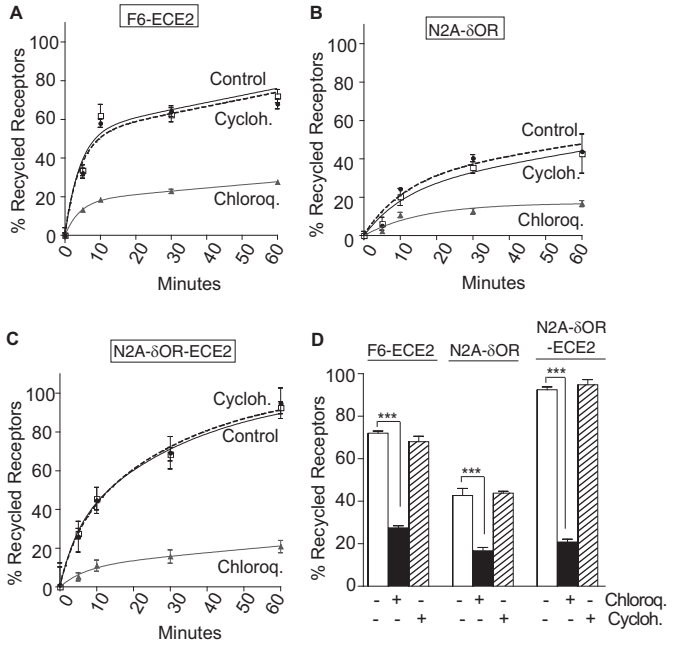


FIGURE 5. **An acidic compartment is necessary for δ opioid receptor recycling.** F6-ECE2 (*A* and *D*), N2A-δOR (*B* and *D*), or N2A-δOR-ECE2 (*C* and *D*) cells were incubated with 1 μM Delt II for 30 min to induce δOR internalization ($t = 0$). The cells were washed and incubated without (*Control*) or with either 100 μM chloroquine (*chloroq*.) or 100 nM cycloheximide (*cycloh*.), and the time-dependent increase in the level of cell surface receptors was monitored by ELISA using antibodies to the epitope tags on the receptor as described under "Experimental Procedures". The percent recycled receptors was calculated by subtracting the percentage of surface receptors at $t = 0$ (30 min internalization) from all time points. *D*, represents % δOR recycled at 60 min in the absence or presence of either chloroquine or cycloheximide. The data represent mean ± S.E. of 3–4 independent experiments in triplicate. ***, $p < 0.001$; *t* test.

