Molecular Pharmacology Fast Forward. Published on March 2, 2006 as doi:10.1124/mol.105.021444
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MOL #21444

Modulation by LL-37 of the responses of salivary glands to purinergic agonists

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Molecular Pharmacology Fast Forward. Published on March 2, 2006 as DOI: 10.1124/mol.105.021444
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Running Title: LL-37 and salivary glands

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List of abbreviations*:* :CRAMP, cathelin-related antimicrobial peptide; hCAP, human cationic antibacterial protein; ; $[Ca^{2+}]_i$, intracellular concentration of calcium; EGFR, receptor for the epidermal growth factor; FPRL1, formyl peptide receptor-like 1; HEPES, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; fura-2/AM, fura-2 acetoxymethyl ester ; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl, 2-oleyl- *sn*-glycero-3-phosphocholine; HBS, HEPES-buffered saline ; LDH, lactate-dehydrogenase; PPI-PLC, polyphosphoinositide-specific phospholipase C; PAR-2, protease-activated receptor type 2; PMSF, phenylmethylsulphonyl fluoride

Composition of the manuscript
Number of pages: 39
Number of Figures: 10
Number of References: 41
Abstract: 204 words
Introduction: 727 words
Materials and Methods: 2,088 words
Results: 2,069 words
Discussion: 1,460 words

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Abstract

The interaction of mice submandibular gland cells with LL-37, a cationic peptide with immunomodulatory properties, was investigated. LL-37 at a concentration which did not affect the integrity of the cells increased the uptake of calcium and activated a calcium-insensitive phospholipase A_2 (PLA_2). The small release of ATP induced by LL-37 could not account for this stimulation since apyrase did not significantly block the response to LL-37. The divalent cation magnesium inhibited the response to LL-37 but this inhibition was probably non-specific since it also inhibited the in vitro bacteriostatic effect of the peptide. The increase of calcium uptake by LL-37 was not affected by KN-62, a rather specific inhibitor of $P2X_7$ receptors in mice. LL-37 also increased the intracellular concentration of calcium ($[Ca^{2+}]_i$) in cells from mice invalidated for these receptors. LL-37 had no effect on the response to carbachol. It inhibited the increase of the ($[Ca^{2+}]_{i.}$) and the activation of phospholipase D by ATP. It potentiated the activation of the PLA_2 by the nucleotide. Finally LL-37 increased the fluidity of the plasma membrane of submandibular gland cells. In conclusion, our results suggest that LL-37 is an autocrine regulator of submandibular gland cells. It does not stimulate mouse $P2X_7$ receptors but modulates their responses.

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Introduction

Cathelicidins are proteins involved in the first phases of our defenses against pathogens. Their isolation relied on the analogy of their N-terminal proregions with cathelin, an inhibitor of cathepsin L originally isolated from bovine leukocytes (Ritonja et al., 1989). This highly conserved proregion (about 100 amino acids) shares many similarities with cystatins, inhibitors of the cysteine proteases. The C-terminal domain of cathelicidins varies among species both in terms of length (12 to 100 amino acids) and of structure. The N- and C-terminal domains are separated by a sequence recognized by proteases. The digestion in the extracellular medium of the propeptides by elastase (Panyutich et al., 1997) or proteinase 3 (Sorensen et al., 2001) releases the C-terminal peptides originally described as antimicrobial but which now prove to be more immunomodulatory than antimicrobial in physiological conditions (Bowdish et al., 2005). Most of the studies on cathelicidins have focused on the C-terminal peptides. These peptides are ubiquitous. They are secreted by macrophages, lymphocytes, epithelial cells, keratinocytes, cells lining the upper respiratory tree, vaginal cells (Bals and Wilson, 2003). LL-37, the only peptide from human origin, is derived from an antibacterial protein of 18 kDa (hCAP-18)[1]. LL-37 and its only analog in mouse, the cathelin-related antimicrobial peptide (CRAMP) are cationic and transform from a random coil in aqueous solution to an amphipathic alpha helix at the contact of a membrane (Yeaman and Yount, 2003). The peptide binds to the bacteria by electrostatic interactions between the positive charges on one side of its alpha helix and the negative

charges of the bacteria. The hydrophobic side of the helix has a detergent effect on the membrane of the bacteria.

LL-37 not only kills bacteria but also binds to endotoxin (Bartlett et al., 2004), is chemotactic for human peripheral blood neutrophils, monocytes and T-lymphocytes through FPRL-1 receptor (Yang et al., 2000) and for mast cells through two other unknown receptors (Niyonsaba et al., 2002). In mast cells, it also promotes the release of histamine (Niyonsaba et al., 2001). It increases the release of chemokines from epithelial cells (Tjabringa et al., 2003) and promotes angiogenesis (Koczulla et al., 2003). It induces keratinocyte migration after epidermal growth factor receptor transactivation (Tokumaru et al., 2005). Binding sites for LL-37 have also been recently described in lung epithelial cells (Lau et al., 2005). It has been reported that LL-37 activates the purinergic $P2X_7$ receptor expressed by monocytes, leading to the release of interleukine-1β (IL-1β) by these cells (Elssner et al., 2004). These conclusions were mostly based on the use of antagonists of the $P2X_7$ receptors. These observations were at variance with the conclusions of Perregaux et al. (2002). These authors also reported that several antibacterial peptides promoted the release of IL-1β from macrophage but, according to them, this was best explained by the decrease in potassium content of the macrophages secondary to the increase in permeability of their plasma membrane.

The purpose of our work was to reconsider the possible interaction between LL-37 and $P2X_7$ receptors. These receptors are present throughout the body and especially in exocrine glands (North, 2002). Considering that the salivary glands express not only $P2X_7$ receptors but also cathelicidin and that the C-terminal peptide of cathelicidin is present in saliva (Van Nieuw Amerongen et al**.,** 2004), this peptide might have an

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autocrine effect on salivary glands and regulate the activity of salivary $P2X_7$ receptors. LL-37, the human peptide, is related (about 65 %) to the peptide from mouse origin (Pestonjamasp et al., 2001) and recent studies have demonstrated the efficacy of LL-37 in murine cell models (Kurosaka et al., 2005; Zughaier et al., 2005). For those reasons we decided to use a murine model which gave us the opportunity to compare the response of control mice and of mice with disrupted $P2X_7$ gene ($P2X_7$ R $^{-/-}$ mice, Solle et al., 2001). Our results show that LL-37 could mimic some of the responses to $P2X_7$ agonists (increase of the uptake of extracellular calcium and activation of PLA_2 activity). LL-37 also modulated the responses to extracellular ATP: it inhibited the increase of the intracellular concentration of calcium ($[Ca^{2+}]_i$) and the activation of phospholipase D (PLD) but potentiated the activation of PLA_2 in response to ATP. LL-37 also increased the $[Ca^{2+}]_i$ in salivary glands from $P2X_7R^{-/-}$ mice. LL-37 could thus elicit some cellular responses independently of $P2X_7$ receptors but also modulated the responses coupled to these receptors.

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Material and Methods

Drugs and animals

The experiments were carried out on male C57Bl/6J control mice and on male $P2X_7R^{-/-}$ mice kindly supplied by Pfizer Inc. (Groton, Ct) and obtained by homologous recombination (Solle et al., 2001). Breeding $P2X_7R^{-/-}$ mice males with females was used to maintain the colony of receptor-deficient animals. Mice used in the experiments were between 10 and 16 weeks of age. The animals were fed *ad libitum* and had free access to water. The care and use of the animals used in this study were approved by the Belgian Ministry of Agriculture in agreement with European regulations.

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was custom synthesized with 99% purity by Genemed Synthesis (South San Francisco, CA). Two batches of LL-37 were used for these experiments. Fura-2/AM and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Molecular Probes (Eugene, OR). Collagenase P and bovine serum albumin (BSA, fraction V) were from Roche Diagnostics (Mannheim, Germany). The glutamine-free amino acids mixture was from Gibco BRL (Paisley, Scotland). Adenosine 5'-triphosphate (ATP), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), leupeptin, pepstatin A, aprotinin, phenylmethylsulphonyl fluoride (PMSF), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and the ATP assay mix were obtained from Sigma Chemical (St Louis, Mo). Isooctane was from Fluka Chemie AG (Buchs, Switzerland), 1-palmitoyl, 2-oleyl- *sn*-glycero-3-phosphocholine (POPC) from Avanti Polar Lipids (Alabaster, AL) and 9,10-[^{3}H]oleic acid from American Radiolabeled Chemicals Inc. (St Louis, Mo). Silicagel-coated TLC plates were from Merck (Darmstadt, Germany). Methanol, ethylacetate and chloroform were from Labscan

Ltd (Dublin, Ireland). The scintillation solution Ecoscint A was from National Diagnostics (Atlanta, GA).

Preparation of a crude cellular suspension from mouse submandibular glands

The mice were anaesthetized and killed with ether. The submandibular glands were immediately dissected and finely minced. The minced tissue was digested in the presence of 0.4-0.5 U per ml collagenase P for 20 minutes at 37°C under constant shaking in 10 ml HEPES-buffered saline (HBS) medium containing (mM): 24.5 HEPES (pH 7.4), 96 NaCl, 6 KCl, 1 $MgCl_2$, 2.5 NaH_2PO_4, 11.5 glucose, 5 sodium pyruvate, 5 sodium glutamate, 5 sodium fumarate, 1% (v:v) glutamine-free amino acids mixture and 0.125% (w:v) bovine serum albumin (BSA). Ten minutes after the beginning of the digestion, the cells were aspirated five times with 10, 5 and 2 ml glass pipettes. At the end of the digestion the crude suspension was mechanically dispersed by gentle pipetting, filtered and washed in an isotonic NaCl solution. The last pellet was resuspended in HBS medium and kept at 4°C until use.

