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Histamine H_3-receptor signaling in cardiac sympathetic nerves: Identification of a novel MAPK-PLA_2-COX-PGE_2-EP_3R pathway

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ABSTRACT

We hypothesized that the histamine H_3-receptor (H_3R)-mediated attenuation of norepinephrine (NE) exocytosis from cardiac sympathetic nerves results not only from a $G\alpha_i$-mediated inhibition of the adenylyl cyclase-cAMP-PKA pathway, but also from a $G\beta\gamma_i$-mediated activation of the MAPK-PLA_2 cascade, culminating in the formation of an arachidonate metabolite with anti-exocytotic characteristics (e.g., PGE_2). We report that in Langendorff-perfused guinea-pig hearts and isolated sympathetic nerve endings (cardiac synaptosomes), H_3R-mediated attenuation of K^+-induced NE exocytosis was prevented by MAPK and PLA_2 inhibitors, and by cyclooxygenase and EP_3-receptor (EP_3R) antagonists. Moreover, H_3R activation resulted in MAPK phosphorylation in H_3R-transfected SH-SY5Y neuroblastoma cells, and in PLA_2 activation and PGE_2 production in cardiac synaptosomes; H_3R-induced MAPK phosphorylation was prevented by an anti-$\beta\gamma$ peptide. Synergism between H_3R and EP_3R agonists (i.e., imetit and sulprostone, respectively) suggested that PGE_2 may be a downstream effector of the anti-exocytotic effect of H_3R activation. Furthermore, the anti-exocytotic effect of imetit and sulprostone was potentiated by the N-type Ca^{2+}-channel antagonist ω-conotoxin GVIA, and prevented by an anti-$G\beta\gamma$ peptide. Our findings imply that an EP_3R $G\beta\gamma_i$-induced decrease in Ca^{2+} influx through N-type Ca^{2+}-channels is involved in the PGE_2/EP_3R-mediated attenuation of NE exocytosis elicited by H_3R activation. Conceivably, activation of the $G\beta\gamma_i$ subunit of H_3R and EP_3R may also inhibit Ca^{2+} entry directly, independent of MAPK intervention. As heart failure, myocardial ischemia and arrhythmic dysfunction are associated with excessive local NE release, attenuation of NE release by H_3R activation is cardioprotective. Accordingly, this novel H_3R signaling pathway may ultimately bear therapeutic significance in hyper-adrenergic states.

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Abbreviations: $[Ca^{2+}]_i$, intraneuronal Ca^{2+}; CBP, clobenpropit; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; EP_3R, EP_3-receptors; H_3R, histamine H_3-receptors; I_{Ca}, Ca^{2+} current; MAFP, methyl arachidonyl fluorophosphonate; MAPK, mitogen activated protein kinase; NE, norepinephrine; PGE_2, prostaglandin E_2; PKA, protein kinase A; PLA_2, phospholipase A_2; VOCC, voltage-operated Ca^{2+}-channels; ω-CTX, ω-conotoxin GVIA

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1. Introduction

Sympathetic nerve terminals in the guinea pig [1,2] and human [3] heart express histamine H_3-receptors (H_3R). H_3R activation reduces norepinephrine (NE) exocytosis and is associated with a marked decrease in the peak intraneuronal Ca^{2+} ($[Ca^{2+}]_i$) response [4]. We recently reported that the H_3R-mediated attenuation of NE exocytosis involves an H_3R-G_i/G_o coupling, adenylyl cyclase inhibition by $G\alpha_i$, decreased cAMP formation and diminished PKA activity [5]. Diminished PKA activity is likely to result in reduced phosphorylation of voltage-operated Ca^{2+}-channels (VOCC), which would be reflected in a decrease in Ca^{2+} current (I_{Ca}). Thus, it is plausible that the H_3R-mediated attenuation of NE exocytosis, and the associated reduction in $[Ca^{2+}]_i$, results from a decreased Ca^{2+} influx via VOCC, due to diminished activity of the adenylyl cyclase-cAMP-PKA pathway.

In addition to adenylyl cyclase inhibition, receptors coupled to pertussis toxin-sensitive heterotrimeric G proteins (e.g., H_3R) are known to stimulate phospholipase A_2 (PLA_2) via the $G\alpha_i$ subunit [6–8]. Furthermore, H_3R couple to the MAPK cascade [9], which contributes to PLA_2 phosphorylation and stimulation of its catalytic activity [10]. PLA_2 activation initiates the arachidonic acid cascade with the ultimate formation of various eicosanoids, including PGE_2. PGE_2 has been shown to inhibit NE release from sympathetic nerves by activating presynaptic EP_3-receptors (EP_3R) [11,12]. Accordingly, we hypothesized that the H_3R-mediated attenuation of NE exocytosis results not only from a decreased adenylyl cyclase-cAMP-PKA function, but also involves another signaling pathway entailing the activation of MAPK and PLA_2, and the eventual formation of an arachidonate metabolite with anti-exocytotic characteristics, most likely PGE_2. We tested this hypothesis both at the subcellular (i.e., cardiac synaptosomes) and whole organ level (i.e., Langendorff-perfused heart).

2. Methods and materials

2.1. Isolated heart

All experiments were approved by the IACUC of Weill Cornell Medical College. Male adult Hartley guinea pigs (350–500 g; Charles River Labs., Wilmington, MA) were anesthetized with CO_2 and rapidly exsanguinated. Hearts were excised and immediately immersed in ice-cold Krebs-Henseleit solution (mM: NaCl, 118; KCl, 4.7; $MgSO_4{\cdot}7H_2O$, 1.2; $NaHCO_3$, 24; KH_2PO_4, 1.1; glucose, 10; $CaCl_2{\cdot}2H_2O$, 2.5) equilibrated with 95% O_2/5% CO_2. Hearts were perfused at constant pressure (40 cm H_2O) in a Langendorff apparatus with warmed Krebs-Henseleit solution (37 °C), containing desipramine (0.1 μM) and atropine (1 μM). ECG was recorded on-line using needle electrodes (400 Hz recording frequency) and analyzed with Powerlab/8SP (AD Instruments, Colorado Springs, CO). Only hearts with a stable sinus rhythm were included in the study. To elicit NE release, two custom-made stainless steel paddles were gently attached to the heart and kept parallel to the intraventricular septum. After 20 min of stabilization, two sequential field stimulations (5 Hz, 2 ms, 5 V, 60 s) using PowerLab/8SP were applied 15 min apart from each other. Coronary effluent was collected before and during stimulation for 2 min. NE overflow into the coronary effluent (i.e., NE exocytosis) was measured by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) [2] and expressed as the ratio between the second and first stimulation (S2/S1). The amount of released NE was very similar during two consecutive stimulations (S2/S1 = 0.992 ± 0.012; $n = 7$). Subsequently, a concentration–response curve (0.03–3 μM, $n = 3$–6) for the imetit-induced attenuation of NE exocytosis was constructed and the IC_{50} was found to be ~0.3 μM. In subsequent experiments the anti-exocytotic effect of imetit (at its IC_{50}) was re-assessed in hearts perfused with the H_3R antagonist clobenpropit (CBP; 50 nM), the PLA_2 inhibitor methyl arachidonyl fluorophosphonate (MAFP; 10 μM) or the EP_3R antagonist ONO-AE_3-240 (10 nM).