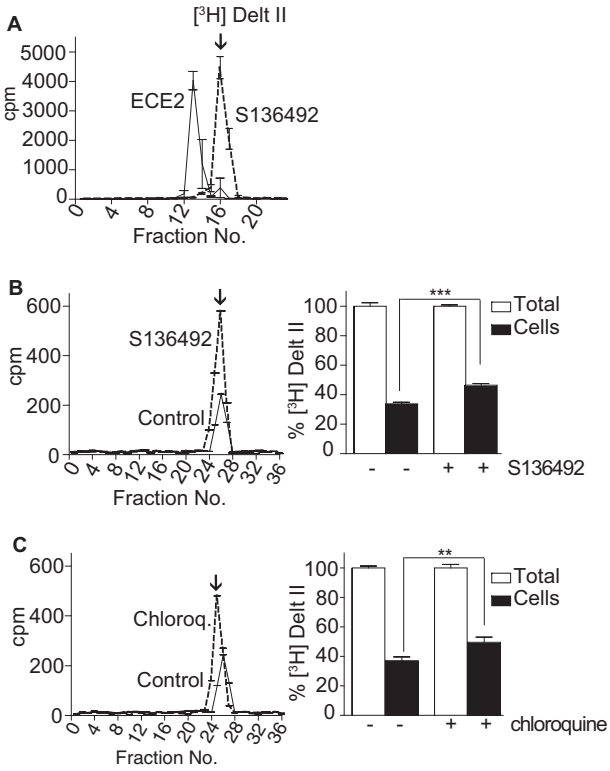


FIGURE 6. **ECE2 processes the endocytosed radiolabeled Delt II in an acidic endocytic compartment.** *A*, purified ECE2 (32.5 ng) was incubated with 10 nM [^{3}H]Delt II at pH 5.5 for 30 min without or with 20 μM S136492 followed by separation of radiolabeled and processed Delt II by thin-layer chromatographic analysis as described in "Experimental Procedures." The *arrow* indicates the position of migration of the intact, full-length [^{3}H]Delt II. *B* and *C*, F6-ECE2 cells were incubated with 10 nM [^{3}H]Delt II for 30 min without or with 20 μM S136492 or 100 μM chloroquine. The cells were subjected to an acid wash to remove surface-bound radiolabel, and cell extracts were analyzed by thin-layer chromatography as described under "Experimental Procedures." The total radioactivity (*Total*) (representing bound radioactivity prior to acid wash) as well as radioactivity left in the acid-washed cells (*Cells*) is shown as mean ± S.E. of three independent experiments in triplicate. The total radioactivity in controls not treated with inhibitors (*i.e.* S136492 and chloroquine) is taken as 100%. A representative graph of the radioactivity in individual fractions of acid-washed cells in the absence or presence of S136492 or chloroquine is shown; the *arrow* indicates the position of full-length [^{3}H]Delt II. **, $p < 0.01$; ***, $p < 0.001$; *t* test.

recycling. Together, these results support the notion that ECE2 activity in an acidic intravesicular compartment regulates receptor recycling; this could involve processing of the peptide agonist.

ECE2 Processes Endocytosed Radiolabeled δ Receptor Peptide Agonist at an Acidic pH—Because we found that ECE2 inhibitors and chloroquine block δOR recycling following internalization induced by the peptidic agonist Delt II, and given that the enzyme exhibits optimal activity at acidic pH (9), we examined whether ECE2 could process Delt II. For this we incubated radiolabeled Delt II with purified ECE2 in the absence or presence of 20 μM ECE2 inhibitor S136492 for 30 min at pH 5.5 and separated the radiolabeled substrate from the product by thin-layer chromatography. We found that purified ECE2 processed radiolabeled Delt II to a product with lower mobility, which was inhibited in the presence of S136492 (Fig. 6*A*).

Next we examined the processing of the peptide agonist following receptor endocytosis in the absence or presence of the ECE2 inhibitor or chloroquine (to examine whether ECE2 and an acidic environment are required for the peptide hydrolysis). For this purpose F6-ECE2 cells were incubated for 30 min with radiolabeled Delt II in the absence or presence of 20 μM S136492 or 100 μM chloroquine and subjected to an acid wash (to remove surface-bound radiolabeled Delt II), and the resulting cell lysate was subjected to fractionation by thin-layer chromatography to examine the fate of radiolabeled ligand retained within the cells. We found higher levels of radioactivity in fraction 26 (which coincides with the mobility of intact, full-length, radiolabeled Delt II) in cells treated with the ECE2 inhibitor or chloroquine as compared with untreated cells (Fig. 6, *B* and *C*). This was not due to different amounts of radiolabeled binding in the presence of the inhibitors, because the total bound radioactivity (representing bound radioactivity prior to acid wash) was found to be the same under all conditions (Fig. 6, *B* and *C*). Taken together, these results indicate that ECE2 processes endocytosed Delt II and that ECE2 activity in an acidic intracellular compartment is required for this process.

ECE2 Modulates δ opioid Receptor Recycling Induced by Peptide Agonists That Are ECE2 Substrates—To test the hypothesis that ECE2 inhibition affects receptor recycling by protecting the endocytosed peptide agonist from being hydrolyzed by ECE2, we used a non-peptide δOR-selective agonist, SNC80, and examined the effect of the inhibitor on recycling following SNC80-mediated receptor endocytosis. We found that in F6-ECE2 cells neither the rate nor the extent of 100 nM or 1