Membrane preparation

The cellular suspension from 3 mice was resuspended in 1 ml ice-cold TEEI buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 2.5 µg/ml aprotinin, 2.5 µg/ml pepstatin A, 2.5 µg/ml leupeptin) and passed through a 27G needle. The suspension was centrifuged at 1,000 x g for 10 minutes at 4°C. The pellet was extracted with 1 ml ice-cold TEEI buffer and centrifuged. This extraction was repeated three times. The four supernatants were pooled and centrifuged at 100,000 x g for 30 minutes at 4°C.

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LDH assay

LDH activity was measured in a spectrophotometer (Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974). Two ml Tris-EGTA-NADH (Tris: 56 mM; EGTA: 5.6 mM; NADH: 170 μM) were added to the cuvette of a spectrophotometer. Fifty μl of the enzyme solution (cellular supernatant or whole-cell extract) were then added. Two min later, 200 μl sodium pyruvate (14 mM) was added. The disappearance of the NADH was monitored at 340 nm for 5 min at 37°C.

ATP assay

The ATP present in the medium was estimated with a bioluminescent assay (Neufeld et al., 1975). Fifty μl of the cellular supernatant were added to a test tube which was transferred to the chamber of a Lumat LB9507 luminometer (EG&G Berthold GmbH,Wildbad, Germany). Known standards of ATP were prepared in the medium used for the incubations of the cells and 50 μl aliquots of these standards were assayed. The ATP assay mix was reconstituted according to the recommendations of the manufacturer and 20 μl of this mix were added to the samples. The emitted light was measured for 15 seconds.

Measurement of the $[Ca^{2+}]_i$

After its isolation, the crude pellet was resuspended in 6 ml fresh HBS medium in the presence of magnesium chloride, amino acids and albumin 0.1%. The suspension was kept at 4°C until use. One ml of this suspension was incubated in 2 ml HBS medium in

the presence of amino acids, 1 mM $MgCl_2$., 0.25 mM $CaCl_2$. and 0.5% (w:v) BSA. The suspension was incubated for 45 minutes at 25°C in the presence of 2 μM fura-2/AM. At the end of the incubation, 10 ml isotonic NaCl were added to the tube which was centrifuged for 1 minute at 500g. The supernatant was discarded and the pellet was resuspended in 4 ml fresh HBS medium, in the absence of amino acids, magnesium chloride or albumin but in the presence of 1 mM calcium chloride. Two ml of this suspension were transferred in the cuvette of a spectrofluorimeter and constantly agitated. The assay was performed at 25°C. The excitation wavelength was switched every second from 340 to 380 nm (slitwidth 8). The light emitted at 510 nm (slitwidth 16) was recorded. At the end of the assay, the traces were calibrated with the successive addition of 0.1 mM digitonin and 40 mM EGTA (pH 8.5 with Tris). The autofluorescence measured after quenching the fluorescence of the fura-2 by the addition of 100 mM $MnCl_2$ was subtracted from all the data before calculation of the ratios. The calcium concentration was estimated by the ratio method as described by Grynkiewicz et al. (1985).

Measurement of the activity of phospholipase A_2 (PLA_2)

Cells from 3 mice were resuspended in 1 ml HBS medium containing the amino acids mixture, 1 mM $CaCl_2$. and 1 mM $MgCl_2$., without albumin. The cells were incubated for 2 hours at 37°C in the presence of 5 μCi ^{3}[H]-oleic acid. At the end of this incubation the cells were washed twice with 10 ml isotonic NaCl. They were resuspended in 2 ml HBS medium containing the amino acids mixture, 0.5 mM calcium chloride, 1 mM magnesium chloride and 0.5% (w/v) BSA. They were incubated for one hour at

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37°C. At the end of this incubation, the cells were washed twice with 10 ml isotonic NaCl. The cells were then resuspended in 9 ml HBS containing the amino acids mixture, 0.5 mM calcium chloride and 0.1% BSA, but no magnesium chloride. Aliquots of 0.5 ml were incubated for 10 minutes at 37°C either in control or in the tested condition. Each condition was tested in triplicates. At the end of the incubation the cells were centrifuged and 0.4 ml of the supernatant was transferred to scintillation vials. In order to estimate the amount of ^{3}[H]-oleic acid present in the medium at the beginning of the incubation (blank values), a similar procedure was applied to non-incubated aliquots of cells. Aliquots of cells were also directly transferred to the scintillation vials to estimate the incorporation of the fatty acid in the cells (total counts). Three ml EcoscintA were added to each vial and the radioactivity was measured in a β-scintillation counter. The blank value was subtracted from the results obtained with the incubated cells and the results were expressed as percent of the radioactivity incorporated in the cells.

Measurement of the activity of phospholipase D (PLD)

The activity of PLD was assayed by measuring the formation of [^{3}H]phosphatidylethanol which is the product of its specific transphosphatidylation reaction in the presence of ethanol (Pochet et al., 2003). Cells from 3 mice were resuspended in 1 ml HBS medium in the presence of 0.5 mM $CaCl_2$. They were incubated for 90 min at room temperature in the presence of 5 μCi/ml [^{3}H] oleic acid. At the end of the labelling period, the cells were washed 3 times with isotonic NaCl. The final pellet was resuspended in 1 ml fresh HBS medium without the tracer, and incubated for 30 min at room temperature. The cells were washed and resuspended in 6.5 ml HBS

medium containing 1 mM $CaCl_2$ but no magnesium. The assays were performed at 37°C under constant shaking. One-ml aliquots of the cellular suspension were preincubated for 5 min with 1.5 % (v/v) ethanol before being incubated with the tested agent for the indicated period. The reaction was stopped by the addition of 3 ml of a mixture of chloroform/methanol (1:2). Phospholipids were extracted with 1 ml chloroform and 1 ml 2.4 N HCl. After 1 hour at 4°C, the two phases were separated by centrifugation at 1,000 g for 10 min. The upper phase was re-extracted with 1 ml chloroform and the 2 lower phases were pooled and washed with 2 ml of a mixture of methanol/1N HCl (1:1). After centrifugation, the upper phase was discarded and the lower phase evaporated to dryness. The lipid extract was dissolved in 50 µl chloroform and 20 µl were spotted onto a thin layer chromatography (TLC) plate. The plate was developed in the upper phase of a mixture of water/ethylacetate/acetic acid/isooctane (10:13:3:2). The lipids were visualized by exposure to iodine vapor and phosphatidylethanol was identified by comparison with an authentic standard. Phospholipids spots were scraped into scintillation vials containing 1 ml methanol. Ten ml Ecoscint A were added to each vial and their radioactivity was determined by ß liquid scintillation. The sum of the radioactivity in these bands was a measure of the incorporation of [^{3}H]-oleic acid in total phospholipids. The PLD activity was expressed as the percentage of the radioactivity present in the phosphatidylethanol spot when compared to the radioactivity in the total phospholipids.

Measurement of the membrane fluidity

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The fluidity of the membranes was estimated by fluorescence anisotropy (Van Laethem et al., 2003). The membrane pellet was resuspended in Hepes-buffered medium (10 mM HEPES pH 7.4 and 150 mM NaCl) and sonicated for 5 sec at 15 μm amplitude. The membranes were incubated in the absence or in the presence of 10 μM LL-37. Aliquots of these two membrane suspensions were labeled with the fluorescent probe DPH by adding the probe in tetrahydrofuran at a 1 μg/ml final concentration and incubating at 37°C for about 15 min. The measurements were performed in a SLM 8000C spectrofluorimeter with Glan-Thompson polarizers placed in T-geometry. Excitation was performed at 360 nm and emission was recorded at 430 nm. For each experiment, 2 ml HS with the labeled vesicles were transferred in a 10x10x45 mm acrylic cuvette (Kartell) placed in a thermostatic chamber. Polarization measurement was made by simultaneously measuring the vertical and horizontal components of the polarized emission. Correction for the background polarization due to sample turbidity was made using unlabeled samples. The ratio of the intensities in the vertically and horizontally polarized detectors was measured with vertically and horizontally polarized excitation, giving respectively the R_{vert} and R_{horiz} ratios. Polarization (P) was calculated as $P=(R_{corr}-1)/(R_{corr}+1)$ where $R = R_{vert}/R_{horiz}$. Anisotropy was derived from P using $r=2P/(3-P)$. Similar measurements were performed on POPC and DPPC multilamellar liposomes prepared as described by Bangham et al. (1965).

Hemolysis of murine red blood cells

Mice were anesthetized with ether. Blood was collected by venipuncture of the inferior vena cave just above the junction of the renal veins and EDTA (2 mg/ml) was

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added. Red blood cells were collected by centrifugation and washed three times with phosphate-buffered saline (PBS). The final pellet was resuspended with PBS at a 10% hematocrit. LL-37 (final concentration 10 µM) or 2 µl Triton X-100 were added to 98 µl of the cellular suspension and the cells were incubated for 30 min at 37°C. At the end of the incubation the tubes were centrifuged and the haemoglobin present in the supernatant was estimated by measuring the absorbance at 540 nm.

Bactericidal and bacteriostatic effect of LL-37

The assay was performed in a 96-well microplate. The bacteria (*E. Coli* strain ATCC 10536) were grown to the mid-logarithmic phase. About ten thousands CFU were incubated in the presence of 100 µl Mueller-Hinton medium and of serial dilutions of LL-37 (concentration range: 30 nM-20 µM). The plate was incubated for 16 h at 37°C. The plate was then scanned with the GS-690 imaging densitometer from BioRad. In order to evaluate the bactericidal property of the peptide, aliquots were removed from these wells and transferred to a Petri dish with the Mueller-Hinton medium. The presence of colonies was examined after an overnight incubation at 37°C.