2.2. Isolation of cardiac synaptosomes

Guinea-pig hearts were isolated as described above and perfused for 15 min in the Langendorff apparatus to ensure that no blood traces remained in the coronary circulation. Hearts were then minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.4. Minced tissue was digested with 40 mg collagenase (Type II, Worthington Biochemicals, Lakewood, NJ) per 10 ml HEPES-buffered saline solution (HBS) per gram of wet heart weight for 1 h at 37 °C. HBS contained 1 mM pargyline to prevent enzymatic destruction of synaptosomal NE. After low-speed centrifugation (10 min at 120 × g at 4 °C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. The homogenate was spun at 650 × g for 10 min at 4 °C and the pellet rehomogenized and respun. The pellet containing cellular debris was discarded, and the supernatants from the last two spins were combined and equally subdivided into 10–12 tubes. Each tube was centrifuged for 20 min at 20,000 × g at 4 °C. This pellet, which contained cardiac synaptosomes, was resuspended in HBS to a final volume of 500 μL in the presence or absence of pharmacological agents in a water bath at 37 °C. Each suspension functioned as an independent sample and was used only once. In every experiment, one sample was untreated (control, basal NE release), and others were incubated with drugs for 10 min. When antagonists were used, samples were incubated with the antagonist for 10 min before incubation with the agonist. Controls were incubated for an equivalent length of time without drugs. NE exocytosis was elicited by incubating samples for 5 min with K^+ (100 mM) (osmolarity was maintained constant by adjusting the NaCl concentration). At the end of the incubation period, each sample was centrifuged for 20 min (20,000 × g at 4 °C). The supernatant was assayed for NE content by high-pressure liquid chromatography (HPLC) with electrochemical detection [2]. The pellet was assayed for protein content by a modified Lowry procedure [2].

2.3. cPLA$_2$ assay

Guinea-pig heart synaptosomes were incubated with the H_3R agonist imetit (100 nM)[13] or the Ca^{2+}-ionophore A23187 (10 μM) for 10 min, either alone or in the presence of the H_3R antagonist clobenpropit [14]. When clobenpropit (50 nM) was

used, samples were incubated with it for 10 min before incubation with imetit or A23187. Controls were incubated for an equivalent length of time without drugs. Following low-speed centrifugation the synaptosomal pellet was isolated and lysed in 10× lysis buffer (Cell Signaling Technology, Danvers, MA). Samples were then centrifuged at 12,000 × *g* at 4 °C and the resulting supernatant was assayed for $cPLA_2$ activity using a $cPLA_2$ assay kit (Cayman Chemical, Ann Arbor, MI), following the manufacturers protocol.

2.4. PGE_2 assay

Following low-speed centrifugation, supernatants from guinea-pig heart synaptosomal preparations were assayed for PGE_2 release using a PGE_2 EIA kit (Cayman Chemical, Ann Arbor, MI), following the manufacturers protocol.

2.5. Detection of p38 and JNK MAPK activation

A human neuroblastoma cell line stably transfected with the H_3R (SH-SY5Y-H_3) was kindly supplied by Dr T.W. Lovenberg, Johnson & Johnson Pharmaceutical R&D, LLC (San Diego, CA) [4]. SH-SY5Y-H_3 cells were maintained in a 1:1 ratio of Eagle's and Ham's F-12 minimal essential medium mixture, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 450 μg/ml geneticin, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C, 5% CO_2. Cell culture media and supplements were purchased from Mediatech Inc. (Herndon, VA). Cells were grown to confluence in six-well plates. MAPK phosphorylation (i.e., an indication of MAPK activation) was elicited by incubating SH-SY5Y-H_3 cells with the H_3R agonist imetit (100 nM), in the absence or presence of the H_3R antagonist clobenpropit (CBP; 50 nM) for 10 min at 37 °C, or with anti-βγ peptide (1 μM) in the presence or absence of imetit. SH-SY5Y-H_3 cells were then lysed (Lysis buffer; Cell Signaling Technology Inc., Beverly, MA). Samples of lysate (15 μg/lane) were prepared with 5× Tris-glycine SDS sample buffer and boiled for 5 min before separation on 10–20% gradient Tris-glycine SDS-polyacrylamide minigels (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at 200 V, 40 mA/gel for 1 h. Gels were then electrotransferred to polyvinylidine difluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA) for 90 min at 200 V, 300 mA, 4 °C. Membranes were blocked for 2 h in blocking buffer [Tris-buffered saline (TBS) containing 0.1% Tween 20, 5% (w/v) non-fat dry milk]. Phospho-p38 antibody (Biosource; Camarillo, CA) (1:1000) and phospho-JNK antibody (Cell Signaling Technology Inc., Beverly, MA) (1:1000) diluted in primary antibody dilution buffer (TBS containing 0.1% Tween 20, 5% bovine serum albumin) were incubated with the PVDF membrane overnight at 4 °C. The PVDF membrane was washed three times with TBST and then horseradish peroxidase-coupled anti-rabbit IgG (Cell Signaling Technology Inc., Beverly, MA) was added at a 1:3000 dilution in blocking buffer for 1 h. The PVDF membrane was then washed three times with TBST and the protein of interest was detected using enhanced chemiluminescence (Millipore, Billerica, MA) followed by exposure to X-ray film (Biomax MR; Eastman Kodak, Rochester, NY). Bands were analyzed by densitometry using Fluorchem™ 8800 (Alpha Innotech, San Leandro, CA).

2.6. Drugs and chemicals

Indomethacin, imetit, ω-conotoxin GVIA, clobenpropit, A23187 and nifedipine were purchased from Sigma–Aldrich Chemical Co (St Louis, MO). MAFP was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PD 98059, SB 202190, SB 202474, SP600125 and N^1-methyl-1,9-pyrazoloanthrone were purchased from CalbioChem (La Jolla, CA). Sulprostone was purchased from Cayman Chemical (Ann Arbor, MI). The anti-βγ peptide was purchased from AnaSpec, Inc. (San Jose, CA). L-798,106 was a gift from Merck Frosst Canada & Co. ONO-AE3-240 was a gift from ONO Pharmaceutical Co. Ltd., Japan. ONO-AE3-240 was dissolved in 100% ethanol and the anti-Gβγ peptide in 10% NH_4OH. All other drugs were dissolved in dimethyl sulfoxide (DMSO). Further dilutions were made with HEPES buffer; at the concentration used, DMSO, ethanol and NH_4OH did not affect NE release.