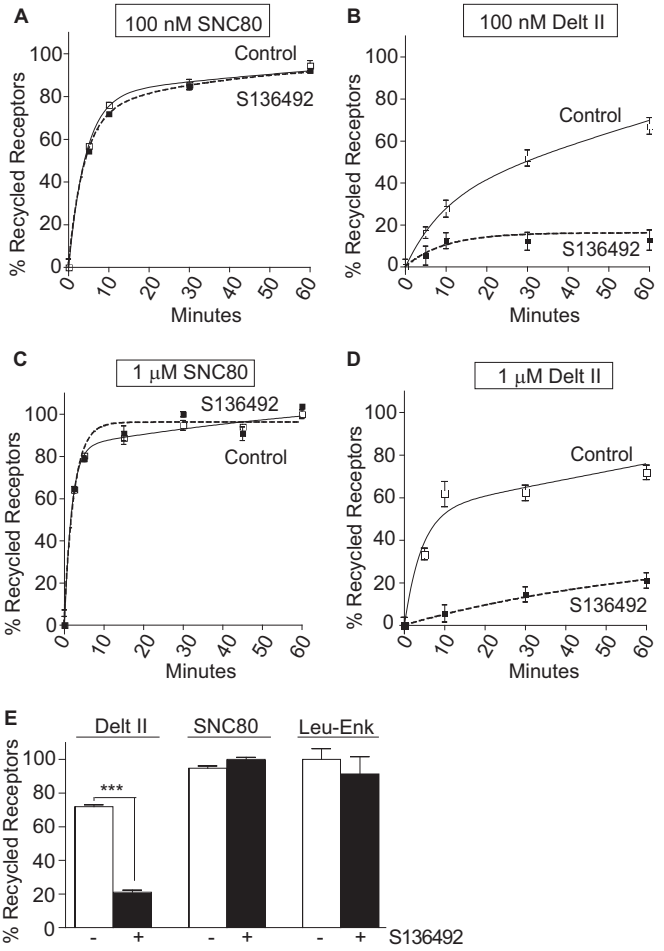


FIGURE 7. **Receptor recycling following internalization by a non-peptidic agonist (SNC80) or a peptide that is not an ECE2 substrate is not blocked by the inhibitor of ECE2.** F6-ECE2 cells were incubated with 100 nM SNC80 (*A*) or Delt II (*B*) or 1 μM SNC80 (*C* and *E*), Delt II (*D* and *E*), or Leu-enkephalin (*Leu-Enk*) (*E*) for 30 min to induce δOR internalization ($t = 0$). The cells were washed and incubated without (*Control*) or with 20 μM ECE2 inhibitor (S136492), and the time-dependent increase in the level of cell surface receptors was monitored by ELISA using antibodies to the epitope tags on the receptor as described in "Experimental Procedures." The percent recycled receptors was calculated by subtracting the percent surface receptors at $t = 0$ (30-min internalization) from all time points. *E*, represents percent δOR recycled at 60 min in the absence or presence of S136492. The data represent mean ± S.E. of 3–5 independent experiments in triplicate. ***, $p < 0.001$; *t* test.

μM SNC80-mediated recycling was affected by the ECE2 inhibitor, whereas 100 nM or 1 μM Delt II-mediated recycling was selectively and significantly impaired ($p < 0.001$) by the inhibitor (Fig. 7). The rate and extent of SNC80-mediated recycling was higher than the Delt-II mediated recycling (Fig. 7). These results suggest that ECE2 specifically modulates receptor recycling induced by peptides that are its substrates. Next we examined the requirement that the peptide agonist has to be a substrate of ECE2 by examining the effect of the ECE2 inhibitor on recycling following δOR endocytosis by Leu-enkephalin, a peptide that is not a substrate of ECE2 (13). We found that receptor recycling in response to Leu-enkephalin is not affected by the inhibitor (Fig. 7*E*). These results support the idea that ECE2 activity regulates receptor recycling by processing peptidic agonist substrates following endocytosis.

Inhibition of ECE2 Activity Impairs Opioid Receptor Resensitization—Next, we directly examined the functional implication of ECE2 activity to receptor signaling by considering the extent to which the ECE2 inhibitor affected cAMP levels, because activation of these receptors leads to inhibition of adenylate cyclase activity (27, 34). We carried out these studies under basal conditions or by preincubating the cells with a combination of forskolin and IBMX to increase basal cAMP levels (27, 34, 47). This was followed by a 30-min treatment with Delt II (mimicking conditions for δOR endocytosis with Delt II in recycling studies). The agonist was removed and the receptor allowed to recycle back to the cell surface for 60 min in the absence or presence of 20 μM S136492, 100 μM chloroquine, or 10 μM captopril (mimicking the conditions for δOR recycling). To assess receptor signaling, cells were then given a second pulse (5-min treatment) of Delt II, and the levels of cAMP were detected. We found that in F6-ECE2 or Neuro2A-δOR-ECE2 cells, a second short pulse of Delt II led to decreases in cAMP levels (*i.e.* receptor signaling) in untreated cells (Fig. 8). This was not seen in cells treated with the ECE2 inhibitor or chloroquine during the recycling phase (Fig. 8). This is consistent with the retention of surface receptors in an intracellular compartment seen with ECE2 inhibition (Figs. 4 and 5). Because in cells not pretreated with forskolin (Fig. 8, *A* and *B*) the basal levels of cAMP were low and not in the linear range of the detection system (*i.e.* 2000–10000 relative luminescence units (*RLU*)), studies were also carried out in the presence of forskolin and IBMX, a treatment routinely used to elevate cAMP levels and one that facilitates rigorous studies with opioid receptor signaling (27, 34, 47). We found that under these conditions of elevated cAMP levels, ECE2 inhibition during recycling also led to a loss of signaling (Fig. 8, *C* and *D*). Taken together these results suggest that by facilitating receptor recycling, ECE2 modulates the resensitization of δOR signaling.

