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Abbreviations : APJ, Putative receptor Protein related to the Angiotensin receptor AT1; AC, Adenylate Cyclase; AngII, Angiotensin II; i.c.v, intracerebroventricular; i.v, intravenous; AVP, Arginine-Vasopressin; NA, Noradrenaline; L-NA, L-Nitro-omega-L-Arginine; NO, Nitric Oxide.

Abstract

Apelin plays a prominent role in body fluid and cardiovascular homeostasis. To explore further upstream the role played by this peptide, non-peptidic agonists and antagonists of the apelin receptor are required. To identify such compounds which do not exist to date, we used an original Fluorescence Resonance Energy Transfer-based assay to screen a GPCR-focused library of fluorescent compounds on the human EGFP-tagged apelin receptor. This led to isolate E339-3D6 that displayed a 90 nmol/L affinity, behaved as a partial agonist with regard to cAMP production and as a full agonist with regard to apelin receptor internalization. Finally, E339-3D6 induced vasorelaxation of rat aorta precontracted with noradrenaline and potently inhibited systemic vasopressin release in water-deprived mice when intracerebroventricularly injected. This compound represents the first non-peptidic agonist of the apelin receptor, the optimization of which will allow to develop a new generation of vasodilator and aquaretic agents.

INTRODUCTION

Apelin, is a bioactive peptide isolated from bovine stomach extracts and identified as the endogenous ligand of the human orphan G protein-coupled receptor (GPCR), APJ (1, 2). Apelin derives from a single 77-amino acid precursor, proapelin which has a fully conserved C-terminal 17-amino acid sequence in all species studied, apelin 17 (K17F), including the pyroglutamyl form of apelin 13 (pE13F) (see Table S1 for sequences) (2-4). Both peptides (K17F and pE13F) naturally occur in rat brain and plasma (5). They exhibit a strong inhibitory activity on forskolin-induced cAMP production in cells expressing the human (3, 6) or the rat apelin receptor (7). These peptides promote phosphorylation of ERKs, Akt and p70 S6 kinase (8). They are also highly potent inducers of apelin receptor internalization (9, 10). Apelin and its receptor are both widely distributed in the brain (4, 7, 11, 12) but are particularly highly expressed in the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei. Dual labeling studies demonstrate that within these two nuclei, apelin and its receptor co-localize with arginine vasopressin (AVP) in magnocellular neurons (5, 10, 13, 14). In lactating rats, characterized by increases in both synthesis and release of AVP, central injection of K17F inhibits the phasic firing pattern of AVP neurons, thereby resulting in decreased AVP release in the blood circulation, and increased aqueous diuresis (5). Moreover, after water deprivation, endogenous levels of AVP and apelin are conversely regulated to optimize systemic AVP release necessary to avoid additional water loss at the kidney level (5, 14). Recently, we also showed that such opposite regulation of plasma apelin and AVP levels by osmotic stimuli exists in humans suggesting that apelin, like AVP, may participate in the maintenance of body fluid homeostasis not only in rodents but also in humans (15).

Apelin and its receptor are also present in the cardiovascular system, i.e. heart, kidney and vessels (16). Systemically administered apelin decreases arterial blood pressure (BP) (4, 9, 10, 17), via a nitric oxide (NO)-dependent mechanism (17). Consistent with these data, apelin

receptor-deficient mice display an exaggerated pressor response to systemic angiotensin II (AngII), suggesting a counter-regulatory effect of apelin on AngII (18). Finally, apelin improves cardiac contractility and reduces cardiac loading (19-21), suggesting a role for apelin in the regulation of cardiovascular functions. In agreement with this hypothesis, when apelin-deficient mice are subjected to chronic pressure overload by surgical constriction of the aorta, they develop severe and progressive heart failure (22).

Given the broad array of physiological actions of apelin, its receptor represents a new interesting target for therapeutic research and drug design. In this context, the development of a nonpeptidic apelin receptor agonist by favouring diuresis and improving the contractile performance of the myocardium whilst reducing peripheral resistances could be particularly useful for the treatment of heart failure.

In order to identify an agonist or antagonist of the apelin receptor, we used in the present study a novel Fluorescence Resonance Energy Transfer (FRET)-based assay to screen a library of fluorescent (lissamine-tagged) nonpeptidic compounds on the human N-terminal EGFP-tagged apelin receptor, stably expressed in HEK-293 cells. This screening led to isolate several hits whose affinities (9×10^{-8} to 10^{-6} M) have been confirmed in classical binding experiments using radiolabelled pE13F. The most potent hit, E339-3D6 (23) displayed an affinity of 9×10^{-8} mol/L, behaved as a partial agonist with regard to cAMP production and as a full agonist with regard to apelin receptor internalization. E339-3D6 could be therefore considered as a biased agonist. Using molecular modeling, we provided evidence that the binding moieties of E339-3D6 and pE13F overlapped within the receptor binding cavity. This novel ligand represents an unique and interesting lead compound with agonist properties on the apelin receptor, inducing *ex vivo* a concentration-dependent vasorelaxation of rat aorta precontracted with noradrenaline (NA) and decreasing *in vivo* systemic AVP release in water-deprived mice, both effects being comparable to those produced by apelin.

MATERIALS AND METHODS

Drugs and Antibodies. Apelin 17 (K17F) and the pyroglutamyl form of apelin 13 (pE13F) were synthesized by NeoMPS (Strasbourg, France), see Table S1 for amino acid sequences.

Animals. Male Swiss mice (18-20 g) and adult male Wistar Kyoto rats (300-350 g) were maintained under 12 h light/dark cycle with free access to food and water and were obtained from Charles River Laboratories (L'Arbresle, France) and from Janvier (Le Genest-St-Isle, France). All animal experiments were carried out in accordance with current institutional guidelines for the care and use of experimental animals.

Chemistry.

The solid phase synthesis of the screened fluorescent compound library has been previously described (24). Solid phase re-synthesis of the best hit, E339-3D6 (23) and of lissamine-undecanoic-apelin-13 were described in the **online data supplement**.

Screening by FRET. A library of 800 fluorescent compounds (lissamine-tagged) were screened by FRET on HEK 293 cells stably expressing the full length EGFP-tagged human apelin receptor or the EGFP-Δ 16 human apelin receptor (See **online data supplement** for details).