3. Results

3.1. MAPK activation plays a role in the H_3R-induced inhibition of NE exocytosis

We first determined whether H_3R stimulation with the selective agonist imetit [13] results in MAPK activation. For this, we used the H_3R-transfected neuroblastoma cell line SH-SY5Y (SH-SY5Y-H_3) [4]. As illustrated in Fig. 1, treatment of SH-SY5Y-H_3 cells with imetit (100 nM) resulted in a marked increase in the phosphorylation of p38 (panel A) and JNK (panel B). The selective H_3R antagonist clobenpropit (50 nM) prevented this effect (Fig. 1A and B). Furthermore, the imetit-induced activation of MAPK was prevented by incubation with the anti-βγ peptide (Fig. 1B). This suggested that the H_3R-coupled Gβγ subunit is involved in the activation of MAPK by imetit. Since these findings suggested that H_3R stimulation results in MAPK activation, we next investigated whether the H_3R-induced inhibition of NE exocytosis is dependent upon MAPK activation. For this, we first assessed whether MAPK inhibition modified the imetit-induced attenuation of NE exocytosis in cardiac synaptosomes. Depolarization of synaptosomes with K^+ (100 mM) resulted in a ~25% increase in endogenous NE release above basal level (Fig. 2A–C). In the presence of imetit (100 nM), K^+-induced NE release was inhibited by ~40–60% (Fig. 2A–C), an effect which we have previously shown to be prevented by clobenpropit [5], indicating that the inhibition of the K^+-induced NE exocytosis is mediated by H_3R activation. As shown in Fig. 2A, the MEK/ERK inhibitor PD98059 [15] diminished the anti-exocytotic effect of imetit by ~30% at 3 μM and abolished it at 10 μM. Similarly, the p38 inhibitor SB202190 [16] diminished the effect of imetit by ~60% at 30 nM and abolished it at 100 nM (Fig. 2B), while the JNK inhibitor SP600125 [17] diminished the effect of imetit by ~30% at 150 nM and abolished it at 200 nM (Fig. 2C). In contrast, no antagonism of imetit occurred with compound SB202474, a pyridinyl imidazole analog of SB202190 that does not bind p38 [18], nor with N^1-methyl-1,9-pyrazoloanthrone, an analog of SP600125 that is over 100-fold less potent than SP600125 at inhibiting JNK [17]. We found that in the presence of SB202474 (10 μM) and N^1-methyl-1,9-pyrazoloanthrone

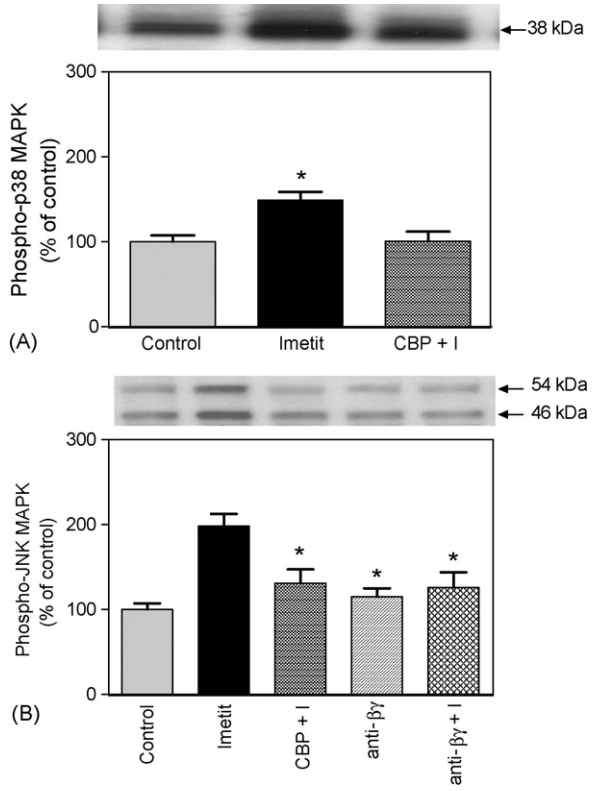

Fig. 1 – H_3R activation increases the phosphorylation of p38 and JNK MAPK. SH-SY5Y-H_3 neuroblastoma cells were incubated in the absence (control) or presence of imetit (100 nM) and clobenpropit (CBP; 50 nM) + imetit (100 nM) (CBP + I) or the anti-βγ peptide (1 μM) + imetit for 10 min at 37 °C. Cells were then lysed and equal amounts of protein (15 μg/lane) were run on SDS-PAGE gel followed by Western blot analysis using antibodies against phospho-p38 MAPK (panel A) or against phospho-JNK MAPK (panel B). As expected, a single band at 38 kDa was visualized for p38 MAPK and double bands at 46 and 54 kDa were detected for JNK MAPK (representing JNK1 and JNK2, respectively). Bars are means (±S.E.M.; $n = 4$–5). $^*P < 0.05$ from control (by ANOVA followed by post hoc Dunnett's test).

(10 μM), imetit inhibited NE exocytosis by 54.0 ± 4.7 (±S.E.M.; $n = 12$) and 53.6 ± 2.3% (±S.E.M.; $n = 4$; NS), respectively, as compared with a 59.8 ± 7.7% inhibition with imetit alone (100 nM). Notably, none of the MAPK inhibitors affected basal NE release or K^+-induced NE exocytosis in the absence of imetit (see legend to Fig. 2).

3.2. *PLA_2 and cyclooxygenase activation plays a role in the H_3R-induced inhibition of NE exocytosis*

Since $cPLA_2$ is a substrate for MAPK, and phosphorylation by MAPK increases the enzymatic activity of $cPLA_2$ [10], we next assessed whether the H_3R-mediated inhibition of NE exocytosis via MAPK entails the activation of $cPLA_2$ in sympathetic nerve endings. Incubation of guinea-pig heart synaptosomes with imetit (100 nM) significantly enhanced the level of $cPLA_2$ activity (~25%; Fig. 3A). Pretreatment with the selective H_3R antagonist clobenpropit (50 nM) [5] prevented the imetit-induced increase of $cPLA_2$ activity, indicating that this increase was dependent on H_3R activation. Similar to imetit, the Ca^{2+}-ionophore A23187 (10 μM) also enhanced $cPLA_2$ activity (~20%), however this effect was not inhibited by clobenpropit (50 nM; Fig. 3A).

Given that the anti-exocytotic effect of H_3R activation was associated with an increase in $cPLA_2$ activity, we questioned whether $cPLA_2$ activation leads to the downstream production of arachidonate metabolites capable of inhibiting NE exocytosis. For this, we determined whether $cPLA_2$ inhibition reduced the anti-exocytotic effect of imetit in guinea-pig heart synaptosomes. Depolarization of synaptosomes with K^+ (100 mM) resulted in a ~30% increase in endogenous NE release above basal level (Fig. 4A–D). In the presence of imetit (100 nM), K^+-induced NE release was inhibited by ~40–60% (Fig. 4A–D), an effect due to H_3R activation since, as mentioned above for Fig. 2, it was prevented by clobenpropit. As shown in Fig. 4A, the PLA_2 inhibitor MAFP (10 μM) prevented the anti-exocytotic effect of imetit in cardiac synaptosomes. Similarly, the cyclooxygenase inhibitor indomethacin (10 μM) also prevented the effect of imetit (Fig. 4B). These results suggested that a cyclooxygenase product of the arachidonic acid cascade might mediate the anti-exocytotic effect of imetit. Notably, neither the PLA_2 inhibitor MAFP nor the cyclooxygenase inhibitor indomethacin affected basal NE release or K^+-induced NE exocytosis in the absence of imetit (see legend to Fig. 4).