Statistical analysis

Results are expressed as means ± s.e.m. of the number of experiments indicated. Statistical significance between various conditions was assessed with Student's *t* test.

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Results

LL-37 is generated by digestion of human cathelicidin by elastase or protease-3. The peptide has 37 amino acids with 2 leucines at its N-terminal side. With its 5 acidic and 11 basic residues, it is positively charged at physiological pH (pI = 10.6). It forms a perfect amphipathic alpha helix between residues 11 and 31 (Agerberth et al., 1995). This peptide is highly toxic for bacteria but has also some deleterious effects on the plasma membrane of eukaryotic cells (Oren et al., 1999). In preliminary experiments, the release of LDH from a cellular suspension of submandibular glands exposed to LL-37 was examined (Figure 1). After a 15-min incubation at 37°C, LL-37 did not promote the efflux of LDH when tested at concentrations up to 10 μM (from 1.8 ± 0.4 % in the absence to 2.9 ± 0.5 % in the presence of 10 μM LL-37, n=3; $P > 0.05$). A higher concentration of LL-37 (20 μM) significantly increased the release of LDH after 15 min (to 11.5 ± 1.9 %, $P < 0.05$, n=3). After a 60-min incubation LL-37 significantly increased the release of LDH at 10 μM (to 7.9 ± 1.0 %, $P < 0.05$) and at 20 μM (23.9 ± 3.1 %, $P < 0.01$). The peptide was then tested at a 10 μM concentration on the haemolysis of murine red blood cells. After a 30-min incubation at 37°C, the haemolysis averaged only 2% of the maximal haemolysis measured with Triton X-100. It was thus decided to test the peptide at concentrations not exceeding 10 μM.

Effect of LL-37 on the $[Ca^{2+}]_i$

Cells from submandibular glands were loaded with fura-2/AM. These cells were exposed to either carbachol (a muscarinic agonist activating a G protein), or ATP or LL-37 (Figure 2, upper panel). In response to 100 μM carbachol, the $[Ca^{2+}]_i$ sharply

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increased from a basal value of 127 ± 8 to 239 ± 21 nM (n=4) 5 seconds after the addition of the agonist. This peak value was followed by a decrease and the $[Ca^{2+}]_i$ stabilized at a plateau of approximately 200 nM, significantly higher than the resting level. This response was typical of the response elicited by a receptor coupled to a G protein and activating a polyphosphoinositide-specific phospholipase C (PPI-PLC). The response to 1 mM ATP was very different. The $[Ca^{2+}]_i$ initially increased from 107 ± 14 nM to 263 ± 22 nM (n=4) after 5 seconds and further increased for the next 3 minutes to reach 428 ± 40 nM (n=4). This response was the consequence of the formation of a non-selective cation channel by the $P2X_7$ receptor. Ten μM LL-37 increased the $[Ca^{2+}]_i$ from 105 ± 6 nM to 152 ± 12 nM (n=8) after 5 seconds and to 236 ± 20 nM after 3 minutes. The response to LL-37 was dose-dependent between 1 and 10 μM (Figure 3).

In the absence of extracellular calcium, carbachol increased the $[Ca^{2+}]_i$ from 27 ± 5 nM to 62 ± 8 nM (Figure 4, insert; n=3, $P < 0.05$) confirming that the muscarinic agonist could mobilize intracellular pools of calcium. ATP had no effect on the $[Ca^{2+}]_i$ confirming that the nucleotide did not stimulate a metabotropic receptor coupled to the hydrolysis of polyphosphoinositides. LL-37 significantly decreased the $[Ca^{2+}]_i$ from 57 ± 2 nM to 44 ± 3 nM after 90 sec (n=3, $P < 0.05$). After a preincubation for 2 min in control conditions and in the absence of calcium, the addition to the medium of 1 mM calcium significantly increased the $[Ca^{2+}]_i$ from 49 ± 2 nM to 113 ± 9 nM (n=6) ($P < 0.05$) within 10 seconds (Figure 4). When the cells had been exposed for 2 minutes to ATP, the addition of calcium to the medium increased the $[Ca^{2+}]_i$ to 165 ± 9 nM after 5 seconds and to 321 ± 30 nM (n= 4) after 3 minutes ($P < 0.001$). LL-37 (10 μM) also increased the uptake of extracellular calcium: the $[Ca^{2+}]_i$ averaged 206 ± 26 nM after 5 seconds and

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remained stable (205 ± 30 nM after 3 minutes (n=3, $P < 0.01$)). From these results it could be concluded that ATP and LL-37 did not mobilize intracellular pools of calcium but rather increased the uptake of extracellular calcium.

Interaction between LL-37 and muscarinic or purinergic agonists on the $[Ca^{2+}]_i$

The interaction between LL-37 and carbachol or ATP was next explored. The cells were exposed for 5 minutes to 10 μM LL-37 before stimulation with either 100 μM carbachol or 1 mM ATP. As shown in the upper panel of Figure 5, the preincubation with LL-37 had no effect on the response to carbachol. The response to 1 mM ATP was inhibited by LL-37 (Figure 5, lower panel). In the absence of LL-37 the variation of the $[Ca^{2+}]_i$ after 2 min exposure to the nucleotide averaged +323 ± 38 nM (n=6) in cells preincubated with DMSO and +198 ± 16 nM (n=4) in cells preincubated with 10 μM LL-37 ($P < 0.05$).

Contribution of ATP and purinergic receptors in the response to LL-37

The time-course of the response to LL-37 and its dependency towards extracellular calcium suggested that an ionotropic purinergic receptor might be involved in the response to the peptide. Considering that cationic peptides can permeabilize the plasma membrane of eukaryotic cells (Oren et al., 1999), we considered the possibility that the response to LL-37 was merely the consequence of the release of ATP induced by the peptide. The cells were incubated with 10 μM LL-37. After centrifugation, the ATP content of the medium was estimated with a luciferin-luciferase mixture (Neufeld et al., 1975). Cells were incubated in a test tube in the chamber of the luminometer and preincubated with the ATP assay mix before the addition of LL-37 (Figure 6). In these

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conditions a significant increase (P = 0.015, n=6) of the light emitted was noted. This increase was transient and significantly lower 15 seconds after the addition of the peptide (P = 0.029, n=6). The contribution of this small release of ATP in the response to a lower (3 μM) concentration of LL-37 was examined in the presence of apyrase or magnesium ion. Apyrase is an ATP diphosphohydrolase which catalyzes the degradation of ATP; magnesium ion forms a complex with ATP and decreases the concentration of ATP^{4-}, the true agonist of P2X receptors. Apyrase had no effect on the increase of the $[Ca^{2+}]_i$ in response to carbachol (data not shown). The enzyme did not significantly affect the acute response to 300 μM ATP (Figure 7, upper panel) but fully suppressed its late effect (from +118 ± 9 nM (n=4) in the absence of apyrase to +34 ± 4 nM (n=5) in the presence of the enzyme, 4 minutes after the addition of ATP, P < 0.0001, non-paired *t* test). The response to 3 μM LL-37 was not inhibited by the enzyme (from +89 ± 19 nM, n=5 in the absence to +56 ± 15 nM, n=4 in the presence of 5 U/ml apyrase, after a 4-min exposure to LL-37, P = 0.2574) (Figure 7, lower panel). As shown in Figure 8, the addition of 5 mM magnesium to the extracellular medium inhibited by more than 80 % the response to 1 mM ATP (from +148 ± 17, n=8 to +27 ± 4 nM , n=7; P < 0.0001) and by 50 % the response to LL-37 (from +81 ± 7 nM, n=3 to +37 ± 14 nM, n=3; P = 0.048). Since the results obtained with apyrase and magnesium were not consistent, we decided to test the effect of magnesium on the "bactericidal" activity of LL-37. Five mM magnesium blocked the bacteriostatic and bactericidal effects of LL-37 (data not shown) confirming that magnesium inhibited the peptide itself. From these results and considering that apyrase did not inhibit the increase of the $[Ca^{2+}]_i$ in response to LL-37, we concluded that released ATP did not mediate the effect of LL-37 on calcium.

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It has been recently reported that LL-37 could activate by itself human $P2X_7$ receptors (Elssner et al., 2004). We thus tested the effect of KN-62, a calmidazolium derivative which is a strong inhibitor of some $P2X_7$ receptors (Virginio et al., 1997). As shown in the upper panel of Figure 9, 10 μM KN-62 inhibited the response to 1 mM ATP by 50% (from +130 ± 23 nM 2 min after ATP in control cells to +65 ±7 nM in cells treated with KN-62, n=4; P = 0.038). KN-62 had no significant effect on the response to LL-37 (Figure 9). These results suggested that the $P2X_7$ receptors were probably not involved in the increase of the $[Ca^{2+}]_i$ in response to LL-37. This was confirmed using cells isolated from salivary glands of $P2X_7R^{-/-}$ mice (Solle et al., 2001). As shown in the lower panel of Figure 2, these cells responded normally to carbachol. Their response to extracellular ATP was deeply blunted and had a totally different time-course when compared to cells from wild-type animals. ATP only transiently increased the $[Ca^{2+}]_i$ from 99 ± 6 nM to 125 ± 8 nM (n=3). The variation of the $[Ca^{2+}]_i$ in response to LL-37 was not affected by the absence of $P2X_7$ receptors (from 92 ± 2 nM to 233 ±14 nM, n=7).