ECE2 Modulates Recycling and Signaling by the Endogenous Opioid Peptide BAM22—To explore the physiological significance of ECE2 inhibition on opioid receptor recycling, we used an endogenous opioid peptide ligand, BAM22, a proenkephalin-derived peptide found in the brain, adrenal medulla, and ileum, as the peptide agonist (48–50). This peptide is also a substrate of ECE2 (13). First we examined whether the ECE2 inhibitor S136492 affected the rate or extent of δOR endocytosis. For this we prelabeled the surface receptors by incubating N2A-δOR cells with anti-*myc* antibodies and examined the extent of internalization with 100 nM BAM22 in the absence and presence of 20 μM S136492 by measuring the extent of decrease in cell surface receptors. We found that the extent of internalization was unaffected by S136492 (Fig. 9*A*), suggesting that ECE2 does not play a role in modulating receptor internalization. When the effect of the inhibitor on recycling in response to BAM22 was examined, we found that the inhibitor significantly and robustly impaired the rate and extent of receptor recycling (Fig. 9*B*). This is consistent with the idea that ECE2 modulates the recycling of receptors internalized by a substrate peptide. When the implication of this effect on signaling was examined, we found that ECE2 inhibition led to a significant decrease ($p < 0.05$) in signaling by δOR (Fig. 9, *C* and *D*). Importantly, because these signaling studies were carried

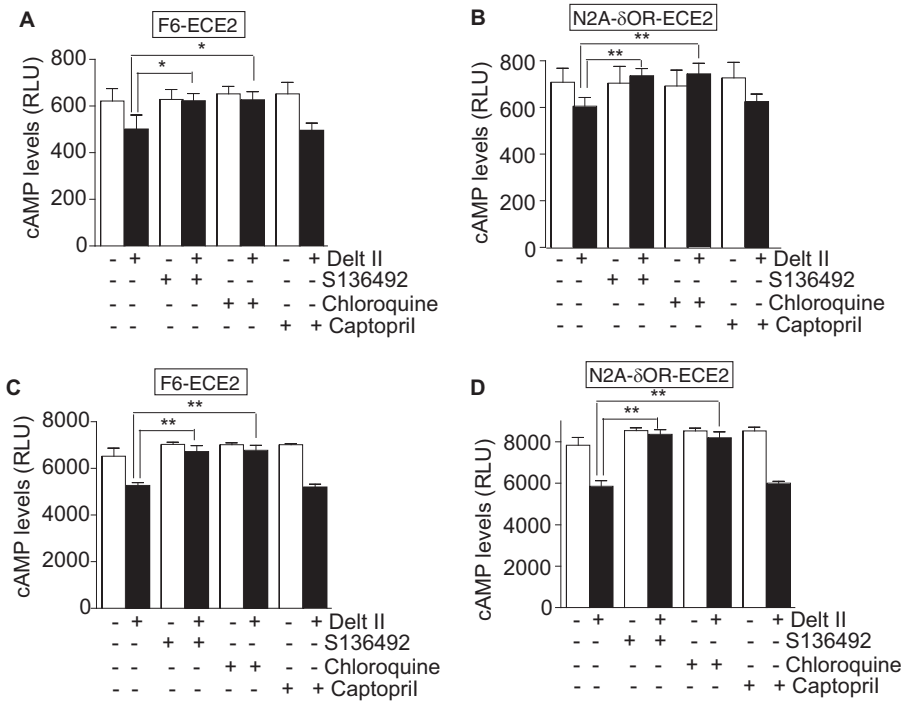


FIGURE 8. **ECE2 activity affects δ opioid receptor resensitization.** F6-ECE2 (*A* and *C*) or N2A-δOR ECE2 (*B* and *D*) cells were preincubated without (*A* and *B*) or with (*C* and *D*) 10 μM forskolin followed by incubation with 1 μM Delt II for 30 min. The cells were washed to remove the agonist and incubated without or with 20 μM ECE2 inhibitor (S136492), 100 μM choloroquine, or 10 μM captopril for 60 min, and the level of cAMP in response to a second pulse (5-min treatment) of Delt II was determined as described under "Experimental Procedures." The relative luminescence units (*RLU*) from three independent experiments carried out in triplicate were expressed as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; *t* test.

out in the continued presence of the drug, the results imply that the ECE2 activity affects receptor desensitization. These studies support a role for ECE2 in modulating receptor resensitization in response to endogenous opioid peptides.