3.3. *Increased PGE_2 production is involved in the H_3R-induced inhibition of NE exocytosis: role of EP_3R at subcellular and whole organ levels*

Among the various cyclooxygenase products of the arachidonate cascade, PGE_2 is known to inhibit NE exocytosis [12]. Accordingly, we determined whether H_3R activation leads to increased PGE_2 production. As shown in Fig. 3B, incubation of guinea-pig heart synaptosomes with imetit (100 nM) or the Ca^{2+}-ionophore A23187 (10 μM) was associated with a ~50% increase in PGE_2 production. The H_3R antagonist clobenpropit (50 nM) again prevented the effect of imetit but not that of the ionophore (Fig. 3B). This suggested that the H_3R-induced attenuation of NE exocytosis associated with PLA_2 activation might be due to the downstream generation of PGE_2.

We next investigated whether PGE_2 may be signaling via EP_3R, which are known to mediate the PGE_2-induced inhibition of NE exocytosis [11,12]. To this end, we used two selective EP_3R antagonists L-798,106 [19] and ONO-AE_3-240 [20]. As shown in Fig. 4C and D, both EP_3R antagonists (L-798,106 at 10 μM and ONO-AE_3-240 at 10 nM) abolished the imetit-induced attenuation of NE exocytosis in cardiac synaptosomes. The EP_3R antagonists did not affect basal NE release or K^+-induced NE exocytosis in the absence of imetit (see legend to Fig. 4).

Our findings in cardiac synaptosomes and cultured cells suggested that stimulation of H_3R results in MAPK and $cPLA_2$ activation, leading to PGE_2 formation, activation of EP_3R on sympathetic terminals and inhibition of NE exocytosis. We next extended this research to the whole heart to verify that this H_3R pathway is operational at the intact organ level. Hearts were isolated from guinea pigs, perfused in a Langendorff apparatus and subjected to electrical field

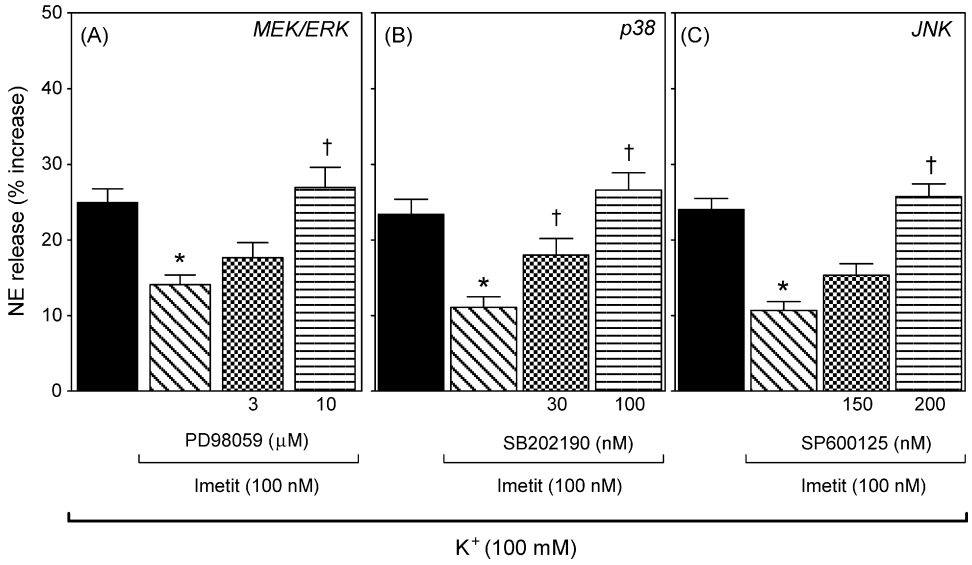

Fig. 2 – MAPK inhibition prevents the H_3R-mediated attenuation of NE exocytosis from guinea-pig heart synaptosomes. The histamine H_3R agonist imetit (100 nM) attenuates the release of endogenous NE elicited by K^+ depolarization (100 mM) of cardiac sympathetic nerve endings *in vitro*. Pretreatment of synaptosomes with the MEK/ERK inhibitor PD98059 (3 and 10 μM; panel A), the p38 inhibitor SB202190 (30 and 100 nM; panel B) and the JNK inhibitor SP600125 (150 and 200 nM; panel C) markedly inhibited the anti-exocytotic effect of imetit. Bars are mean increases in NE release above own basal level (±S.E.M.; n = 8–17). * and †, significantly different from K^+ alone and imetit, respectively ($P < 0.01$ by ANOVA followed by post hoc Dunnett's test). Basal NE release was 1.27 ± 0.06 pmol/mg protein; it increased to 1.57 ± 0.04 pmol/mg with K^+ (100 mM; n = 41). In the presence of PD98059 (10 μM), SB202190 (100 nM) or SP600125 (200 nM), basal NE level was 1.27 ± 0.04, 1.29 ± 0.02 and 1.28 ± 0.03 pmol/mg (n = 4, 4, 4), respectively, while K^+-induced NE exocytosis was 1.32 ± 0.06, 1.39 ± 0.03 and 1.37 ± 0.03 (n = 4, 4, 4), respectively.

stimulation to elicit NE release from sympathetic nerves. As shown in Fig. 5A, activation of H_3R with imetit caused a concentration-dependent decline in NE overflow into the coronary effluent. In the 30 nM–3 μM concentration range, imetit inhibited NE exocytosis by ~15–35%. Fig. 5B shows that the anti-exocytotic effect of imetit (300 nM) was prevented not only by H_3R blockade with clobenpropit (50 nM), but also by PLA_2 blockade with MAFP (10 μM) and by EP_3R blockade with ONO-AE_3-240 (10 nM). This suggests that in the intact organ, as well as in synaptosomes, inhibition of NE exocytosis by H_3R activation involves the PLA_2-dependent generation of an endogenous EP_3R agonist (e.g., PGE_2) capable of inhibiting NE release.

To further strengthen the case for downstream EP_3R involvement in the H_3R-mediated anti-exocytotic effect of imetit, we next assessed whether the EP_3R agonist sulprostone

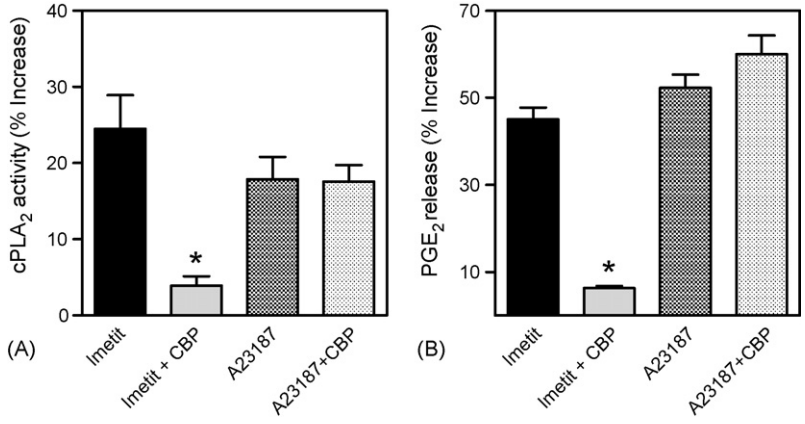

Fig. 3 – Stimulation of H_3R enhances $cPLA_2$ activity and PGE_2 production in guinea-pig heart synaptosomes. The H_3R agonist imetit (100 nM) and the Ca^{2+} ionophore A23187 (10 μM) enhance $cPLA_2$ activity (Panel A) and PGE_2 production (Panel B) in cardiac sympathetic nerve endings. The H_3R antagonist clobenpropit (CBP; 50 nM) antagonizes the effects of imetit, but not those of the ionophore. Bars are mean increases above control (±S.E.M.; n = 3 and 4, for A and B, respectively). *Significantly different from imetit alone ($P < 0.01$ by one-way ANOVA with Bonferroni post hoc test). Basal $cPLA_2$ activity was 20.48 ± 4.4 nmol/min/μg protein. Basal PGE_2 level was 154.95 ± 14.64 pg/mg protein.