Membrane Preparations and Radioligand Binding Experiments. Membranes from CHO or HEK 293 cells stably expressing respectively, the rat apelin receptor-EGFP and the EGFP-Δ 16 human apelin receptor, were prepared as previously described (25). Membrane preparations (5 µg/assay) were incubated for 1 h at 20°C with 2 x 10^{-10} mol/L [^{125}I]pE13F in binding buffer alone or in presence of K17F, pE13F or E339-3D6 at various concentrations. Reaction was stopped and filtered on Whatman GF/C filters. After washing radioactivity was

counted. Saturation-binding curves were obtained by incubating membrane proteins with [^{125}I]pE13F at different concentrations (See **online data supplement** for details).

cAMP Assay. The cAMP assay was performed as previously described (7) (**online data supplement** for details).

Internalization Assay. CHO cells stably expressing the rat apelin receptor-EGFP or $AT1_A$-EGFP were seeded at 20 % confluency on glass coverslips coated with polylysine (WI; 0.01 %) (Sigma-Aldrich, St Quentin, France). Internalization was performed by incubating the cells at 37 °C for various times with 10^{-7} mol/L K17F or lissamine-apelin 13 or with different concentrations of E339-3D6 as previously described (9). Cells were then mounted in Mowiol for confocal microscopic analysis (See **online data supplement** for details).

Quantification of Internalization by Digital Image Analysis. Quantification of the extent of ligand-induced rat apelin receptor-EGFP internalization was performed by confocal microscopy coupled to digital image analysis as previously described (9, 26) (See **Supplementary Methods** for details).

Aortic Rings Preparation and Isometric Tension Recording. According to (27, 28), rat aortic rings were equilibrated in physiological salt solution for 120 min under a resting tension of 2 g. After checking the integrity of the endothelium, cumulative concentration–response curves to K17F (10^{-12} to 10^{-6} mol/L), E339-3D6 (10^{-12} to 10^{-6} mol/L) or Ach (10^{-10} to 10^{-4} mol/L) were constructed after precontraction with NA (3×10^{-6} mol/L) in presence or in absence of endothelium. Each concentration of the drug was added every 15 min (See **online data supplement** for details).

Intracerebroventricular Injections in Mice and AVP Radioimmunoassay. K17F (1 µg) and E339-3D6 (from 0.03 to 2 µg) were administrated by i.c.v. route in conscious mice with free access to water or deprived of water for 24 h as previously described (5). Animals were killed 1 min after the injection, and trunk blood (0.5-1 mL) was collected in chilled tubes containing 50 µL of 0.3 mol/L EDTA pH 7.4. AVP concentrations were determined as previously described (5) from 0.2 mL of plasma by using a specific vasopressin-[Arg^8] RIA kit (Biovalley, Marne la Vallée, France).

Data and Statistical Analysis. Values are given as means ± standard error (S.E.M). One-way ANOVA or ANOVA for repeated measures followed by a Fisher Protected Least Significance (PLSD) or by a Student's unpaired *t*-test (peptide versus vehicle) were used to assess the significance of the results.

RESULTS

Identification of Apelin Receptor Ligands by a Screening Approach Based on FRET

When the project started, no convenient radioactive binding assay was available for the apelin receptor. An alternative approach using FRET between fluorescently tagged proteins and collections of fluorescent molecules just emerged (24, 29-31). We thus decided to adapt this technique to set up a screening assay for apelin receptor. We used the human truncated apelin receptor EGFP-tagged (EGFP-Δ 16 human apelin receptor) at the N-terminal part as an energy donor and ligands carrying a lissamine fluorophore as energy acceptors. The principle of the assay consisted in identifying molecules from the library that reduced EGFP fluorescence emission as a result of FRET. We screened a library of 800 nonpeptidic compounds (24, 29) on cells expressing the EGFP-Δ 16 human apelin receptor. We identified three hit compounds inducing FRET including the compound E339-3D6. It displayed the

highest affinity for the EGFP-Δ 16 human apelin receptor (K_d = 9 ± 1 x 10^{-8} mol/L), as assessed by determining the amplitude of EGFP extinction at different concentrations of E339-3D6 (**Figure 1A**,**B**, **Table S1**). Finally, FRET was also detected with the non truncated human apelin receptor, although less intensely (data not shown).

Binding Affinity of E339-3D6

The affinity of E339-3D6 for the apelin receptor was confirmed by determining its ability to displace [^{125}I]-pE13F (2 x 10^{-10} mol/L) binding on membrane preparations from HEK 293 cells or CHO cells stably expressing respectively the EGFP-Δ 16 human apelin receptor and the rat apelin receptor tagged at its C-terminal part with EGFP previously characterized (5). The total binding for a concentration of [^{125}I]-pE13F of 2 x 10^{-10} mol/L was 302 ± 27 fmol/mg protein, and the specific binding was 290 ± 23 fmol/mg protein. We first determined the apparent dissociation constant (K_d) of pE13F (K_d = 1 x 10^{-10} mol/L) by Scatchard analysis of the saturation curves (data not shown). E339-3D6, K17F and pE13F dose-dependently inhibited specific binding to the EGFP-Δ 16 human apelin receptor with *Ki* values of 3.9 ± 0.7 x 10^{-7} mol/L, 5.9 ± 1.6 x 10^{-11} mol/L and 2.9 ± 0.4 x 10^{-10} mol/L, respectively and to the rat apelin receptor with *Ki* values of 3.8 ± 0.4 x 10^{-7} mol/L, 5.0 ± 0.8 x 10^{-11} mol/L and 2.1 ± 0.9 x 10^{-10} mol/L, respectively (**Table S1**). Corresponding Hill coefficient values were close to unity, compatible with a single-site competitive model.

Effects of E339-3D6 on Forskolin-Induced cAMP Production

Incubation of CHO cells stably expressing the rat apelin receptor-EGFP with increasing concentrations of E339-3D6 (10^{-8} to 10^{-4} mol/L), K17F (10^{-12} to 10^{-7} mol/L) and pE13F (10^{-12} to 10^{-7} mol/L) resulted in a concentration-dependent inhibition of forskolin-induced cAMP production (**Figure 1C**) with IC_{50} of 6.4 ± 1.3 x 10^{-7} mol/L, 8.3 ± 1.2 x 10^{-11} mol/L and 2.3 ± 1.2 x 10^{-10} mol/L, respectively. The maximal inhibitory effect of E339-3D6 (8135 ± 685

fmoles/100,000 cells, n=5) corresponding to 60% of inhibition of forskolin-induced cAMP production (20136 ± 1361 fmoles/100,000 cells, n=10) occured for a concentration equal or superior to 10^{-5} mol/L and was significantly different from those induced by K17F (1410 ± 260 fmoles/100,000 cells, n=10, $p<0.001$) and pE13F (2477 ± 602 fmoles/100,000 cells, n=5, $p<0.005$). The maximal inhibitory effects of K17F (93 %) and pE13F (87 %) on forskolin-induced cAMP production occured for concentrations equal or superior to 10^{-8} mol/L. Under basal conditions, E339-3D6, K17F and pE13F applied separately at the concentrations of 10^{-4} mol/L or 10^{-7} mol/L, did not significantly decrease basal cAMP levels (data not shown).