Finally, we directly examined the extent to which the ECE2 inhibitor affects δOR desensitization. Because ECE2 inhibition leads to attenuated recycling, the continued presence of the inhibitor is likely to lead to receptor desensitization. We determined the level of δOR signaling in F6-ECE2 or N2A-δOR cells treated with 1 μM Delt II for 0–120 min in the absence or presence of 20 μM S136492, 100 μM chloroquine, 10 μM captopril, or 100 nM cycloheximide. Under these conditions, both in F6-ECE2 cells and in N2A-δOR cells, treatment with either the ECE2 inhibitor S136492 or chloroquine, but not captopril or cycloheximide, led to a significant decrease ($p < 0.001$ for F6-ECE2 and $p < 0.01$ for N2A-δOR cells) in signaling as compared with the control, implying that ECE2 inhibition leads to increased δOR desensitization (Fig. 10). These results, taken together with our finding that the inhibition of ECE2 activity does not affect receptor endocytosis (Fig. 9) but impairs recycling (Figs. 4, 5, 7, and 9), are consistent with the idea that by blocking receptor recycling to the surface, the inhibitor affects receptor signaling. Together these data support a key role for ECE2 activity in modulating receptor function by post-endocytic peptide processing, which in turn affects the extent of receptor recycling and signaling.

ECE2 Modulates Recycling and Signaling by δ opioid Receptors in Primary Cortical Neurons—Next we examined whether ECE2 regulates δOR function in an endogenous system and used primary cortical neurons, because they have been shown to express BAM22, a proenkephalin-derived peptide (48–50). First, we examined the co-localization of ECE2 with δOR using antibodies that recognize native ECE2 and native δ receptors (δOR selectivity of these antibodies was characterized using δOR knock-out mouse tissue (31)). We detected co-localization of ECE2 with δOR in untreated cells (Fig. 11*A*). The extent of intracellular co-localization increased following treatment with BAM22 (Fig. 11*B*). This increase in co-localization was reduced during receptor recycling (Fig. 11*C*). As expected, treatment with the ECE2 inhibitor during recycling led to retention of the receptors and ECE2 in the intracellular compartment (Fig. 11*D*). These results show that, similar to the recombinant system, the recycling of native receptors in an endogenous system is modulated by ECE2.

Next we examined the specific role of ECE2 activity on receptor recycling in primary cortical neurons. For this, cortical neurons were treated with 1 μM Delt II or 100 nM BAM22 for 30 min, the agonist was removed, and the cells were incubated for 60 min without or with 20 μM S136492, 100 μM chloroquine, 10 μM captopril, 100 nM cycloheximide, 10 μM thiorphan, or 100 nM bafilomycin (a vacuolar type H^+-ATPase inhibitor that prevents endosomal acidification (28)). Surface receptors were quantified by ELISA using a δOR-specific antibody (29, 31). We found that recycling of δOR internalized in response to either Delt II or BAM22 was substantially and significantly blocked by ECE2 inhibition and by agents that increase the intracellular pH such as chloroquine or bafilomycin and not by thiorphan or captopril, inhibitors of neprilysin or angiotensin-converting enzyme, or by cycloheximide (a protein synthesis inhibitor) used as a control (Fig. 12). We also examined the effect of ECE2

inhibition on receptor signaling in primary cortical neurons. For this purpose, cortical neuronal receptors internalized in response to Delt II or BAM22 were allowed to recycle in the absence or presence of 20 μM S136492 for 60 min. The level of signaling in response to a second 5-min treatment with agonists was determined by measuring cAMP levels. We found that, as with the recombinant cell lines, in primary neurons there is a significant decrease in cAMP levels in response to Delt II or BAM22, and this is significantly dampened (attenuated) by the ECE2 inhibitor (Fig. 12*B*). These results suggest that by impairing receptor recycling, the inhibitor affects the resensitization process, supporting the idea that in this native system ECE2 affects receptor function by affecting recycling in response to an endogenous opioid peptide and thus modulating receptor resensitization.