Effect of LL-37 on the activity of phospholipases and on the fluidity of the plasma membrane

The $P2X_7$ receptor regulates the activity of various phospholipases in salivary glands (Alzola et al., 1998; Pochet et al., 2003). The activity of PLD was estimated by measuring the production of Peth in cells labeled with [^{3}H]-oleic acid and incubated in the presence of ethanol. As shown in the upper panel of Figure 10, the purinergic agonist and carbachol increased the production of Peth 3.5- and 2-fold respectively. By itself 10 μM LL-37 had no effect on the concentration of this phospholipid (90 ± 5%, n=5 when

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compared to control). LL-37 inhibited the activation of PLD by ATP from 348 ± 16% (n=4) in the absence to 196 ± 16% (n=4) in the presence of LL-37. The peptide had no effect on the stimulation of PLD by carbachol (Figure 10, upper panel).

To measure the activity of PLA_2, the cells were labeled with [^{3}H]-oleic acid and after a wash-out period, the release of the fatty acid in the medium was estimated. As shown in the lower panel of Figure 10, carbachol had no significant effect on this activity. Extracellular ATP increased the release of oleic acid 2.5-fold. Removal of the extracellular calcium had no effect on the basal release of oleic acid but inhibited by 60% (from + 170% to + 70%) the stimulation by ATP. LL-37 also promoted the release of oleic acid. In the presence of extracellular calcium the response to 10 μM LL-37 was very similar to the response to ATP. Removal of calcium had no effect on the response to LL-37 (from 7.1 ± 0.8% (n=5) in the presence of calcium to 7.4 ± 0.5% (n=4) in the absence of the ion). The combination of the two stimuli further increased the release of oleic acid. The synergism was better observed in the absence of calcium: more than 15% of the total oleic acid incorporated in the lipids was released when ATP and LL-37 were added together. Activation of the muscarinic receptors did not affect the release of oleic acid either in basal conditions or in the presence of LL-37. The effect of LL-37 on the fluidity of the plasma membrane was next tested. Membranes were isolated and incubated with a fluorescent probe (DPH). The movement of the probe was estimated by measuring the anisotropy at various temperatures in control membranes or in membranes preincubated with 10 μM LL-37. The anisotropy of micelles DPPC at 20°C (0.315 ± 0.008, n=3) was much higher than the anisotropy of micelles of POPC (0.117 ± 0.009, n=3). The anisotropy decreased at higher temperatures in both micelles (data not shown).

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This result is consistent with the fact that the presence of an unsaturated fatty acid or a high temperature increases the fluidity of the micelles. By comparison, the anisotropy of control membranes was intermediate (0.183 ± 0.006 at 20°C, n=6). The pretreatment with LL-37 significantly decreased the anisotropy (0.163 ± 0.004 at 20°C, n=6, $P < 0.05$ when compared to control membranes). This small effect was consistent in the 6 experiments and significant at every temperature tested.

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Discussion

Our results show that in submandibular glands from control mice, LL-37 increased the $[Ca^{2+}]_i$ and stimulated a calcium-insensitive PLA_2. These responses were observed at concentrations of LL-37 which had no effect on the response to carbachol. This last result confirmed that LL-37 did not affect the integrity of the muscarinic receptors, their coupling to a PPI-PLC, the filling state of intracellular pools of calcium and their mobilization by inositol phosphates. LL-37 did not activate a receptor coupled to the activation of a PPI-PLC since LL-37 did not mobilize intracellular pools of calcium but only increased the uptake of extracellular calcium.

These responses to LL-37 were not secondary to the release of ATP. Indeed, the measurement of the ATP content in the incubation medium revealed that LL-37 evoked a very small and transient release of ATP. It must be stressed however that these measurements probably underestimate the concentration of ATP at the vicinity of the purinergic receptors. The contribution of ATP in the response to LL-37 was evaluated by the addition of apyrase in the incubation medium. This enzyme which degrades ATP to AMP and pyrophosphate strongly inhibited the response to exogenous ATP. It did not inhibit the response to LL-37. Similar results were obtained with cells incubated in the presence of glucose and hexokinase (data not shown). These results are not consistent with those of Elssner et al. (2004) who also reported that LL-37 could indeed provoke an efflux of ATP from lipopolysaccharide-primed monocytes but that apyrase inhibited the release of IL-1β by these cells in response to LL-37.

Since apyrase (and hexokinase) did not inhibit the response to LL-37 and since Elssner et al. (2004) reported that the peptide could stimulate $P2X_7$ receptors, the effect

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of KN-62, a rather specific antagonist of these receptors was tested on the response to LL-37. This calmidazolium derivative inhibited the response to ATP without affecting the response to LL-37. This is at variance with the results of Elssner et al. (2004) who reported that KN-62 could block the activation of macrophages by LL-37 and suggests that, in mouse salivary glands, the response to LL-37 was not mediated by $P2X_7$ receptors. Our results were confirmed in mice lacking the $P2X_7$ receptor. ATP only slightly increased the $[Ca^{2+}]_i$ in cells from the salivary glands of these mice and this small response was strictly dependent on the presence of calcium in the medium suggesting that it involved another P2X receptor (Pochet et al., in preparation). Cells from $P2X_7R^{-/-}$ mice which were thus devoid of $P2X_7$ receptors normally responded to LL-37 with respect to the $[Ca^{2+}]_i$. This result confirmed that the mouse $P2X_7$ receptors were not required to observe an increase of the $[Ca^{2+}]_i$ in response to LL-37.

It has been reported that LL-37 could increase the $[Ca^{2+}]_i$ in other cells like mast cells (Niyonsaba et al., 2001) or neutrophils (Yang et al., 2000). In these systems, LL-37 activated a PPI-PLC and mobilized intracellular pools of calcium after activating a plasma membrane receptor coupled to a GTP-binding protein like FPRL1 (Yang et al., 2000) or another receptor not yet defined (Niyonsaba et al., 2002). This mechanism is very unlikely in our model: LL-37 did not mobilize intracellular pools of calcium since it did not increase the $[Ca^{2+}]_i$ in a calcium-free medium. More recently Tjabringa et al. (2003) reported that LL-37 could stimulate EGFR in epithelial cells. This stimulation was blocked by G6001, an inhibitor of metalloproteases. They concluded that LL-37 might activate a metalloprotease and release an endogenous activator of EGFR. The response to LL-37 was not affected by either an activator or an inhibitor of the PAR-2, the protease-

activated receptor expressed by the salivary glands (J.P. Dehaye, unpublished results) suggesting that this receptor was not involved in the response to LL-37. From all these negative results it can be concluded that the uptake of calcium induced by LL-37 in mouse submandibular gland was not secondary to the activation of a murine purinergic or protease-activated receptor or a receptor coupled to a G protein activating a PPI-PLC.

Salivary glands express various phospholipases A_2 (Alzola et al., 1998) and D (Pochet et al., 2003) among which some are activated by an increase of the $[Ca^{2+}]_i$. Both ATP and LL-37 increased a PLA_2 activity. The activation of PLA_2 by ATP is absent in cells from $P2X_7R^{-/-}$ mice confirming that $P2X_7$ receptors are involved in this response (E. Kabré, personal communication). The response to LL-37 was not mediated by these receptors since it was present in cells from $P2X_7R^{-/-}$ mice (E. Kabré, personal communication). The activation by LL-37 was not secondary to the increase of the $[Ca^{2+}]_i$ since it could still be observed in the absence of extracellular calcium. In these ionic conditions, the combination of ATP and LL-37 led to a much larger increase of the PLA_2 activity. The effect of LL-37 on the activity of PLA_2 has never been measured directly but it has been previously reported that LL-37, at the opposite of human defensin, did not promote the release of prostaglandins from mast cells (Niyonsaba et al., 2001). Yet the modulation of the PLA_2 activity by amphipathic peptides is a well-known phenomenon: mellitin, a strong activator of PLA_2, shares with LL-37 a high content of basic residues and the formation of an amphipathic alpha helix (Bucki et al., 2004). More recently it has been reported that antibacterial peptides with a high affinity for phosphatidylcholines can activate a secretory PLA_2 (Zhao and Kinnunen, 2003). LL-37 had no effect on the activity of the phospholipase D. This result was unexpected considering that other

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cationic antimicrobial peptides like mellitin or dermaseptin are activators of PLD respectively in human monocytic leukemia cells (Saini et al., 1999) and in polymorphonuclear leukocytes (Ammar et al., 1998). LL-37 specifically interfered with the purinergic regulation of PLD: it inhibited by nearly 50% the response to ATP without affecting the response to carbachol.

The previous results emphasized the fact that the interactions between LL-37 and purinergic responses were complex. By itself, LL-37 did not reproduce all the responses to ATP (it did not activate a PLD). When combined with ATP, LL-37 inhibited some responses (increase of the $[Ca^{2+}]_i$ and activation of PLD) but potentiated others (activation of PLA_2). These contradictory effects of LL-37 on cellular responses induced by $P2X_7$ receptors exclude a direct activation of the receptor by LL-37. The responses to LL-37 might be explained by the formation of a pore in the plasma membrane, the subsequent influx of calcium and efflux of potassium responsible for the activation of a PLA_2 (Andrei et al., 2004). The interaction of the peptide with the lipid phase of the plasma membrane might also account for its effects on the regulation by ATP of phospholipase A_2 (activation) and PLD (inhibition). Indeed according to Rao (1992), membrane active peptides activate PLA_2 and inhibit PLD activity when tested on unilamellar vesicles and these responses are best explained by a modification of the packaging of the lipid. It should be noted that LL-37 slightly increased the fluidity of the plasma membrane. LL-37 might thus regulate the cellular function by modifying the physico-chemical state of the membrane rather than by interacting with a plasma membrane receptor. Such a model has been proposed by Zughaier et al. (2005). According to these authors the synergy between endotoxin and LL-37 on the release of

reactive oxygen species is mediated by a better interaction of the peptide with the plasma membrane and not by a direct interaction with TRL4, the receptor for lipopolysaccharides. This model is in agreement with the general model proposed by Perregaux et al. (2002) who proposed that the cationic peptides activate macrophages by perturbing their plasma membrane. It is also consistent with the recent results of Lau et al. (2006). These authors reported that concentrations of LL-37 similar to the concentrations used in our study could induce apoptotic lesions in thymocytes. We observed that LL-37 provoked a small release of ATP and a slight efflux of calcium when the cells were incubated in a calcium-free medium. A significant release of LDH was observed at high concentrations of LL-37. From these results we cannot exclude that the responses of salivary glands to LL-37 were secondary to proapoptotic lesions provoked by the peptide.