Capacity of E339-3D6 to Trigger Apelin Receptor Internalization

Confocal microscope analysis of CHO cells stably expressing the rat apelin receptor-EGFP in resting conditions displayed intense apelin receptor-EGFP fluorescence at the plasma membrane as previously described (9) (**Figure 2A**). Incubation with 10^{-7} mol/L K17F or 10^{-7} mol/L fluorescent apelin 13 (i.e. lissamine-apelin 13: Lissamine-SO_2-NH-$(CH_2)_{10}$-CO-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe-OH) (**Figure 2A, S1**) for 20 min resulted in the internalization of the apelin receptor-ligand complexes (**Figure S1A** arrowheads). We then tested the capacity of E339-3D6 to trigger internalization of apelin receptor-EGFP. Incubation of CHO cells with increasing concentrations of E339-3D6 (10^{-7} to 10^{-4} mol/L) for 20 min resulted in the dose-dependent internalization of the rat apelin receptor-EGFP (**Figure 2B**). The maximal internalization occurred for E339-3D6 concentrations comprised between 3 x 10^{-6} mol/L and 10^{-4} mol/L. Moreover, since E339-3D6 was fluorescent due to the presence of lissamine, its cellular localization was followed during the internalization process. After 20 min of incubation with 3 x 10^{-6} mol/L and 10^{-4} mol/L E339-3D6, an extensive overlap was observed in labeling for the apelin receptor-EGFP and E339-3D6 within cytoplasmic vesicles, indicating that the internalization of the apelin receptor implies apelin receptor/E339-3D6 complex formation (**Figure 2B**). In contrast,

incubation of CHO cells stably expressing the rat angiotensin II receptor type 1A fused at its C-terminus with EGFP (AT1a-EGFP) with 10^{-4} mol/L E339-3D6 neither induced internalization of the AT1a-EGFP receptor nor the internalization of E339-3D6 itself (**Figure 2C**). The time-course of the rat apelin receptor-EGFP internalization induced by 3 x 10^{-5} mol/L E339-3D6 (**Figure S1B**) showed the appearance of fluorescent vesicles located under the plasma membrane after 5 min of incubation. E339-3D6-induced endocytosis was more pronounced after 10 min of incubation and maximal after 20 min. After 45 min of incubation, vesicles fused and moved in clusters to the perinuclear region of cells with a slight sorting to the plasma membrane **(Figure S1B).** To evaluate if E339-3D6 and K17F induce the same maximal extent of internalization, we quantified at a maximal dose of K17F (10^{-7} mol/L) and E339-3D6 (10^{-4} mol/L), the extent of ligand-induced rat apelin receptor-EGFP internalization by confocal microscopy coupled to digital image analysis (**Figure 3**) as previously described (26). First, the confocal analysis showed that as well for K17F as for E339-3D6, 100% of analyzed cells (100 cells for each condition) exhibited a profile of internalization (**Figure 3A**). Secondly, quantification of membrane/intracellular fluorescent ratios (M/I) induced by 10^{-7} mol/L K17F (M/I = 1.07 ± 0.15, number of cells analyzed=17) or 10^{-4} mol/L E339-3D6 (M/I = 1.03 ± 0.11, number of cells analyzed=17) indicated that E339-3D6 and K17F induced the same maximal extent of internalization (not statistically different, p=0.95) (**Figure 3B,C**).

Comparison of the Binding Properties of E339-3D6 versus the Apelin Peptide, pE13F, by Molecular Modeling

The *in silico* models of the human apelin receptor/pE13F and human apelin receptor/E339-3D6 complexes obtained after molecular simulation, revealed that both ligands bind in the same way within the receptor pocket. Both, pE13F and E339-3D6, exhibited a preferred elongated conformation when docked within the receptor cavity. In the complexes, the C-terminal phenylalanine of pE13F and the lissamine chromophore of E339-3D6 were

surrounded by aromatic side-chains of the receptor cavity forming a stable network of π-π interactions (**Figure 4**). In fact, E339-3D6 missing lissamine has much lower affinity for the receptor (data not shown). However, lissamine itself even at a high concentration of 10^{-4} mol/L did not inhibit specific binding to the rat apelin receptor-EGFP and was not able to induce apelin receptor internalization (data not shown).

Vasorelaxing Effects of E339-3D6 on Isolated Rat Aorta

In aortic rings precontracted with 3 x 10^{-6} mol/L NA, a concentration-dependent relaxation was induced by Ach, K17F and E339-3D6. All these effects were almost abolished in the absence of endothelium (**Figure 5**). The sensitivity (pD_2) to K17F and E339-3D6 (8.1 ± 0.3 and 8.6 ± 0.4, respectively) were significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding value for Ach (6.8 ± 0.4) whereas the maximal relaxations (% of induced tone) were similar (E_{max} of 92 ± 2, and 102 ± 1 and 94 ± 5, respectively).

Effects of Intracerebroventricular Injection of E339-3D6 on Systemic Vasopressin Release in Conscious Mice Deprived of Water for 24 h

Water deprivation of mice for 24 h significantly increases plasma AVP levels (87.6 ± 14.2 pg/mL , n=11 *versus* 33.2 ± 3.3 pg/mL , n=11; $P< 0.005$) (**Figure 6**). As previously described (10), i.c.v. injection of K17F in water-deprived mice at the dose of 1 µg (468 pmol) (significantly decreased plasma AVP levels (48.5 ± 7.3 pg/mL) compared with water-deprived mice injected with saline (87.6 ± 14.2 pg/mL) ($P< 0.05$) (**Figure 6**). I.c.v. injection of E339-3D6 in increasing doses (from 0.03 to 2 µg corresponding to 21 to 1422 pmol) to water-deprived mice induced a dose-dependent decrease in plasma AVP levels with an ED_{50} of 0.09 µg (64 pmol) (**Figure 6**, inset). The maximal decrease in AVP release induced by 1 µg (711 pmol) E339-3D6 (- 67 %) was similar to that observed with 1 µg (468 pmol) K17F (- 72 %) (**Figure 6**).

DISCUSSION

The endogenous peptide apelin improves cardiac contractility and decreases cardiac loading *in vivo* (19, 20). Together with its ability to decrease arterial BP and systemic AVP release inducing an increase in aqueous diuresis (4, 5, 10), this suggests a prominent role for the apelin system in body fluid and cardiovascular homeostasis. Therefore the development of a specific and selective apelin receptor agonist offers the possibility of exploring the role played by this peptide further upstream in cardiovascular diseases and water retention and/or hyponatremic disorders and might be useful for the treatment of heart failure. The present study identifies the first nonpeptidic agonist for the apelin receptor and describes its pharmacological properties *in vitro* as well as its *in vivo* biological effects.