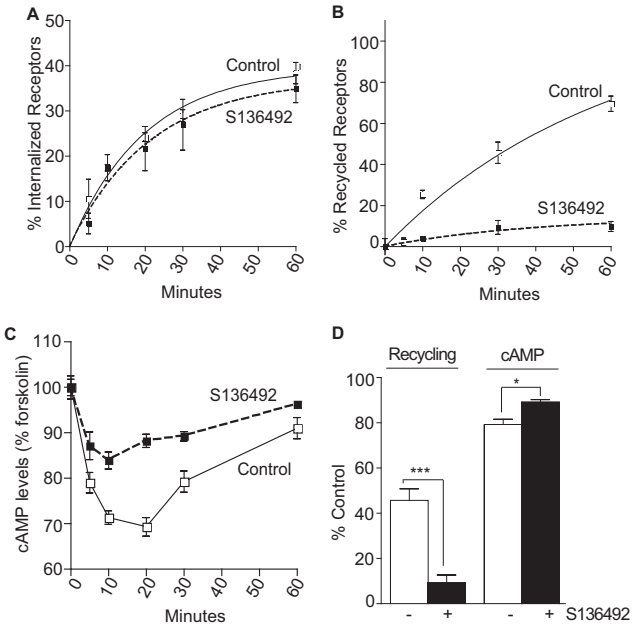


FIGURE 9. **ECE2 activity affects BAM22-mediated δ opioid receptor recycling and signaling.** *A*, surface receptors in N2A-δOR cells were prelabeled by incubation with anti-*myc* antibodies ($t = 0$) after which cells were incubated with 100 nM BAM22 for up to 60 min without (*Control*) or with the ECE2 inhibitor (20 μM S136492). Cell surface receptors were monitored using anti-mouse IgG coupled to HRP as described under "Experimental Procedures." The level of surface receptors at $t = 0$ was taken as 100% of the surface receptors. The percent internalized receptors were calculated as 100 − % surface receptors at each time point. *B*, N2A-δOR cells were incubated with 100 nM BAM22 for 30 min ($t = 0$), and then cells were washed and incubated for the indicated time periods without (control) or with the ECE2 inhibitor (20 μM). The level of cell surface receptors was monitored by ELISA as described under "Experimental Procedures." The percent recycled receptors was calculated by subtracting the percentage of surface receptors at $t = 0$ (30 min internalization) from all time points. *C*, N2A-δOR cells were incubated with 100 nM BAM22 and 10 μM forskolin in the absence (control) or presence of 20 μM ECE2 inhibitor (S136492), for the indicated periods, and the level of cAMP was determined as described under "Experimental Procedures." The relative level of cAMP accumulated is shown, where cAMP in the absence of ligand treatment but the presence of forskolin is taken as 100%. *D*, represents % δOR recycled and cAMP levels at 30 min in the absence or presence of S136492. The data in *A–D* represent mean ± S.E. of three independent experiments in triplicate. *, $p < 0.05$; ***, $p < 0.001$; *t* test.

DISCUSSION

In this study we explored a role for ECE2 in regulating δOR function by affecting receptor recycling and thus influencing signaling. Using selective inhibitors of ECE2 and related metallopeptidases, we show that ECE2 specifically modulates the recycling and resensitization of peptidic δOR agonists that are its substrates. The fact that an acidic compartment is required for this process is consistent with the acidic pH optimum for ECE2. Furthermore, impairment of receptor recycling and resensitization by the ECE2 inhibitor suggests that ECE2 activity by processing a substrate peptide agonist facilitates receptor recycling, thereby playing a central role in the resensitization process.

According to the classic models of receptor desensitization/resensitization, following activation the receptor is phosphorylated by G protein receptor kinases, which leads to the recruitment of β-arrestins to the phosphorylated receptor and clathrin-coated pit mediated endocytosis (51). The endocytosed receptor dissociates from the associated β-arrestin upon acidification in the endocytic vesicle, and this then allows dephosphorylation of the receptor and recycling to the cell surface (52). Although many studies have explored the fate of the endocytosed receptor using various candidate receptors, relatively few studies have focused on the fate of the agonist co-endocytosed with the receptor. Studies using peptide agonists, at least in the case of opioid receptors, have shown that the co-endocytosed agonist is recycled back to the surface or processed in an acidic compartment, depending on the length of agonist treatment (17, 53). Relatively few studies have focused on the enzymes that are responsible for post-endocytic peptide agonist degradation, although a number of peptidases have been shown to be capable of hydrolyzing neuroendocrine peptides

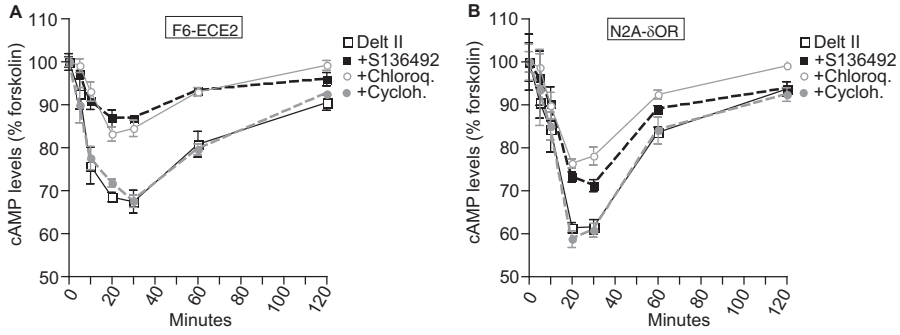


FIGURE 10. **Effect of ECE2 inhibition on δ opioid receptor desensitization.** F6-ECE2 (*A*) or N2A-δOR (*B*) cells were incubated with 1 μM Delt II and 10 μM forskolin in the absence (control) or presence of 20 μM ECE2 inhibitor, 100 μM chloroquine (*Chloroq.*), or 100 nM cycloheximide (*Cycloh.*) for the indicated periods, and the level of cAMP was determined as described under "Experimental Procedures." The data represents mean ± S.E. of three independent experiments in triplicate.