In conclusion, our results show that LL-37 regulates the activity of submandibular gland cells. This result implies that salivary glands contribute to the host defense mechanisms not only by secreting LL-37 (Murakami et al., 2002; Woo et al., 2003) but also by the cellular responses like the activation of PLA_2 locally triggered by the peptide. The response of submandibular glands to LL-37 does not require the expression of $P2X_7$ receptors but the peptide can nevertheless modulate the responses to this receptor probably by its effect on the properties of the plasma membrane.

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Acknowledgements

The authors wish to thank Dr C. Gabel from Pfizer Inc. for his generous gift of the $P2X_7R^{-/-}$ mice. The scintillation counter was a gift from SOPAR PHARMA (Belgium).

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Molecular Pharmacology Fast Forward. Published on March 2, 2006 as DOI: 10.1124/mol.105.021444
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Footnotes

This work was supported by grant n° 3.4506.00 from the Fonds de la Recherche Scientifique Médicale of Belgium and a grant from the Emile Defay Fund to JPD and by "Fundación benefico-docente Jesus Gangoiti-Barrera" and grants 9/UPV00042.310-15941/2004 from the University of the Basque to A.M. S.P. is a postdoctoral researcher of the Fonds National de la Recherche Scientifique of Belgium.

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Legend to figures

***Figure 1*: Effect of LL-37 on the release of LDH from submandibular gland cells**

A crude cellular suspension was prepared from mice submandibular glands and incubated for 15 or 60 min in the presence of various concentrations of LL-37. At the end of the incubation the cells were centrifuged and the LDH activity of the supernanatnt was estimated. An aliquot of the cellular suspension was homogenized and its LDH activity was assayed (total activity of the cell extract). The results are expressed as percentage of the total LDH activity released in the medium during the experiment. They are the means ± s.e.m. of 3 experiments. * $P < 0.01$; ** $P < 0.005$.

***Figure 2*: Effect of carbachol, ATP and LL-37 on the $[Ca^{2+}]_i$ in cells from mice submandibular glands**

A crude cellular suspension was prepared from submandibular glands of $P2X_7R^{+}/^{+}$ (upper panel) or $P2X_7R^{-}/^{-}$ (lower panel) mice. The cells were loaded with fura-2/AM and after washing they were resuspended in HBS medium, in the absence of amino acids, $MgCl_2$ and BSA but in the presence of 1 mM $CaCl_2$. They were transferred in the cuvette of a spectrofluorimeter and incubated at 25°C under constant agitation. Two min after the start of the incubation, the cells were exposed to either 100 μM carbachol, 1 mM ATP or 10 μM LL-37. At the end of the incubation the traces were calibrated as described in Materials and Methods. Results are the means ± s.e.m. of 3 to 8 experiments.

***Figure 3*: Effect of various concentrations of LL-37 on the $[Ca^{2+}]_i$**

Cells from submandibular glands loaded with fura-2 were exposed to 0, 1, 3 or 10 μM LL-37. Results are the variation of the $[Ca^{2+}]_i$ one min before and one min after the addition of DMSO or LL-37 to the medium. Results are the means ± s.e.m. of 4 to 11 experiments. * $P < 0.05$; ** $P < 0.01$.

***Figure 4*: Effect of ATP and LL-37 on the mobilization of intracellular pools of calcium and on the uptake of extracellular calcium**

Cells from submandibular glands loaded with fura-2 were resuspended in a calcium-free medium. Thirty sec after the beginning of the measurement, 100 μM EGTA was added to the medium (A) and 30 sec later the cells were exposed to either DMSO or to 1 mM ATP or 10 μM LL-37 (B). Two min later 1 mM $CaCl_2$ was added to the medium (Ca). Insert: Cells were incubated in the same conditions but were exposed to 100 μM carbachol at 1 min. Results are the means ± s.e.m. of 3 experiments.

***Figure 5*: Effect of LL-37 on the variation of the $[Ca^{2+}]_i$ in response to carbachol and ATP**

Cells from submandibular glands loaded with fura-2 were resuspended in a medium containing 1 mM $CaCl_2$. Two min after the beginning of the measurement, the cells were exposed to either 0.5% DMSO (open symbols) or to 10 μM LL-37 (closed symbols). Five min later 100 μM carbachol (Cb, upper panel) or 1 mM ATP (lower panel) was added to the medium. The results are expressed as the variation of the $[Ca^{2+}]_i$ when compared to $[Ca^{2+}]_i$ at 6 min. They are the means ± s.e.m. of 3 to 6 experiments.

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***Figure 6*: Effect of LL-37 on the release of ATP**

Cells from submandibular glands were resuspended in a medium without calcium. Aliquots (190 µl) were transferred to a test tube containing 10 µl of the ATP assay mix. The test tube was placed in the chamber of the luminometer and the light emitted was integrated for 15 sec (-15 → 0). Ten µl DMSO or LL-37 (final concentration: 10 µM) were added and the light was measured for the next 30 seconds (0 → +15 and +15 → +30). Results are the means ± s.e.m. of 6 experiments. * $P < 0.05$

***Figure 7*: Effect of apyrase on the variation of the $[Ca^{2+}]_i$ in response to ATP or LL-37**

Cells from submandibular glands loaded with fura-2 were resuspended in the presence of 1 mM $CaCl_2$ in the absence (open symbols) or in the presence (closed symbols) of 5 U/ml apyrase. One min after the start of the measurement, the cells were exposed to 300 µM ATP (upper panel) or 3 µM LL-37 (lower panel). Results are expressed as the variation of the $[Ca^{2+}]_i$ when compared to the initial $[Ca^{2+}]_i$. They are the means ± s.e.m. of 4 or 5 experiments.

***Figure 8*: Effect of magnesium on the variation of the $[Ca^{2+}]_i$ in response to ATP or LL-37**

Cells from submandibular glands loaded with fura-2 were resuspended in the presence of 1 mM $CaCl_2$ in the absence (open symbols) or in the presence (closed symbols) of 5 mM $MgCl_2$. One min after the start of the measurement the cells were exposed to 1 mM ATP (upper panel) or 3 µM LL-37 (lower panel). Results are expressed as the variation of the

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$[Ca^{2+}]_i$ when compared to the initial $[Ca^{2+}]_i$. They are the means ± s.e.m. of 3 to 8 experiments.

***Figure 9*: Effect of KN-62 on the variation of the $[Ca^{2+}]_i$ in response to ATP or LL-37**

Cells from submandibular glands loaded with fura-2 were resuspended in the presence of 1 mM $CaCl_2$ and preincubated for 10 minutes in the absence (open symbols) or in the presence (closed symbols) of 10 μM KN-62. One min after the start of the measurement, the cells were exposed to 1 mM ATP (upper panel) or 3 μM LL-37 (lower panel). Results are expressed as the variation of the $[Ca^{2+}]_i$ when compared to the initial $[Ca^{2+}]_i$. They are the means ± s.e.m. of 4 experiments.

***Figure 10*: Effect of ATP, carbachol and LL-37 on the PLD and PLA_2 activities of submandibular cells.**

Cells from mice submandibular glands were labelled with [^{3}H]oleic acid (5 μCi/ml) for 90 minutes and allowed to equilibrate for 30 minutes. ***Upper panel (PLD):*** The cells were resuspended in HBS medium with 1 mM $CaCl_2$ but without magnesium. One-ml aliquots were preincubated with 1.5% ethanol for 5 minutes. Cells were then incubated for 30 minutes at 37°C in the presence of 100 μM carbachol or 1 mM ATP, in the absence or in the presence of 10 μM LL-37. At the end of the incubation, the lipids were extracted, separated by TLC and [^{3}H]-phosphatidylethanol (Peth) formation was measured. ***Lower panel (PLA_2):*** The cells were resuspended in HBS medium without magnesium but with 1 mM $CaCl_2$ or without $CaCl_2$ but with 100 μM EGTA. The cells

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were incubated at 37°C for 10 minutes in the presence of 0.5% DMSO or 1 mM ATP or 100 μM carbachol or 10 μM LL-37. The radioactivity present in the medium at the beginning of the experiment was estimated in non-incubated samples and subtracted from the incubated samples. An aliquot of cells was counted to estimate the total radioactivity of cells. Results are expressed as percent of control (PLD) or as the total radioactivity in the cells (PLA_2) and are the means ± s.e.m. of 3 to 5 experiments (upper panel) or of 3 experiments (lower panel). * $P < 0.05$ when compared to the control condition (absence of ATP); ** $P < 0.01$ when compared to the control condition (absence of LL-37).