At the beginning of our search for APJ receptor ligands, there was no available binding assay suitable for medium or high throughput screening. As an alternative, we decided to use a novel emerging strategy based on FRET (31, 32). The human apelin receptor was expressed fused to EGFP on its N-terminal. In parallel, libraries of fluorescent, non-peptide compounds were synthesized (24, 29). The fluorophore, lissamine, was chosen for its ability to absorb at EGFP emission wavelength via FRET. About 800 tagged molecules were thus prepared and screened on the EGFP-apelin receptor. As previously validated, such an assay allows the detection of molecules binding to the tagged receptor on orthosteric or allosteric sites (30, 33, 34) without need of a radioligand. This method is of particular interest for orphan receptors and when no classical binding assay is easily accessible. In the particular case of the human apelin receptor, by using this new screening approach, we have isolated three hit compounds including the compound E339-3D6 (23). E339-3D6 displayed an affinity of 9×10^{-8} mol/L for the human truncated apelin receptor determined by FRET and a *Ki* of 3.9×10^{-7} mol/L for the human truncated apelin receptor and a *Ki* of 3.8×10^{-7} mol/L for the rat apelin receptor determined by radioligand binding assay. E339-3D6 exhibits a high affinity and selectivity for

both rat and human apelin receptor. For instance, the molecule does not lead to FRET detection on the following human GPCRs, vasopressin V1a, oxytocin OTR, muscarinic m1R, chemokine CXCR4 and CCR5, melanocortin MC3 nor on rat takykinin NK2 receptors (data not shown). E339-3D6 provides an original research probe that validate the FRET-based strategy that could be applied to other GPCRs, including orphan receptors.

E339-3D6 inhibits forskolin-stimulated cAMP accumulation but the maximal response was only 60% of the maximal responses elicited by the natural full agonists K17F and pE13F, showing that E339-3D6 displays partial agonist activity with regard to cAMP production. In agreement with the partial agonist behavior, the binding sites for E339-3D6 and pE13F are overlapping as shown by the docking of these compounds in the 3D model of the apelin receptor. In the α2-adrenergic receptor, full and partial agonists having different intrinsic efficacy were shown to induce and/or stabilize distinct conformational states of the receptor (35, 36). This suggests that pE13F and E339-3D6 could stabilize different conformations of the apelin receptor, pE13F would exclusively bind the active conformation of the receptor whereas E339-3D6 would bind both active and inactive conformations of the receptor, thus generating quantitatively different amounts of active receptors.

However, confocal analysis of CHO cells stably expressing the rat apelin receptor-EGFP stimulated by K17F or E339-3D6 at maximal concentrations, showed that E339-3D6 and K17F induced the same maximal extent of internalization. This shows that E339-3D6 is a full agonist with regard to internalization. Since the apelin receptor is internalized by the early endosomes via a clathrin-dependent mechanism, probably involving the perinuclear recycling compartment (9), it could be concluded that E339-3D6 would be a biased agonist and even an imperfect bias where selectivity for different signaling pathways is a matter of degree as reviewed by Violin and Lefkowitz (37).

This is in line with the concept that different ligands can stabilize distinct receptor conformations that may differ in their signaling partner preference (38-40), thus inducing

different biological responses. Indeed, we previously showed that K16P (the C-terminal phenylalanine-deleted fragment of K17F) and K17F bind the apelin receptor with similar affinities and equally inhibit forskolin-induced cAMP production, whereas in contrast to K17F (9), K16P does not induce apelin receptor internalization and does not decrease arterial BP (9). This indicates that a specific receptor conformation inducing internalization is required to decrease arterial BP. In the 3D model of the apelin receptor, the C-terminal phenylalanine in pE13F is positioned in an aromatic pocket within the receptor binding site. Interestingly, we found that the lissamine chromophore in E339-3D6 is similarly embedded, which could account in part for the full agonist activity of E339-3D6 with regard to apelin receptor internalization.

Since the apelin induced-decrease in BP was proposed to occur through a NO-mediated arterial vasodilatation (17, 18), we first evaluated *ex vivo,* the effect of E339-3D6 on rat aortic vascular tone. To our best knowledge, the effects of K17F on vascular responsiveness of non-obese normotensive rats were poorly investigated. In db/db mice, apelin 36 restores the altered aortic vascular responsiveness to Ach and AngII by potentiating phosphorylation of Akt and eNOS (41). Furthermore, apelin 13 produced relaxation in normal human splanchnic arteries via NO release after activation of apelin receptors located in the endothelium (42). More recently, apelin 36 and pE13F administration in man was shown to cause NO-dependent arterial vasodilation (16). Moreover, we recently reported that K17F caused NO-dependent vasorelaxation of rat renal glomerular arterioles precontracted with AngII (43). In the present work, K17F and E339-3D6 induced a similar concentration- and endothelium-dependent vasorelaxation of NA-precontracted aortic rings from normotensive rats. Since the apelin induced-decrease in BP was proposed to occur through a NO-mediated arterial vasodilatation (17, 18), the apelin- and E339-3D6-induced aorta vasodilatation could be also mediated by endocytosis of the apelin receptor. Since E339-3D6 is full agonist with regard to internalization, this could explain in part why E339-3D6 has a maximal vasorelaxant effect

equal to K17F. Nevertheless, the vasorelaxant action of E339-3D6, even if its affinity for the apelin receptor is weaker than that of the endogenous peptide K17F, could be also related to higher metabolic stability and biological activity of E339-3D6 as compared to K17F, as expected from its non-peptidic structure.

Interestingly, both K17F and E339-3D6 exhibited a significantly more potent vasorelaxant effect than Ach in our experimental conditions, as their pD_2 were significantly higher than that of Ach despite similar maximal relaxant effects. The endothelium-dependent vasorelaxant effect of E339-3D6, similar to that of K17F, is in agreement with the presence of apelin receptor binding sites in human aorta (44) and mRNA apelin receptor expression in endothelial cells lining large conduit vessels of various organs (16, 43). E339-3D6 is the first apelin receptor agonist decreasing arterial vascular tone in normotensive rats, suggesting that E339-3D6 could constitute a lead compound for the future development of a new class of vasodilator agents.