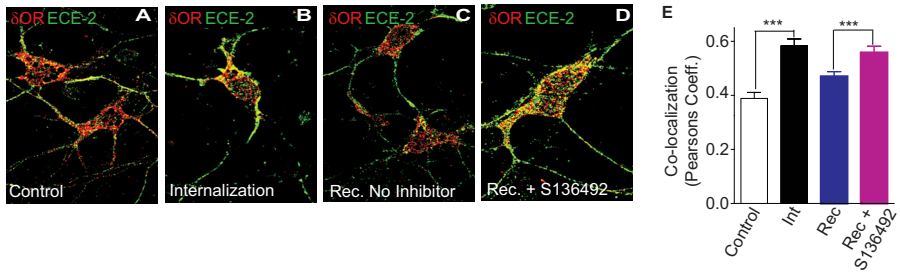


FIGURE 11. **Effect of ECE2 inhibition on co-localization of δ opioid receptors with ECE2 in primary cortical neurons.** *A–D*, primary cortical neurons were stained with antibodies that recognize native δOR (*red*) and native ECE2 (*green*) as described under "Experimental Procedures." Cells were treated without (*A*) or with 100 nM BAM22 (*B*) for 30 min, washed and stained immediately (*Int*), or stained following incubation for 60 min in medium (*Rec*) in the absence (*C*) or presence of 20 μM S136492 (*Rec + S136492*) (*D*). *E*, Pearson's coefficient analysis shows that agonist treatment for 30 min leads to an increase in co-localization of δOR with ECE2, which is reduced during recycling but not following inhibition of ECE2 activity during recycling. $n = 10$ fields (10–15 cells/field); ***, $p < 0.001$; *t* test.

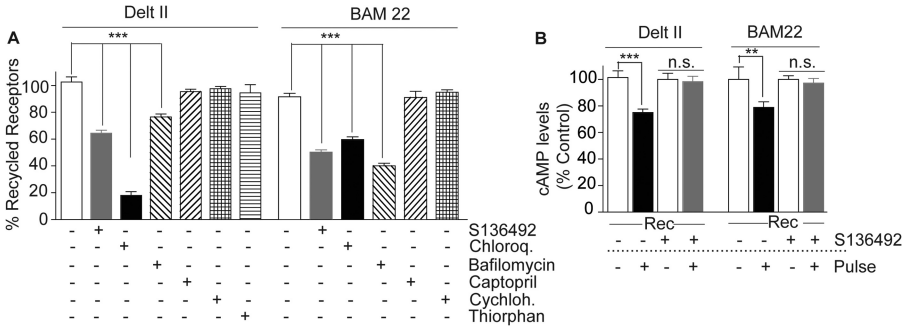


FIGURE 12. **Effect of ECE2 inhibition on δ opioid receptor recycling and signaling in primary cortical neurons.** *A*, primary cortical neurons were incubated with 1 μM Delt II or 100 nM BAM22 for 30 min ($t = 0$). Cells were washed and incubated for 60 min in medium alone or with 20 μM S136492, 100 μM chloroquine (*Chloroq.*), 100 nM bafilomycin, 10 μM captopril, 100 nM cycloheximide (*Cychlo.*), or 10 μM thiorphan. Cells were washed and surface receptors quantified using ELISA with a δOR-specific antibody that recognizes native receptors. The percent recycled receptors was calculated by subtracting the percentage of surface receptors at $t = 0$ (30-min internalization) from all time points. The data represent mean ± S.E. of three independent biological replicates and three technical replicates. *B*, primary neurons incubated with 1 μM Delt II or 100 nM BAM22 for 30 min were washed and incubated for 60 min (*Rec*) in medium alone or with 20 μM S136492. Cells were then given a second 5-min treatment with Delt II or BAM22 (*Pulse*), and cAMP levels were determined using the DiscoveRx kit as described under "Experimental Procedures." The relative level of cAMP is shown as mean ± S.E. of three independent experiments in triplicate, where cAMP levels prior to the second drug pulse (5-min treatment) were taken as 100%. **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant; one-way ANOVA (*A*) and *t* test (*B*).