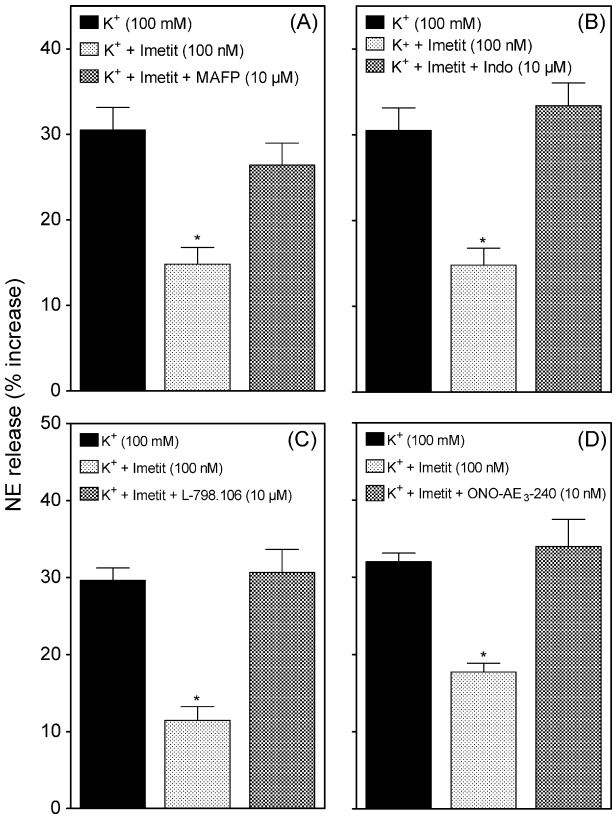

Fig. 4 – Inhibition of PLA_2 and cyclooxygenase, and blockade of EP_3R prevent the H_3R-mediated attenuation of NE exocytosis in guinea-pig heart synaptosomes. The H_3R agonist imetit (100 nM) attenuates the release of endogenous NE from K^+-depolarized (100 mM) sympathetic nerve endings. Incubation of synaptosomes with the PLA_2 inhibitor MAFP (10 μM; panel A), the cyclooxygenase inhibitor indomethacin (10 μM; panel B), and the EP_3R antagonists L-798,106 (10 μM; panel C) and ONO-AE_3-240 (10 nM; panel D), prevented the anti-exocytotic effect of imetit. Synaptosomes treated with MAFP or indomethacin were isolated from hearts previously perfused with these agents. Bars are mean increases in NE release above own basal level (±S.E.M.; $n = 12$). *Significantly different from K^+ alone ($P < 0.01$ by ANOVA with post hoc Dunnett's test). Basal NE release was 1.27 ± 0.04 pmol/mg protein ($n = 44$); it increased to 1.66 ± 0.04 pmol/mg with K^+ (100 mM; $n = 36$). In the presence of MAFP (10 μM) or indomethacin (10 μM), basal NE release was 1.24 ± 0.04 and 1.24 ± 0.05 pmol/mg ($n = 12$ and 12), respectively, while K^+-induced NE exocytosis was 1.5 ± 0.07 and 1.43 ± 0.07 pmol/mg ($n = 12$ and 12), respectively. In the presence of the EP_3R antagonists L-798,106 (10 μM) or ONO-AE_3-240 (10 nM), basal NE level was 1.27 ± 0.05 and 1.47 ± 0.03 pmol/mg ($n = 12$ and 12), respectively, while K^+-induced NE exocytosis was 1.44 ± 0.05 and 1.7 ± 0.04 pmol/mg ($n = 12$ and 12), respectively.

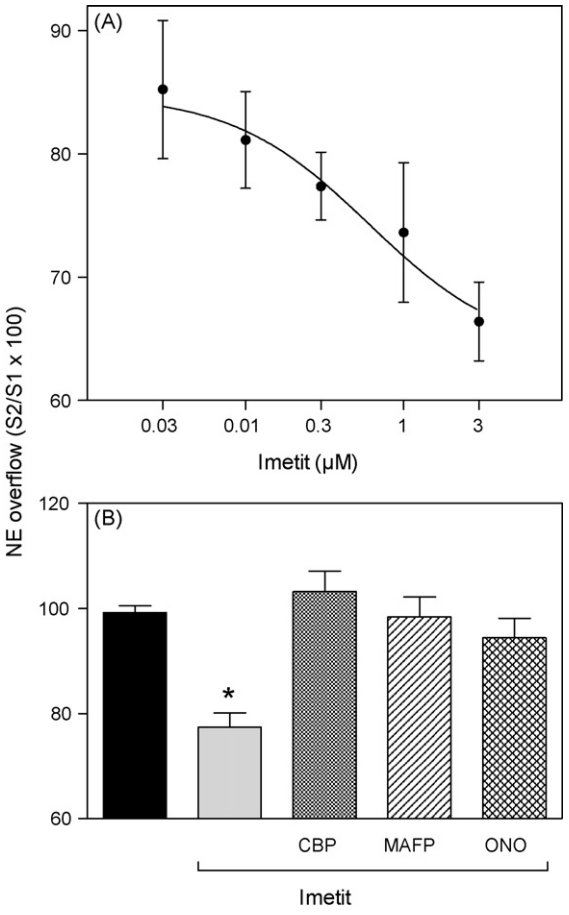

Fig. 5 – Inhibition of PLA_2 and blockade of EP_3R prevent the H_3R-mediated attenuation of NE exocytosis in guinea-pig hearts ex vivo. NE was released into the coronary effluent by transmural electrical field stimulation of sympathetic nerve terminals. Panel A: concentration–response curve for the anti-exocytotic effect of imetit (IC_{50} ~300 nM). Points are mean NE overflow values (i.e., NE exocytosis) expressed as the ratio between the second and first stimulation (S2/S1 × 100; ±S.E.M.; $n = 5–7$). Panel B: the anti-exocytotic effect of imetit (300 nM) is prevented by perfusion of the hearts with the H_3R antagonist clobenpropit (CBP; 50 nM), the PLA_2 inhibitor MAFP (10 μM) and the EP_3R antagonist ONO-AE_3-240 (10 nM). Bars are mean NE overflow values (±S.E.M.; $n = 5–7$). *Significantly different from transmural electrical field stimulation in the absence of drugs (control; $P < 0.01$ by ANOVA with post hoc Dunnett's test).