Another important part of our study addresses the question whether central administration of E339-3D6, like apelin, is able to decrease systemic AVP in 24h-water deprived mice. E339-3D6 significantly decreased dehydration-induced AVP release and its potency did not differ from that of the natural ligand K17F (10). These data suggest that E339-3D6 similarly to K17F, when i.c.v injected, is able to rapidly reach the hypothalamic structures involved in AVP release and, by acting on apelin receptors expressed by magnocellular vasopressinergic neurons, inhibits the phasic electrical activity of these neurons. This subsequently induces a decrease in AVP release in the blood circulation. As previously shown for the central action of apelin (5), we can hypothesize that an apelin receptor agonist such as E339-3D6, by counteracting AVP actions would have aquaretic effects. In this context, apelin receptor agonists would be particularly interesting for the treatment of water retention and/or hyponatremia, avoiding the excessive loss of sodium and potassium commonly found with the use of diuretics.

In conclusion, we described the discovery of the first nonpeptidic specific agonist of the apelin receptor, E339-3D6, by an original and efficient screening approach. We demonstrated *ex vivo* its ability to produce vasorelaxation of rat aorta as well as its *in vivo* potential to inhibit water deprivation-induced AVP release in the blood circulation. Such compound would be useful for a better understanding of the physiopathological roles of apelin and its receptor and to evaluate the therapeutic potential of apelin receptor agonists in different animal models of pathologies. This ligand represents a unique and very interesting lead compound for which a medicinal chemistry program has been undertaken in order to optimize its affinity and its bioavailability. Optimization of the structure towards a compound meeting the clinical candidate status requirements will also be conducted in order to develop new agonists of the apelin receptor which could represent a new generation of vasodilator and aquaretic agents.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

REFERENCES

1. O'Dowd, B. F., Heiber, M., Chan, A., Heng, H. H., Tsui, L. C., Kennedy, J. L., Shi, X., Petronis, A., George, S. R., and Nguyen, T. (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 136, 355-360
2. Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M. X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C., Kurokawa, T., Onda, H., and Fujino, M. (1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 251, 471-476
3. Habata, Y., Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Hinuma, S., Kitada, C., Nishizawa, N., Murosaki, S., Kurokawa, T., Onda, H., Tatemoto, K., and Fujino, M. (1999) Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum. *Biochim Biophys Acta* 13, 25-35
4. Lee, D. K., Cheng, R., Nguyen, T., Fan, T., Kariyawasam, A. P., Liu, Y., Osmond, D. H., George, S. R., and O'Dowd, B. F. (2000) Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 74, 34-41
5. De Mota, N., Reaux-Le Goazigo, A., El Messari, S., Chartrel, N., Roesch, D., Dujardin, C., Kordon, C., Vaudry, H., Moos, F., and Llorens-Cortes, C. (2004) Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. *Proc Natl Acad Sci U S A* 101, 10464-10469
6. Medhurst, A. D., Jennings, C. A., Robbins, M. J., Davis, R. P., Ellis, C., Winborn, K. Y., Lawrie, K. W., Hervieu, G., Riley, G., Bolaky, J. E., Herrity, N. C., Murdock, P., and Darker, J. G. (2003) Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin. *J Neurochem* 84, 1162-1172

7. De Mota, N., Lenkei, Z., and Llorens-Cortes, C. (2000) Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. *Neuroendocrinology* 72, 400-407

8. Masri, B., Morin, N., Cornu, M., Knibiehler, B., and Audigier, Y. (2004) Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. *Faseb J* 22, 22

9. El Messari, S., Iturrioz, X., Fassot, C., De Mota, N., Roesch, D., and Llorens-Cortes, C. (2004) Functional dissociation of apelin receptor signaling and endocytosis: implications for the effects of apelin on arterial blood pressure. *J Neurochem* 90, 1290-1301

10. Reaux, A., De Mota, N., Skultetyova, I., Lenkei, Z., El Messari, S., Gallatz, K., Corvol, P., Palkovits, M., and Llorens-Cortes, C. (2001) Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. *J Neurochem* 77, 1085-1096

11. O'Carroll, A. M., Selby, T. L., Palkovits, M., and Lolait, S. J. (2000) Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochim Biophys Acta* 21, 72-80

12. Reaux, A., Gallatz, K., Palkovits, M., and Llorens-Cortes, C. (2002) Distribution of apelin-synthesizing neurons in the adult rat brain. *Neuroscience* 113, 653-662

13. O'Carroll, A. M., and Lolait, S. J. (2003) Regulation of rat APJ receptor messenger ribonucleic acid expression in magnocellular neurones of the paraventricular and supraopric nuclei by osmotic stimuli. *J Neuroendocrinol* 15, 661-666

14. Reaux-Le Goazigo, A., Morinville, A., Burlet, A., Llorens-Cortes, C., and Beaudet, A. (2004) Dehydration-induced cross-regulation of apelin and vasopressin immunoreactivity levels in magnocellular hypothalamic neurons. *Endocrinology* 145, 4392-4400

15. Azizi, M., Iturrioz, X., Blanchard, A., Peyrard, S., De Mota, N., Chartrel, N., Vaudry, H., Corvol, P., and Llorens-Cortes, C. (2008) Reciprocal regulation of plasma apelin and vasopressin by osmotic stimuli. *J Am Soc Nephrol* 19, 1015-1024

16. Japp, A. G., and Newby, D. E. (2008) The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. *Biochem Pharmacol* 75, 1882-1892

17. Tatemoto, K., Takayama, K., Zou, M. X., Kumaki, I., Zhang, W., Kumano, K., and Fujimiya, M. (2001) The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept* 99, 87-92

18. Ishida, J., Hashimoto, T., Hashimoto, Y., Nishiwaki, S., Iguchi, T., Harada, S., Sugaya, T., Matsuzaki, H., Yamamoto, R., Shiota, N., Okunishi, H., Kihara, M., Umemura, S., Sugiyama, F., Yagami, K., Kasuya, Y., Mochizuki, N., and Fukamizu, A. (2004) Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. *J Biol Chem* 279, 26274-26279

19. Ashley, E. A., Powers, J., Chen, M., Kundu, R., Finsterbach, T., Caffarelli, A., Deng, A., Eichhorn, J., Mahajan, R., Agrawal, R., Greve, J., Robbins, R., Patterson, A. J., Bernstein, D., and Quertermous, T. (2005) The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. *Cardiovasc Res* 65, 73-82

20. Berry, M. F., Pirolli, T. J., Jayasankar, V., Burdick, J., Morine, K. J., Gardner, T. J., and Woo, Y. J. (2004) Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation* 110, II187-193

21. Szokodi, I., Tavi, P., Foldes, G., Voutilainen-Myllyla, S., Ilves, M., Tokola, H., Pikkarainen, S., Piuhola, J., Rysa, J., Toth, M., and Ruskoaho, H. (2002) Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res* 91, 434-440