including opioid peptides (2, 45, 54, 55). Among them, an enzyme named enkephalinase (EC 3.4.24.11), originally thought to be solely responsible for enkephalin degradation, was soon shown to be able to degrade a number of other neuropeptides, and because it exhibited activity at neutral pH it was renamed "neutral endopeptidase" or neprilysin (6, 8, 56). This enzyme was later shown to process peptides post-secretion in the extracellular space, and its neutral pH optimum is consistent with such a role (6, 8, 56). Another peptide-processing enzyme is ECE1, a member of the metalloendopeptidase family that was originally identified as the enzyme that converts big-endothelin to endothelin (6). However, ECE1 exhibits broad peripheral distribution with its highest expression in the liver and kidney (12), suggesting a role for ECE1 in the processing of additional peptide substrates. Consistent with this, a number of recent studies have found ECE1 to be able to process a variety of neuropeptides (57); these include substance P (43), somatostatin (39), calcitonin gene-related peptide (CGRP) (42), neurokinin (38), and corticotrophin-releasing factor (37), which are co-endocytosed with the cognate receptors. Inhibition of ECE1 activity leads to intracellular retention of the receptors, a concomitant decrease in G protein-mediated signaling by the cell surface receptors, and an increased β-arrestin-mediated signaling by the intracellular receptors (37–39, 42, 43, 58). These studies imply that ECE1 activity, by hydrolyzing the endocytosed peptide, modulates receptor function by affecting receptor trafficking. However, none of these studies include opioid peptides, and hence it is not clear whether this enzyme plays a role in opioid peptide processing. Our finding that ECE2, a closely related member of this family, is involved in opioid peptide processing, resulting in the modulation of receptor recycling and resensitization, supports an emerging role for this subfamily of metalloendopeptidases in regulating neuroendocrine peptide receptor function by post-endocytic peptide processing.

Processing of opioid peptides by ECE2 is likely to be of physiological significance as suggested by its distribution, which overlaps with the distribution of opioid peptide precursors such as proenkephalin (11, 59), and its selective substrate specificity in processing opioid peptides (13). A previous study that analyzed the substrate specificity of ECE2 *in vitro* revealed that among the opioid peptides tested, Dyn B-13, BAM22, BAM18, and peptide E were selectively processed by ECE2 (13), and it is interesting to note that BAM22 and BAM18 were processed to yield BAM12 (13). A number of studies have reported that these peptides are present *in vivo* (1, 60–64) and that they exhibit differential physiological activities (60, 61, 65). For example, BAM12 has been shown to exhibit a 10-fold lower analgesic effect than BAM22 (60, 61, 65). This is thought to be due to differential receptor selectivity by these peptides, BAM12 being

κ opioid receptor-selective and BAM22 being μ opioid receptor-selective (62–64, 66). One could envision a possible *in vivo* scenario where BAM22, which is co-endocytosed with the μ opioid receptor, is processed in an endocytic compartment to BAM12, which is released during receptor recycling and could then activate nearby κ opioid receptors. Such a notion would be consistent with the involvement of ECE2 in the BAM22-mediated receptor recycling seen in the present study.

An additional mechanism by which ECE2 activity is likely to enhance the repertoire of opioid receptor signaling is by processing the ligands that act on G protein-coupled receptor heteromers, an example of which would be BAM22. Such an idea is supported by studies with the sensory neuron-specific receptor (SNSR)-δOR heteromer (67). BAM22 is a dual "bivalent" activity ligand that is able to individually activate δOR via its N-terminal domain or SNSR-3 and -4 via its C-terminal domain (15). However, in the context of the heteromer, BAM22 elicits a poor response; the processing of this peptide to BAM12 and BAM (13–22) restores the response by each ligand activating individual receptors (67). Thus, the processing of BAM22 by enzymes such as ECE2 appears to be critical in modulating signaling by this heteromer, supporting an important role for ECE2 in regulating heteromer activity and related antinociceptive responses (68).

The present study exploring nonconventional roles for peptide-processing enzymes such as ECE2 questions the fundamental dogma in the field. It is generally accepted that neuroendocrine peptides are generated presynaptically via the processing of large precursors in the *trans*-Golgi network and are released upon stimulation to act on the postsynaptic receptors. Based on the findings of the present study, one can easily envision a scenario where the neuropeptides are generated and released postsynaptically. For example, a neuropeptide following the activation of its cognate receptor is co-endocytosed and selectively processed by ECE2 in endosomes, and the products are released to the synaptic cleft where they would activate "their" cognate receptors, thereby initiating a second wave of signaling by a distinct set of receptors. Alternately, it is possible that some endogenous peptides are biased agonists with differential signaling activities; for peptides that exhibit arrestin-mediated signaling, inhibition of processing is likely to have a major impact on the extent of signaling by those peptides. If this leads to the desired physiological response, the selective ECE2 inhibitor could, in such cases, serve as a new kind of therapeutic agent for the treatment of pain and related disorders.

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