would act synergistically with imetit in attenuating NE exocytosis. Shown in Fig. 6A and B are the concentration–response curves for the anti-exocytotic effects of imetit and sulprostone, respectively. The inhibition of K^+-induced NE release ranged between ~5 and ~60% with imetit 3–100 nM (Fig. 6A) and between ~3 and ~50% with sulprostone 10–300 nM (Fig. 6B). When imetit and sulprostone were combined, each at the lowest concentration tested (i.e., 3 and 10 nM, causing a ~10 and ~5% inhibition of NE release, respectively), the inhibition of NE release increased to ~45%; i.e., an

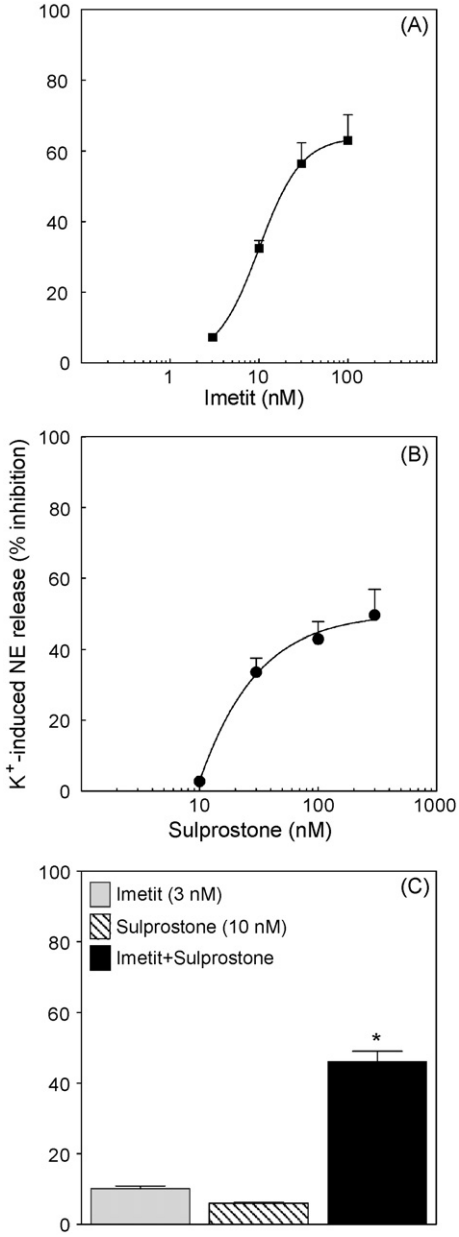

Fig. 6 – H_3R and EP_3R agonists act synergistically to attenuate NE exocytosis from guinea-pig heart synaptosomes. Panels A and B: concentration–response curves for the anti-exocytotic effect of imetit and sulprostone, respectively. Panel C, inhibition of NE exocytosis from cardiac synaptosomes by subthreshold concentrations of imetit (3 nM) and sulprostone (10 nM) administered either alone or in combination; note that a significant attenuation of NE release occurs when imetit is combined with sulprostone (*significantly different from the sum of imetit + sulprostone, $P < 0.001$ by unpaired t-test). Points and bars represent mean percent inhibition of K^+-induced (100 mM) NE exocytosis (±S.E.M.; $n = 8–16$). Basal NE release was 1.36 ± 0.05 pmol/mg protein ($n = 36$). In the presence of imetit (100 nM) NE release was 1.32 ± 0.08 pmol/mg protein ($n = 12$), while in the presence of sulprostone (300 nM) NE release was 1.39 ± 0.05 pmol/mg protein ($n = 8$).

inhibition three-fold greater than the arithmetic sum of the two single inhibitions (Fig. 6C). The synergism between imetit and sulprostone provided further support for the notion that a downstream EP_3R agonist such as PGE_2 is involved in the H_3R-mediated anti-exocytotic effect of imetit. As expected, neither PLA_2 inhibition with MAFP, nor cyclooxygenase inhibition with indomethacin, significantly modified the sulprostone-induced attenuation of NE exocytosis [Absolute values for NE release in pmol/mg (not shown in Fig. 6 or its legend) were ($n = 4$): basal, 1.42 ± 0.05; K^+ (100 mM), 1.71 ± 0.03; K^+ + sulprostone (300 nM), 1.38 ± 0.05; K^+ + MAFP (10 μM) + sulprostone, 1.5 ± 0.06; K^+ + indomethacin (10 μM) + sulprostone, 1.5 ± 0.07].

3.4. *Mechanism(s) of PGE_2-mediated anti-exocytotic effect of H_3R activation*

We next questioned whether the PGE_2-mediated anti-exocytotic effect of H_3R activation might result from an inhibition of Ca^{2+} influx into sympathetic nerve terminals. Since the synergism between the anti-exocytotic effects of imetit and sulprostone suggested a similar mechanism of action, we compared the effects of imetit and sulprostone, each in combination with the selective N-type and L-type Ca^{2+}-channel inhibitors, ω-conotoxin GVIA and nifedipine, respectively. As shown in Fig. 7A and B, when a subthreshold anti-exocytotic concentration of imetit (3 nM) or sulprostone (10 nM) was used in combination with a subthreshold concentration of ω-conotoxin (0.1 nM) or nifedipine (0.1 μM), a marked synergistic anti-exocytotic effect was observed. ω-CTX (0.1 nM) and nifedipine (0.1 μM) did not significantly affect basal NE release (see legend to Fig. 7). These findings suggest that a decrease in Ca^{2+} influx through both N- and L-type Ca^{2+}-channels is likely to be involved in the PGE_2/EP_3R-mediated attenuation of NE exocytosis elicited by H_3R activation.

EP_3R and H_3R are coupled to a G_i protein [12,21]. The $G\beta\gamma_i$ subunit is known to inhibit Ca^{2+} entry [22]; thus, the $G\beta\gamma_i$ subunit could play a role in the H_3R-mediated attenuation of NE exocytosis. To test this hypothesis we used a phosducin-like membrane-permeable Gβγ-blocking peptide [23]. As shown in Fig. 8, the H_3R agonist imetit (100 nM) and the EP_3R agonist sulprostone (300 nM) each caused a ~60% attenuation of K^+-induced NE exocytosis from guinea-pig heart synaptosomes. The inhibitory effects of imetit and sulprostone were prevented by the anti-βγ peptide (1 μM) (Fig. 8). Notably, the anti-βγ peptide (1 μM) had no effect on basal NE release (see legend to Fig. 8). Thus, Ca^{2+}-channel blockade via the βγ-subunit of the G_i-protein may mediate the H_3R- and EP_3R-induced inhibition of NE exocytosis in cardiac sympathetic nerve terminals.