22. Kuba, K., Zhang, L., Imai, Y., Arab, S., Chen, M., Maekawa, Y., Leschnik, M., Leibbrandt, A., Markovic, M., Schwaighofer, J., Beetz, N., Musialek, R., Neely, G. G., Komnenovic, V., Kolm, U., Metzler, B., Ricci, R., Hara, H., Meixner, A., Nghiem, M., Chen, X., Dawood, F., Wong, K. M., Sarao, R., Cukerman, E., Kimura, A., Hein, L., Thalhammer, J., Liu, P. P., and Penninger, J. M. (2007) Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload. *Circ Res* 101, e32-42

23. Llorens Cortes, C., Guillier, F., and Franchet, C. (2008) APJ receptor ligands and uses thereof. *Patent EP 1 903 052*

24. Hibert, M., Franchet, C., Galzi, J. L., Pattus, F., and Guillier, F. (2006) Collection of traceable compounds and uses thereof. *WO/2006/003329*

25. Iturrioz, X., Vazeux, G., Celerier, J., Corvol, P., and Llorens-Cortes, C. (2000) Histidine 450 plays a critical role in catalysis and, with Ca2+, contributes to the substrate specificity of aminopeptidase A. *Biochemistry* 39, 3061-3068

26. Lenkei, Z., Beaudet, A., Chartrel, N., De Mota, N., Irinopoulou, T., Braun, B., Vaudry, H., and Llorens-Cortes, C. (2000) A highly sensitive quantitative cytosensor technique for the identification of receptor ligands in tissue extracts. *J Histochem Cytochem* 48, 1553-1564

27. Puybasset, L., Bea, M. L., Ghaleh, B., Giudicelli, J. F., and Berdeaux, A. (1996) Coronary and systemic hemodynamic effects of sustained inhibition of nitric oxide synthesis in conscious dogs. Evidence for cross talk between nitric oxide and cyclooxygenase in coronary vessels. *Circ Res* 79, 343-357

28. Trochu, J. N., Leblais, V., Rautureau, Y., Beverelli, F., Le Marec, H., Berdeaux, A., and Gauthier, C. (1999) Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol* 128, 69-76

29. Hibert, M., Franchet, C., Galzi, J. L., Pattus, F., and Guillier, F. (2006) Collections of traceable compounds and uses thereof. *WO/2006/003330*

30. Ilien, B., Franchet, C., Bernard, P., Morisset, S., Weill, C. O., Bourguignon, J. J., Hibert, M., and Galzi, J. L. (2003) Fluorescence resonance energy transfer to probe human M1 muscarinic receptor structure and drug binding properties. *J Neurochem* 85, 768-778

31. Vollmer, J. Y., Alix, P., Chollet, A., Takeda, K., and Galzi, J. L. (1999) Subcellular compartmentalization of activation and desensitization of responses mediated by NK2 neurokinin receptors. *J Biol Chem* 274, 37915-37922

32. Galzi, J. L., and P., A. (1998) Use of a fluorescent protein for detecting interaction between a target protein and its ligand. *Patent WO 1998/55873*

33. Bonnet, D., Ilien, B., Galzi, J. L., Riche, S., Antheaune, C., and Hibert, M. (2006) A rapid and versatile method to label receptor ligands using "click" chemistry: Validation with the muscarinic M1 antagonist pirenzepine. *Bioconjug Chem* 17, 1618-1623

34. Galzi, J. L., Hibert, M., Bourguignon, J. J., and Maillet, E. (2003) Method for isolating an allosteric effector of a receptor. *WO/2003/107004*

35. Ghanouni, P., Gryczynski, Z., Steenhuis, J. J., Lee, T. W., Farrens, D. L., Lakowicz, J. R., and Kobilka, B. K. (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *J Biol Chem* 276, 24433-24436

36. Swaminath, G., Xiang, Y., Lee, T. W., Steenhuis, J., Parnot, C., and Kobilka, B. K. (2004) Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* 279, 686-691

37. Violin, J. D., and Lefkowitz, R. J. (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* 28, 416-422

38. Drake, M. T., Violin, J. D., Whalen, E. J., Wisler, J. W., Shenoy, S. K., and Lefkowitz, R. J. (2008) beta-arrestin-biased agonism at the beta2-adrenergic receptor. *J Biol Chem* 283, 5669-5676

39. Galandrin, S., and Bouvier, M. (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* 70, 1575-1584

40. Shukla, A. K., Violin, J. D., Whalen, E. J., Gesty-Palmer, D., Shenoy, S. K., and Lefkowitz, R. J. (2008) Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. *Proc Natl Acad Sci U S A* 105, 9988-9993

41. Zhong, J. C., Yu, X. Y., Huang, Y., Yung, L. M., Lau, C. W., and Lin, S. G. (2007) Apelin modulates aortic vascular tone via endothelial nitric oxide synthase phosphorylation pathway in diabetic mice. *Cardiovasc Res* 74, 388-395

42. Salcedo, A., Garijo, J., Monge, L., Fernandez, N., Luis Garcia-Villalon, A., Sanchez Turrion, V., Cuervas-Mons, V., and Dieguez, G. (2007) Apelin effects in human splanchnic arteries. Role of nitric oxide and prostanoids. *Regul Pept* 144, 50-55

43. Hus-Citharel, A., Bouby, N., Frugiere, A., Bodineau, L., Gasc, J. M., and Llorens-Cortes, C. (2008) Effect of apelin on glomerular hemodynamic function in the rat kidney. *Kidney Int* 74, 486-494

44. Katugampola, S. D., Maguire, J. J., Kuc, R. E., Wiley, K. E., and Davenport, A. P. (2002) Discovery of recently adopted orphan receptors for apelin, urotensin II, and ghrelin identified using novel radioligands and functional role in the human cardiovascular system. *Can J Physiol Pharmacol* 80, 369-374

FIGURE LEGENDS

Figure 1. *In vitro* pharmacological characterization of E339-3D6 (*A*) structure of compound E339-3D6. (*B*) detection of E339-3D6 binding to the EGFP-Δ 16 human apelin receptor. Fluorescence of cells expressing EGFP-Δ 16 human apelin receptor was monitored at 510 nm (excitation 470 nm) as a function of time. After 30 sec recording, E339-3D6 (6×10^{-7} mol/L) was added to the cells leading to the time-dependent decline of EGFP emission. At time 210 sec, an excess of pE13F (5×10^{-6} mol/L) was added enabling the E339-3D6 dissociation and the initial fluorescence emission recovery. (*C*) Effects of K17F, pE13F and E339-3D6 on forskolin-induced cAMP production in CHO cells stably expressing the rat apelin receptor-EGFP. cAMP production was induced by treatment of cells with 10^{-5} mol/L forskolin. The effects of various concentrations of K17F, pE13F and E339-3D6 on forskolin-induced cAMP production were then evaluated. Data are expressed in fmoles of cAMP produced/100,000 cells. Means ± SEM of three to ten separate experiments.