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Figure 1

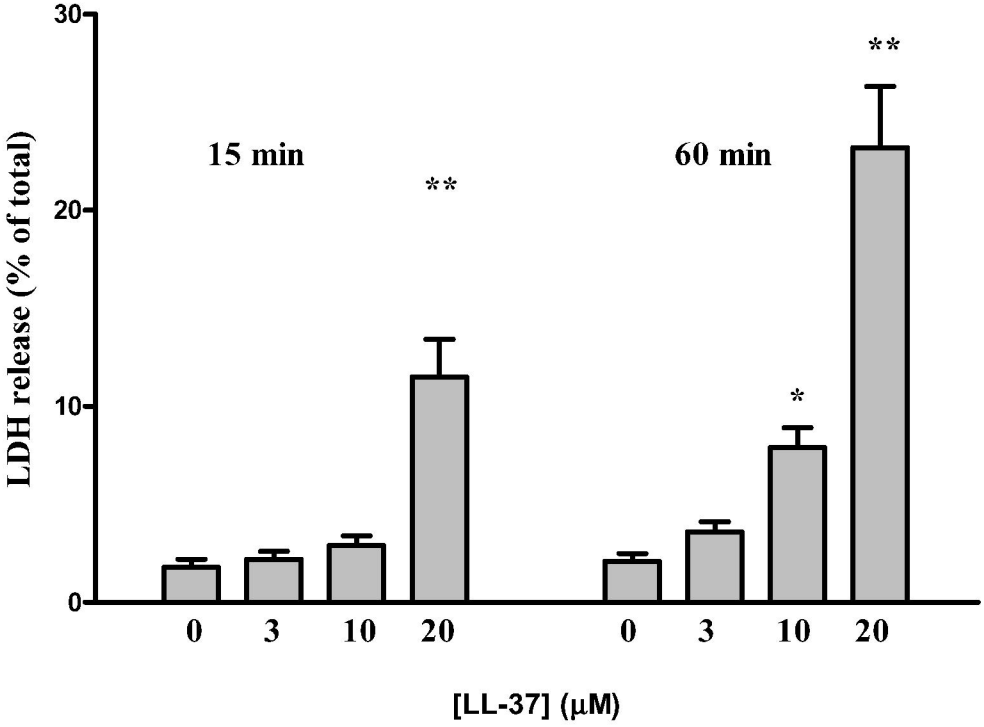

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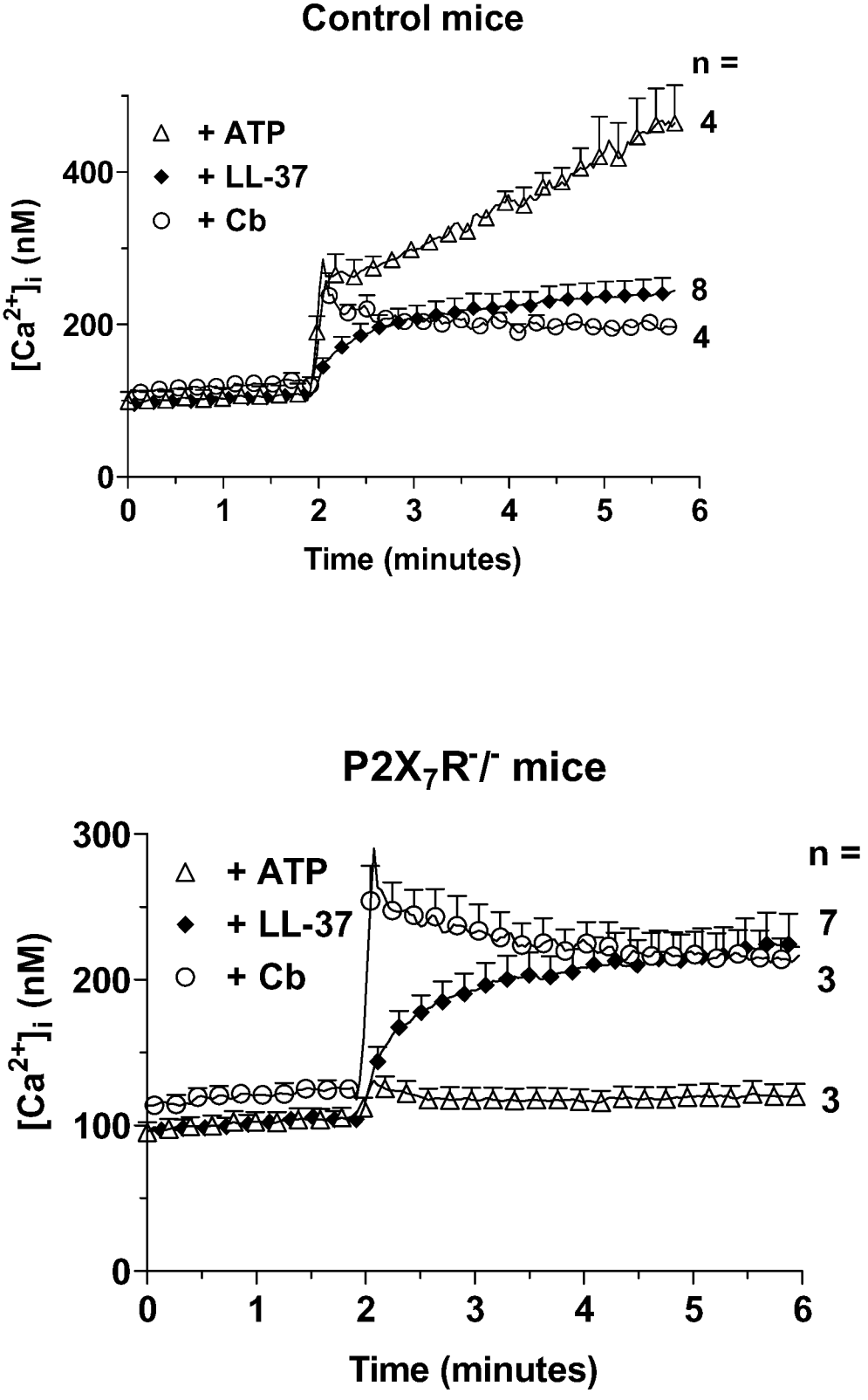
Figure 2



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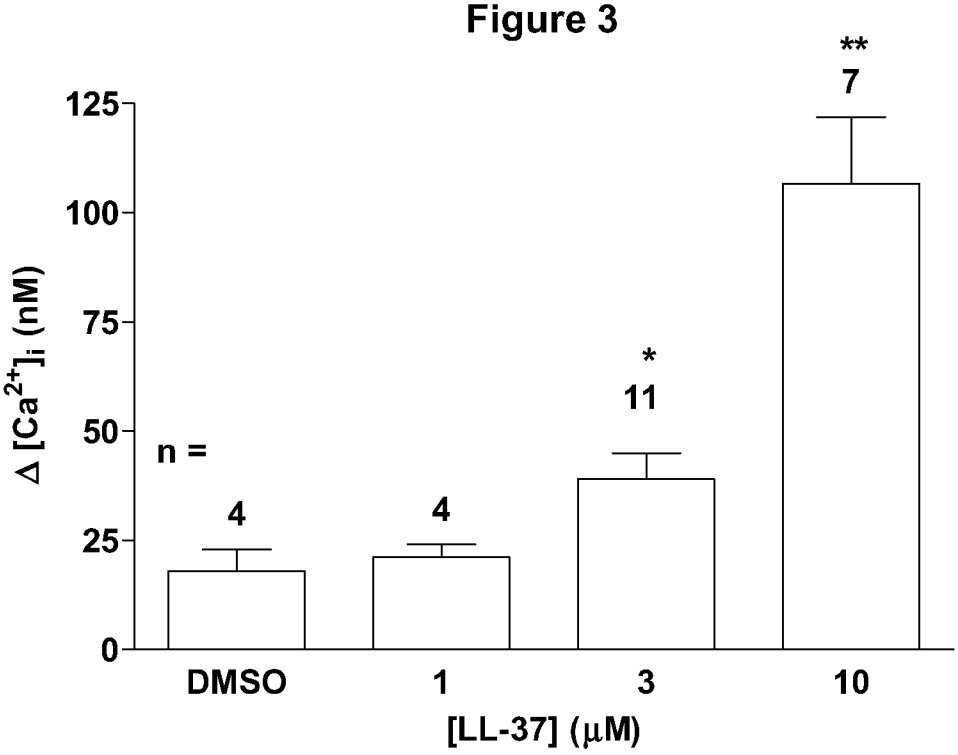