4. Discussion

In this study, we report on a new signaling pathway involved in the H_3R-mediated inhibition of NE exocytosis from cardiac sympathetic nerve terminals. This pathway entails the activation of MAPK and subsequent phosphorylation of $cPLA_2$. The downstream formation of arachidonic acid metabolites

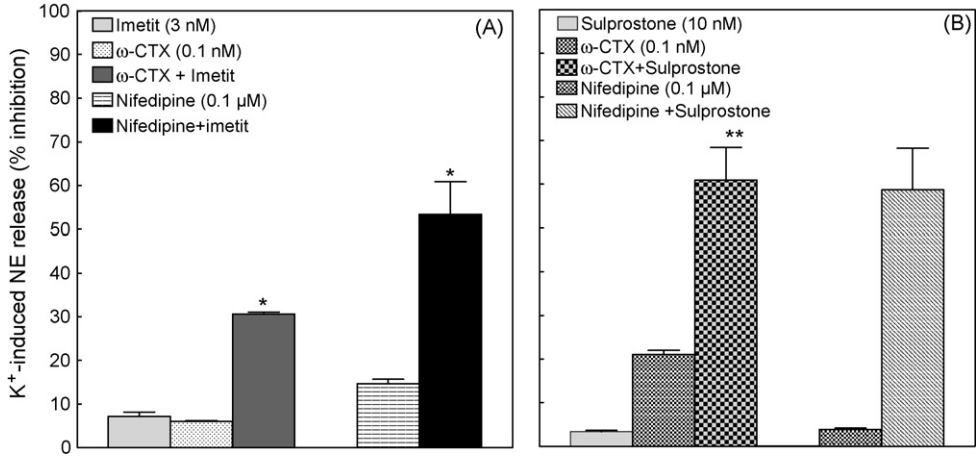

Fig. 7 – N- and L-type Ca^{2+}-channel antagonists act synergistically with H_3R and EP_3R agonists to attenuate NE exocytosis in guinea-pig heart synaptosomes. Panel A, inhibition of NE exocytosis from cardiac synaptosomes by subthreshold concentrations of imetit (3 nM), ω-conotoxin GVIA (ω-CTX; 0.1 nM) and nifedipine (0.1 μM) administered either alone or in combination. Panel B, inhibition of NE exocytosis from cardiac synaptosomes by subthreshold concentrations of sulprostone (10 nM), ω-CTX (0.1 nM) and nifedipine (0.1 μM) administered either alone or in combination. Note that a significant attenuation of NE release occurs when imetit or sulprostone is combined with ω-CTX or nifedipine (*significantly different from the sum of imetit + ω-CTX, imetit + nifedipine, sulprostone + ω-CTX, sulprostone + nifedipine; $P < 0.01$ by unpaired *t*-test). Bars represent mean percent inhibition of K^+-induced (100 mM) NE exocytosis (±S.E.M.; $n = 12$). Basal NE release was 1.23 ± 0.05 pmol/mg protein ($n = 24$). In the presence of ω-CTX (0.1 nM) or nifedipine (0.1 μM) NE release was 1.32 ± 0.09 and 1.18 ± 0.04 pmol/mg protein, respectively ($n = 6$ and 8).

inhibits NE exocytosis, most likely by decreasing Ca^{2+} entry into sympathetic nerve terminals.

Two observations had attracted our attention. Firstly, the finding that H_3R are positively coupled to the MAPK cascade in COS-7 cells [9], and secondly, that H_3R activation results in PLA_2 activation [24]. Given that H_3R are G_i-coupled [12,21], and that the $\beta\gamma_i$ subunit is responsible for the activation of MAPK by G_i-coupled receptors [25], we assumed that H_3R would activate MAPK via $\beta\gamma_i$. Indeed, we found that imetit activates MAPK in SH-SY5Y-H_3 cells, an optimal model of sympathetic nerve endings and that the anti-βγ peptide prevents this effect of imetit (see Fig. 1). Whether H_3R-coupled $G\alpha_i$ contributes to MAPK activation [26] remains to be tested at this point. Since MAPK activation contributes to PLA_2 phosphorylation and stimulation of its catalytic activity [10], we hypothesized that PLA_2 activation initiates the arachidonate cascade, leading to the downstream formation of PGE_2, which is in large part responsible for the H_3R-induced attenuation of NE exocytosis. Our findings are consistent with this hypothesis.

Individual pharmacological inhibition of each MAPK pathway (i.e., MEK/ERK, p38 and JNK) significantly attenuated the anti-exocytotic effect of imetit. Therefore, all three MAPK pathways are likely to be involved in the H_3R-mediated attenuation of NE exocytosis. Whether these pathways complement and/or supplement each other in contributing to the H_3R-induced anti-exocytotic effect is uncertain at this time. Nonetheless, our findings suggest that the H_3R-mediated MAPK activation results in PLA_2 phosphorylation and formation of arachidonate metabolites that play a major role in the attenuation of NE exocytosis. Indeed, we found that activation of H_3R in cardiac sympathetic nerve terminals is associated with an increase in PLA_2 activity and PGE_2 formation, and that these downstream events are selectively prevented by H_3R blockade. Moreover, the H_3R-induced attenuation of NE exocytosis in cardiac sympathetic nerve endings was abolished by PLA_2 or cyclooxygenase inhibition, as well as by blockade of EP_3R. This suggests that PGE_2 is the cyclooxygenase metabolite of the arachidonate cascade involved in the anti-exocytotic effect elicited by the H_3R-initiated activation of the MAPK-PLA_2 pathway.

This conclusion is further supported by our findings in the whole heart *ex vivo*. In these experiments, the transmural stimulation of sympathetic nerves elicited NE exocytosis, which was attenuated by activation of H_3R. The H_3R-mediated attenuation of NE exocytosis was prevented by PLA_2 inhibition and by EP_3R blockade. These results demonstrate that the H_3R-initiated activation of the MAPK-PLA_2 pathway also functions at the organ level and is not purely characteristic of isolated sympathetic nerve terminals. Interestingly, at concentrations of 100 and 300 nM, imetit was an equieffective inhibitor of NE exocytosis in synaptosomes and isolated heart, respectively. These differences in imetit concentration probably reflect technical differences between the two preparations and the different methods used to elicit NE exocytosis. In any event, the anti-exocytotic effect of imetit was prevented in the isolated heart by the same concentrations of CBP, MAFP and ONO-AE_3-240 found to be effective in the synaptosomes.

Additionally, we discovered that imetit and the EP_3R agonist sulprostone act synergistically to attenuate NE exocytosis elicited by K^+ depolarization of sympathetic nerve terminals. This suggests that imetit and sulprostone share a common mechanism of action and favors the hypothesis that a downstream EP_3R agonist such as PGE_2 is involved in the H_3R-mediated anti-exocytotic effect of imetit. Furthermore,

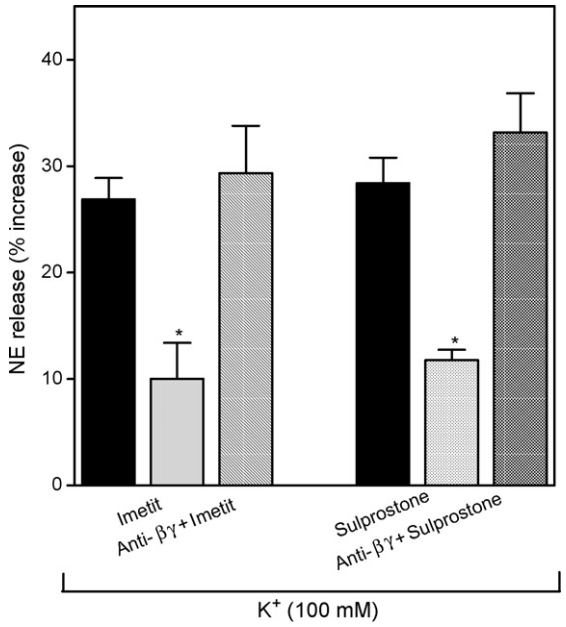

Fig. 8 – A membrane-permeable Gβγ-blocking peptide inhibits the H_3R- and EP_3R-induced attenuation of NE release from guinea-pig heart synaptosomes. The EP_3R and H_3R agonists sulprostone (300 nM) and imetit (100 nM) each attenuates the release of endogenous NE from K^+-depolarized (100 mM) sympathetic nerve endings. Pretreatment of isolated synaptosomes with the phosducin-like anti-Gβγ peptide (1 μM) prevents the anti-exocytotic effect of each imetit and sulprostone. Bars are mean increases in NE release above own basal level (±S.E.M.; $n = 8$). *Significantly different from K^+ alone ($P < 0.01$ by ANOVA with post hoc Dunnett's test). Basal NE release was 1.73 ± 0.05 pmol/mg protein ($n = 8$). In the presence of the anti-Gβγ peptide (1 μM) NE release was 1.84 ± 0.08 pmol/mg protein ($n = 4$).