Figure 2. Effects of K17F and E339-3D6 on rat apelin receptor-EGFP internalization in CHO cells. (*A*) CHO cells stably expressing the rat apelin receptor-EGFP (in green) were treated with K17F (10^{-7} mol/L) for 20 min. (*B*) CHO cells stably expressing the rat apelin receptor-EGFP (green) were treated for 20 min with E339-3D6 (from 10^{-7} to 10^{-4} mol/L) (red). Overlay was visualized in yellow. (*C*) CHO cells stably expressing the rat AT1a receptor-EGFP (green) were treated for 20 min with 10^{-4} M of E339-3D6 (red). Each panel is representative of three separate experiments.

Figure 3. Quantification of rat apelin receptor-EGFP internalization. (*A*) Confocal images of CHO cells stably expressing the rat apelin receptor-EGFP incubated without (Control), with K17F (10^{-7} mol/L) or with E339-3D6 (10^{-4} mol/L) for 20 min. (*B*) Example of gray-scale conversion and median filtering of a cell with twelve radial measurement lines outlined. Example plots of gray-scale density distribution along the first four radial measurement lines

for a cell. The mean density value of the first 30 pixels (shaded), representing the plasma membrane, yields the M value and the mean density of the remaining intracellular pixels yields the I value. The mean of the 12 M values and the 12 I values is used to calculate the M/I ratio for each cell. (*C*) Histogram of M/I ratio as a function of K17F and E339-3D6 concentrations. The results are expressed as means ± SEM. Statistical differences were assessed using Student's t comparison test, with a threshold of significance set at $P \leq 0.05$.

Figure 4. Docking of pE13F or E339-3D6 into the human apelin receptor 3D model. The optimized positions of both pE13F (orange) and E339-3D6 (pink) ligands with the human apelin receptor. The side chains of the residues of the apelin receptor interacting with the ligands are displayed in green.

Figure 5. Effects of E339-3D6 on rat aorta vascular tone. Cumulative concentration-response curves of K17F (circles), E339-3D6 (triangles) and acetylcholine (Ach) (squares) in rat aorta with (full symbols) or without (open symbols) endothelium precontracted by NA (3×10^{-6} mol/L). Data are shown as means ± SEM of three to five independent experiments.

Figure 6. Effects of i.c.v. injection of E339-3D6 in mice on water deprivation-induced systemic AVP release. After 24 h of water deprivation, mice received i.c.v. 10 µL saline or K17F (1 µg) or increasing amounts of E339-3D6 (from 0.03 to 2 µg) and were compared with mice with free access to water that received i.c.v. 10 µL saline. Plasma AVP levels were determined 1 min after injection by RIA. Histogram represents the mean ± SEM of plasma AVP levels in pg/mL , *$p < 0.05$, **$p < 0.005$ versus control. Inset, represents the sigmoidal curve of the E339-3D6 dose-response on AVP release in conscious water-deprived mice.

Figure 1

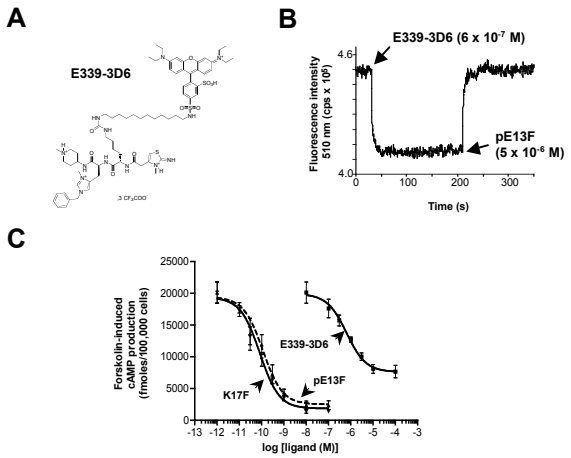

A
E339-3D6
B
E339-3D6 (6 x 10-7 M)
pE13F
(5 x 10-6 M)
Fluorescence intensity
510 nm (cps x 10³)
4.6
4.0
0
100
200
300
Time (s)
C
Forskolin-induced
cAMP production
(fmoles/100,000 cells)
25000
20000
15000
10000
5000
0
E339-3D6
pE13F
K17F
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3
log [ligand (M)]

Figure 2

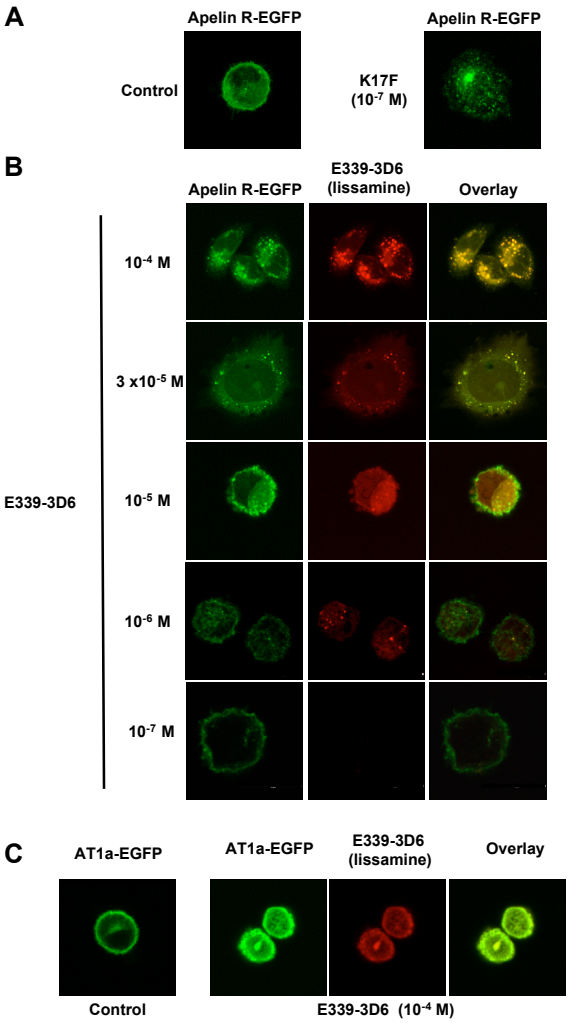

A
Apelin R-EGFP
Control
Apelin R-EGFP
K17F
(10-7 M)
B
Apelin R-EGFP
E339-3D6
(lissamine)
Overlay
E339-3D6
10-4 M
3 x10-5 M
10-5 M
10-6 M
10-7 M
C
AT1a-EGFP
AT1a-EGFP
E339-3D6
(lissamine)
Overlay
Control
E339-3D6 (10-4 M)