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Figure 4

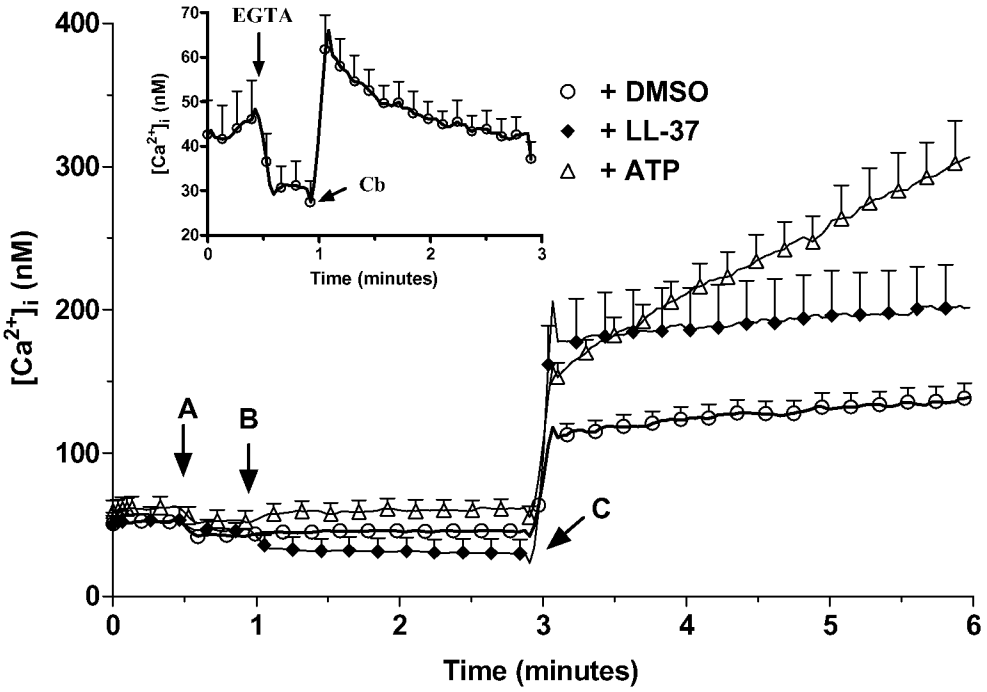

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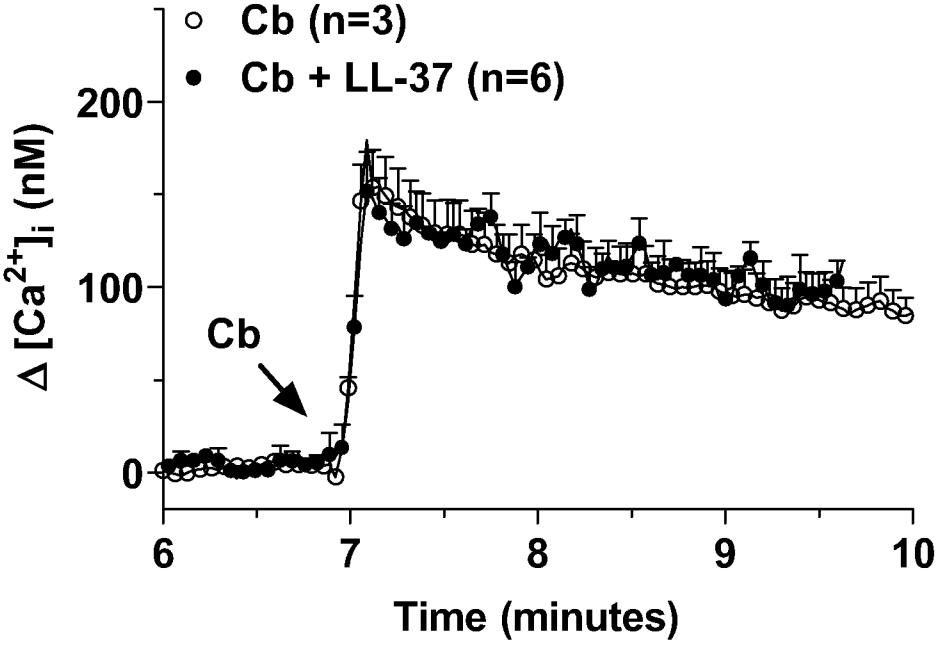
Figure 5



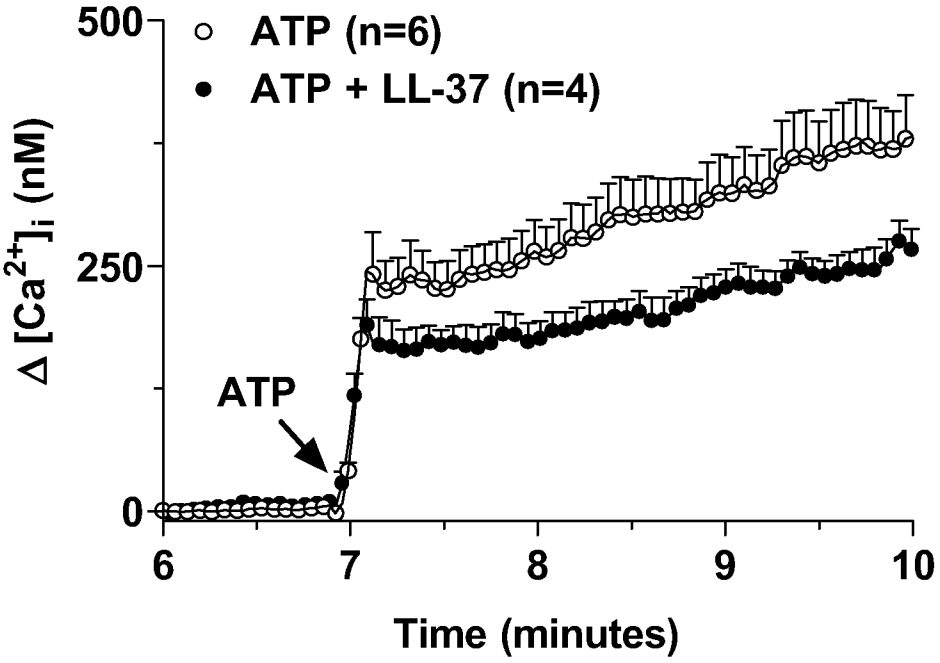


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Figure 6

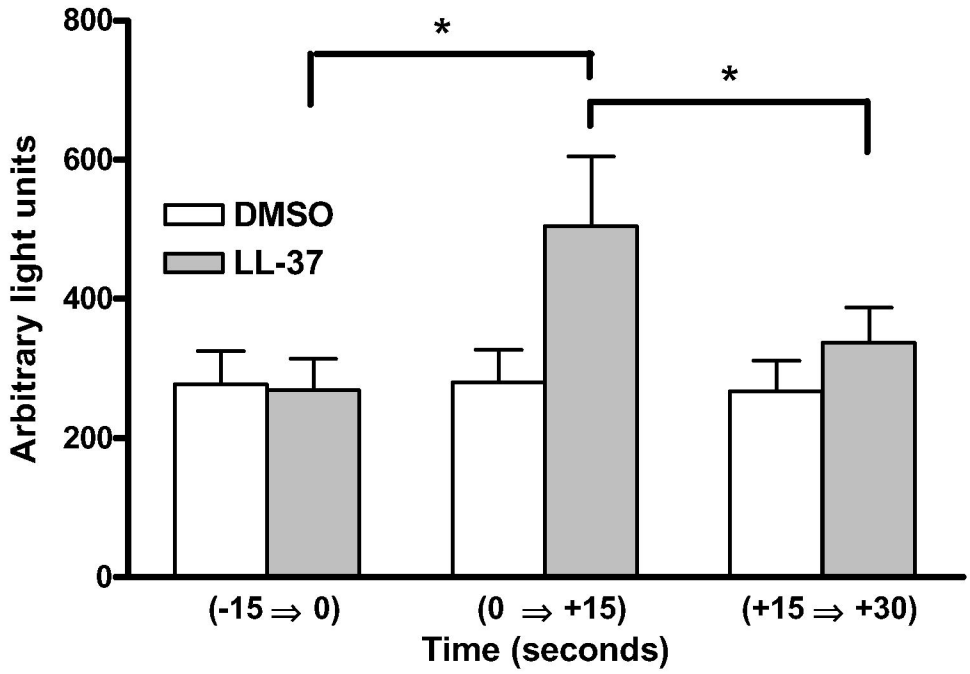

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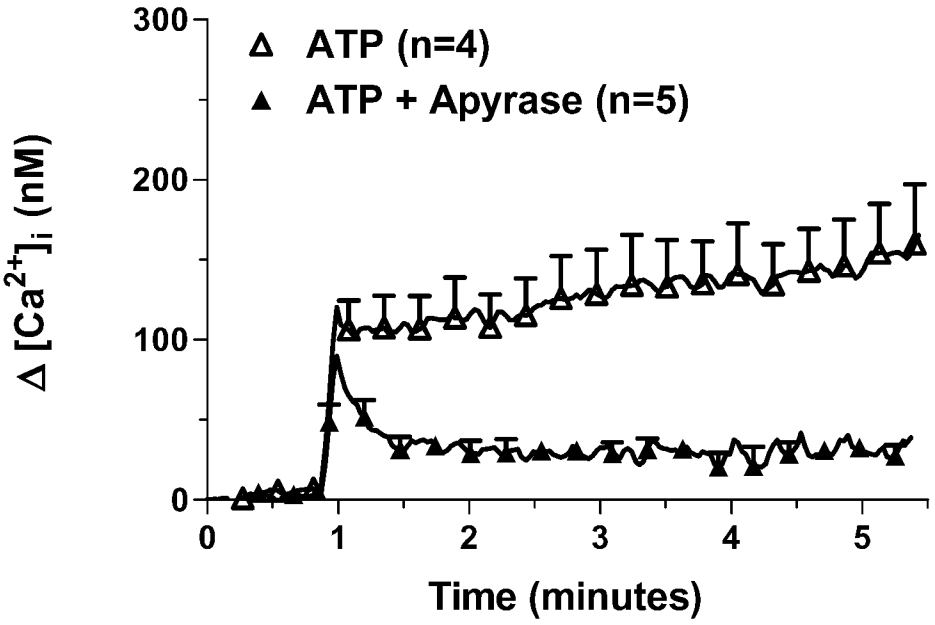
Figure 7