we found that both imetit and sulprostone acted synergistically with the Ca^{2+}-channel blockers ω-conotoxin GVIA and nifedipine in inhibiting NE exocytosis. This suggests that a decrease in Ca^{2+} influx through both N- and L-type Ca^{2+}-channels is likely to be involved in the PGE_2/EP_3R-mediated attenuation of NE exocytosis elicited by H_3R activation.

Although the N-type Ca^{2+}-channels are the dominant Ca^{2+} entry pathway triggering sympathetic transmitter release [27,28], it is possible that L-type Ca^{2+}-channels may also participate in NE exocytosis and be inhibited by H_3R and EP_3R activation [5]. H_3R and EP_3R are both coupled to a G_i protein [12,21]. The $G\beta\gamma_i$ subunit is known to inhibit Ca^{2+} entry via N-type Ca^{2+}-channels [22,29]. Thus, we hypothesized that signaling via the $G\beta\gamma_i$ subunit could represent a common mechanism of action in the attenuation of NE exocytosis by H_3R and EP_3R. Indeed, we found that the membrane-permeable Gβγ-blocking peptide [23], which prevented the imetit-induced MAPK activation in SH-SY5Y-H_3 cells, significantly attenuated the inhibition of K^+-induced NE exocytosis elicited by imetit and sulprostone in cardiac sympathetic nerve endings. This suggests that neuronal Ca^{2+}-channel blockade via the $G\beta\gamma_i$-subunit plays a role in the H_3R- and EP_3R-mediated inhibition of NE exocytosis and that MAPK is an important upstream step in this pathway.

While it is well established that the Gβγ dimer inhibits N-type Ca^{2+}-channel activity [29], Gβγ may also inhibit presynaptic L-type Ca^{2+}-channels [30,31]. Further, $G\beta\gamma_i$ is known to decrease adenylyl cyclase activity [32]. Thus, it is possible that in addition to the attenuation of adenylyl cyclase by $G\alpha_i$ [5], $G\beta\gamma_i$ will also play a role in the H_3R-mediated decrease in cAMP. A decrease in intracellular cAMP would diminish PKA activity and phosphorylation of L-type Ca^{2+} channels resulting in a decreased Ca^{2+} influx. This could explain mechanistically why nifedipine synergized with both imetit and sulprostone in their anti-exocytotic effect. In addition, the H_3R-mediated attenuation of NE exocytosis may also result from an inhibition of Ca^{2+} influx by direct coupling of the $G\beta\gamma_i$ subunit

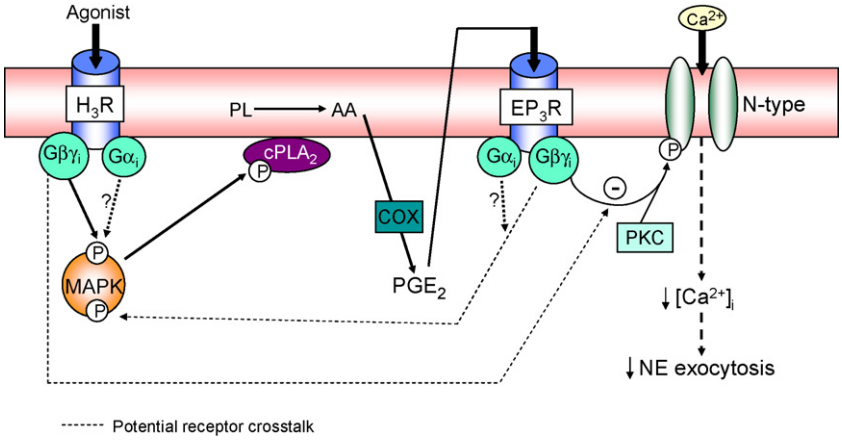

Fig. 9 – Proposed MAPK-PLA_2-PGE_2-EP_3R signaling pathway involved in the H_3R-mediated attenuation of NE exocytosis in cardiac sympathetic nerve terminals. Stimulation of H_3R on sympathetic nerve endings results in the $G\beta\gamma_i$-mediated intraneuronal activation of the MAPK cascade. MAPK activation phosphorylates $cPLA_2$, which is then translocated to the cellular membrane, with the consequent formation of arachidonic acid (AA) from membrane phospholipids (PL), and the subsequent production of PGE_2 via cyclooxygenase (COX). PGE_2 activates EP_3R on the neuronal membrane, and the $G\beta\gamma_i$ subunit of EP_3R inhibits Ca^{2+} entry, thus attenuating NE exocytosis. The $G\beta\gamma_i$ subunit of H_3R may also directly inhibit Ca^{2+} entry without MAPK intervention.

to N-type Ca^{2+}-channels [33–35]. At any rate, our findings suggest that the H_3R $G\beta\gamma_i$-subunit plays a pivotal role in the inhibition of NE exocytosis, most likely via the MAPK-PLA_2-PGE_2-EP_3R pathway, but conceivably also via direct coupling to the N-type Ca^{2+}-channels.

In conclusion, we have identified a novel-signaling pathway (see Fig. 9) whereby stimulation of H_3R on sympathetic nerve endings results in the intraneuronal activation of the MAPK cascade. Activated MAPK phosphorylates $cPLA_2$, which is then translocated to the cellular membrane, with the consequent formation of arachidonic acid from membrane phospholipids, and the subsequent production of PGE_2 via cyclooxygenase. PGE_2 then activates EP_3R on the neuronal membrane, and the $G\beta\gamma_i$ subunit of EP_3R inhibits Ca^{2+} entry, thus attenuating NE exocytosis. This novel pathway likely functions in concert with the traditional $H_3R/G\alpha_i$-induced inhibition of adenylyl cyclase [5], which leads to a decreased phosphorylation of Ca^{2+}-channels, diminished Ca^{2+} entry and thus, attenuation of NE exocytosis. Conceivably, the $G\beta\gamma_i$ subunit of H_3R could also directly inhibit Ca^{2+} entry without MAPK intervention. Potential H_3R/EP_3R cross-talk is likely; thus, MAPK could be activated by $G\beta\gamma_i$ and $G\alpha_i$ from both H_3R and EP_3R, and the N-type Ca^{2+}-channel could be inhibited by $G\beta\gamma_i$ from both H_3R and EP_3R.

Conflict of interest

None.

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