Figure 3

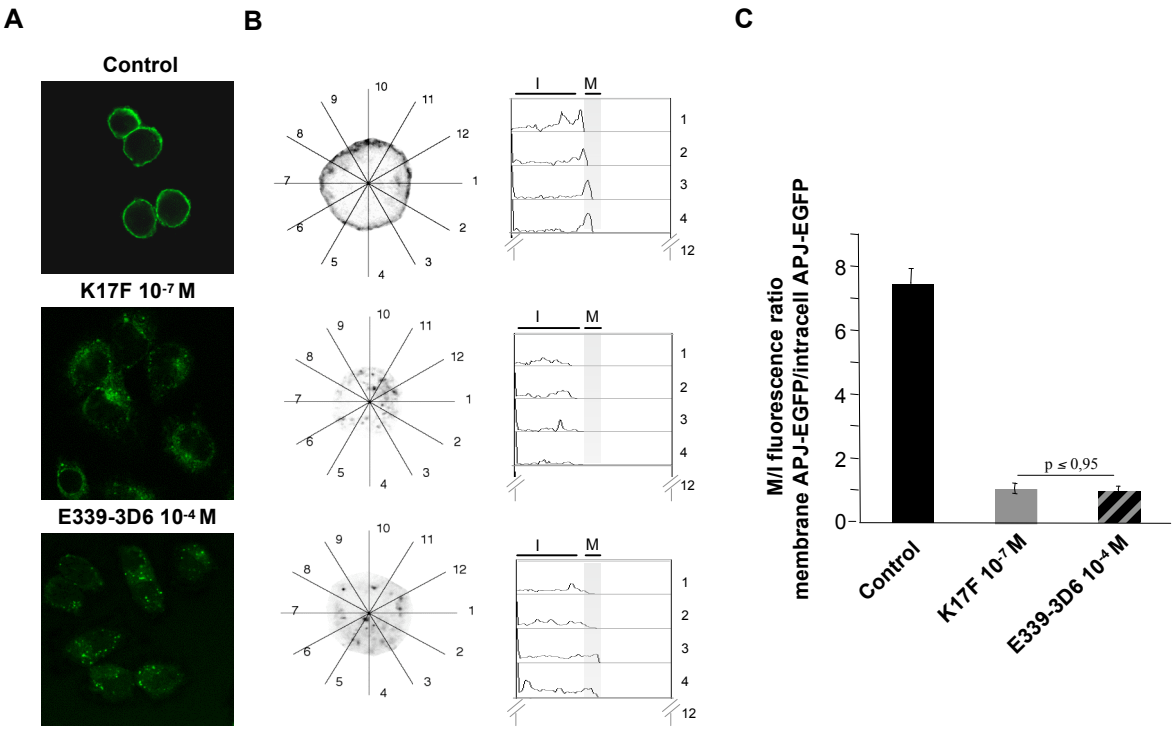

A
B
C
Control
K17F 10-7 M
E339-3D6 10-4 M
I
M
M/I fluorescence ratio
membrane APJ-EGFP/intracell APJ-EGFP
p ≤ 0,95
Control
K17F 10-7 M
E339-3D6 10-4 M

Figure 4

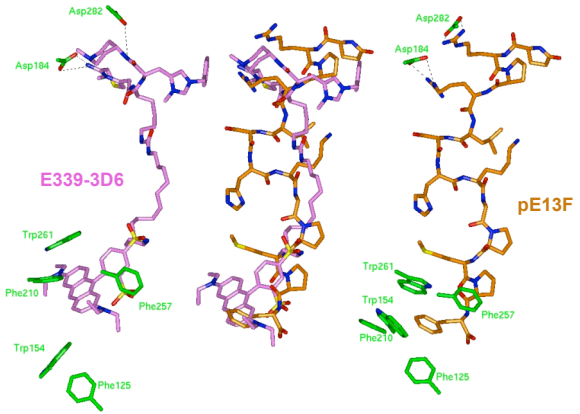

Asp282
Asp184
E339-3D6
Trp261
Phe210
Phe257
Trp154
Phe125
Asp282
Asp184
pE13F
Trp261
Trp154
Phe257
Phe210
Phe125

Figure 5

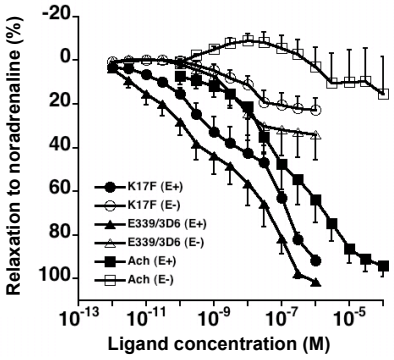
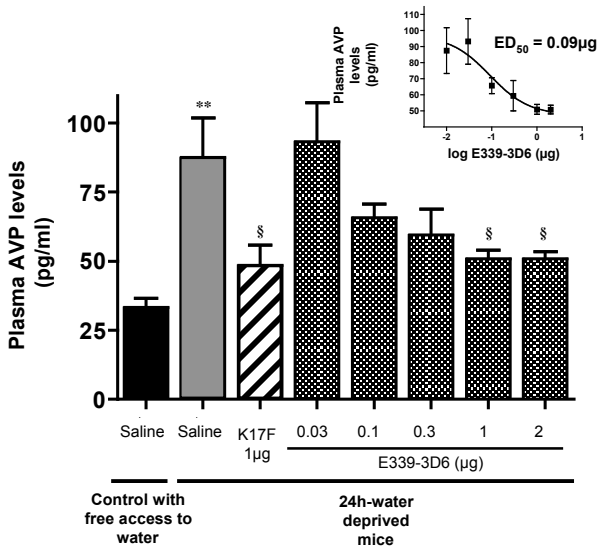

Relaxation to noradrenaline (%)
-20
0
20
40
60
80
100
K17F (E+)
K17F (E-)
E339/3D6 (E+)
E339/3D6 (E-)
Ach (E+)
Ach (E-)
10-13
10-11
10-9
10-7
10-5
Ligand concentration (M)

Figure 6


Plasma AVP levels (pg/ml)
100
75
50
25
0
**
§
§
§
Saline
Saline
K17F 1µg
0.03
0.1
0.3
1
2
E339-3D6 (µg)
Control with free access to water
24h-water deprived mice
Plasma AVP levels (pg/ml)
ED50 = 0.09µg
log E339-3D6 (µg)