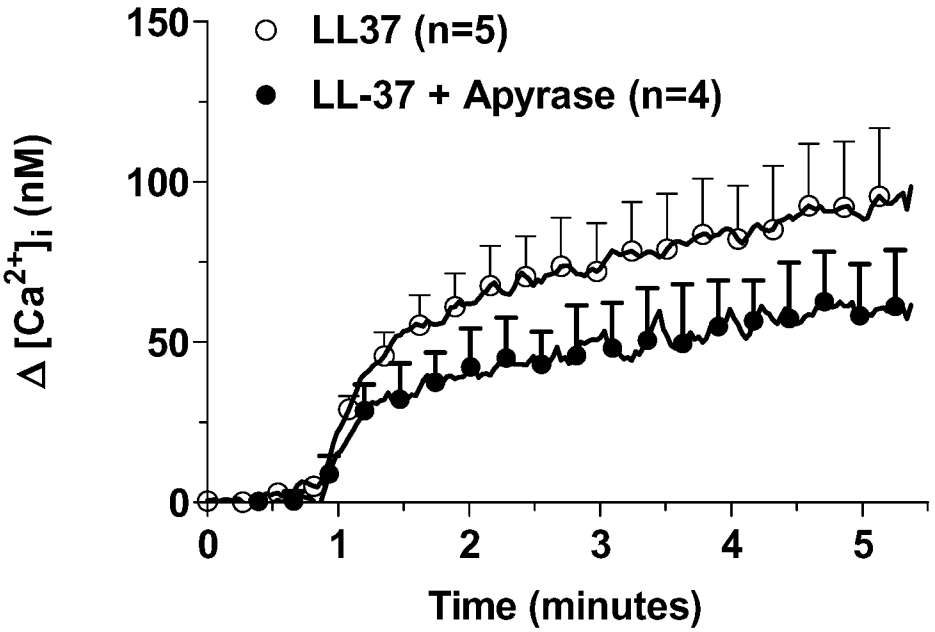


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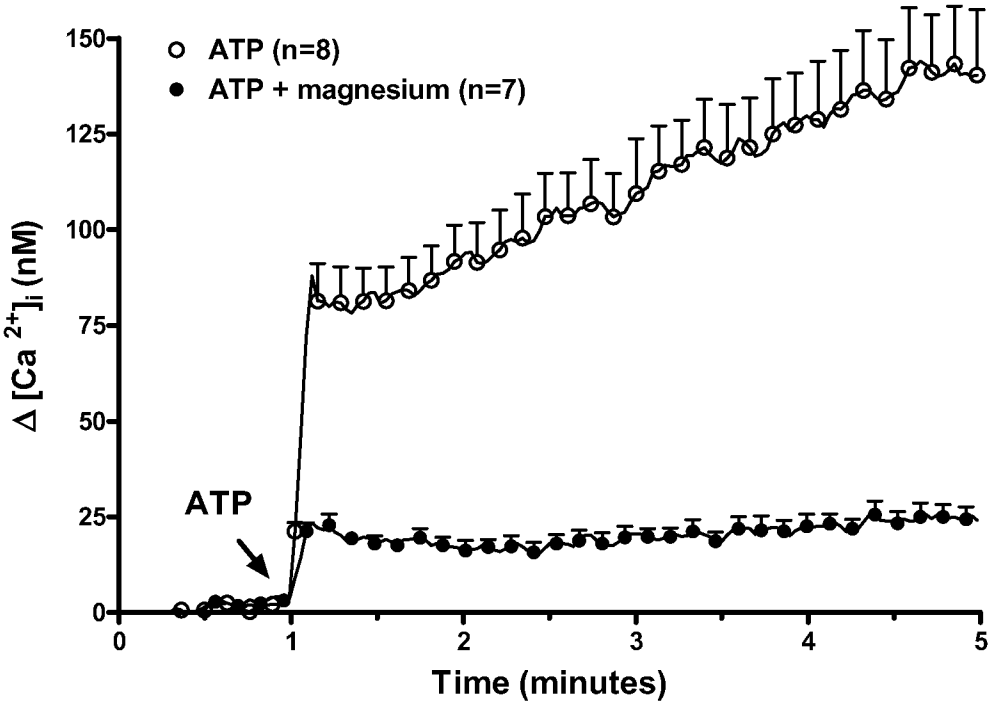
Figure 8



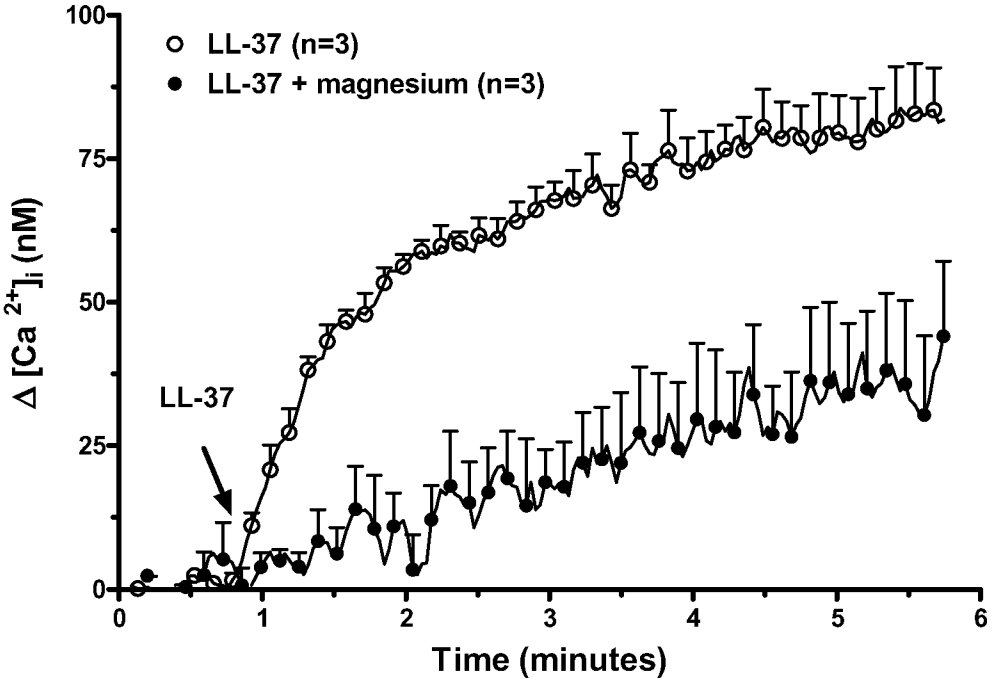


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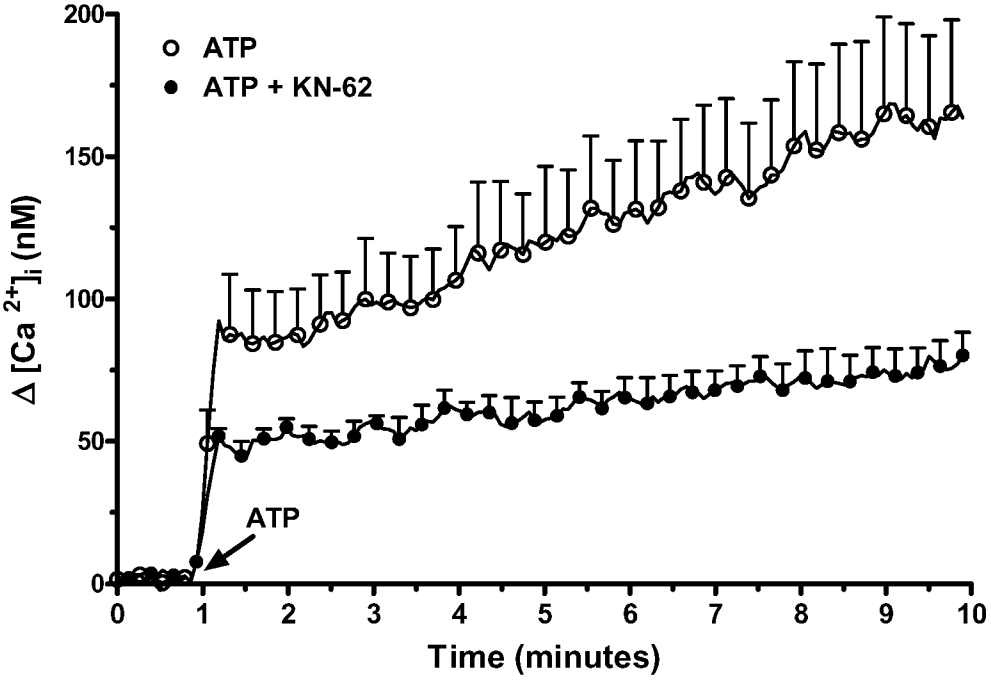
Figure 9



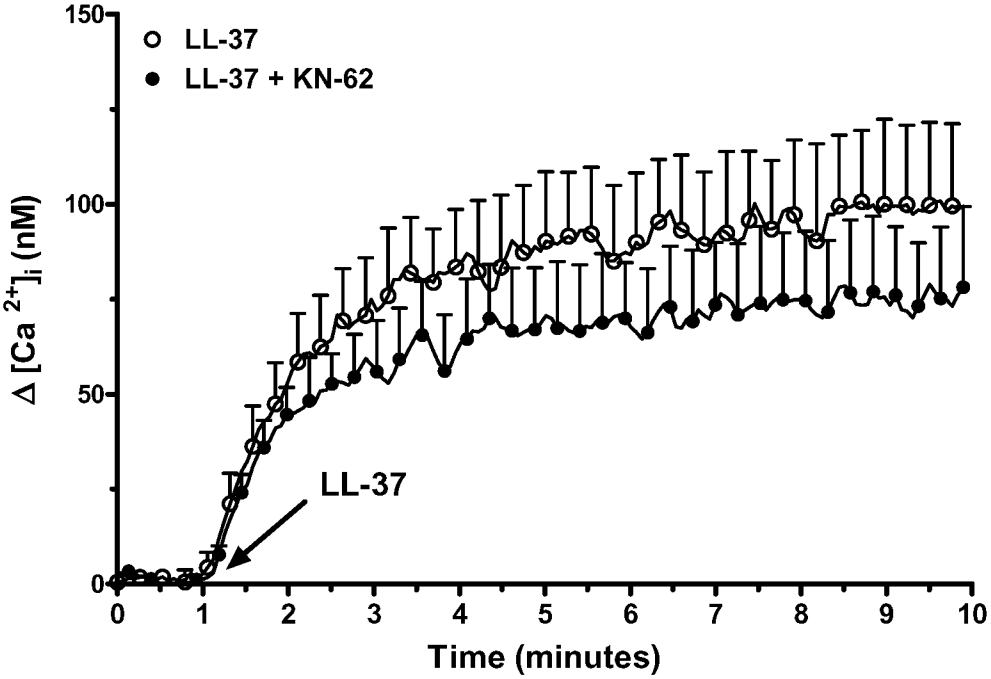


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Figure 10