Histamine H3-Receptor-Induced Attenuation of Norepinephrine Exocytosis: A Decreased Protein Kinase A Activity Mediates a Reduction in Intracellular Calcium

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ABSTRACT

We had reported that activation of presynaptic histamine H3-receptors inhibits norepinephrine exocytosis from depolarized cardiac sympathetic nerve endings, an action associated with a marked decrease in intraneuronal Ca2+ that we ascribed to a decreased Ca2+ influx. An H3-receptor-mediated inhibition of cAMP-dependent phosphorylation of Ca2+ channels could cause a sequential attenuation of Ca2+ influx, intraneuronal Ca2+ and norepinephrine exocytosis. We tested this hypothesis in sympathetic nerve endings (cardiac synaptosomes) expressing native H3-receptors and in human neuroblastoma SH-SY5Y cells transfected with H3-receptors. Norepinephrine exocytosis was elicited by K+ or by stimulation of adenylyl cyclase with forskolin. H3-receptor activation markedly attenuated the K+ - and forskolin-induced norepinephrine exocytosis; pretreatment with pertussis toxin prevented this effect. Similar to forskolin, 8-bromo-cAMP elicited norepinephrine exocytosis but, unlike forskolin, it was unaffected by H3-receptor activation, demonstrating that inhibition of adenylyl cyclase is a pivotal step in the H3-receptor transductional cascade. Indeed, we found that H3-receptor activation attenuated norepinephrine exocytosis concomitantly with a decrease in intracellular cAMP and PKA activity in SH-SY5Y-H3 cells. Moreover, pharmacological PKA inhibition acted synergistically with H3-receptor activation to reduce K+ -induced peak intracellular Ca2+ in SH-SY5Y-H3 cells and norepinephrine exocytosis in cardiac synaptosomes. Furthermore, H3-receptor activation synergized with N- and L-type Ca2+ channel blockers to reduce norepinephrine exocytosis in cardiac synaptosomes. Our findings suggest that the H3-receptor-mediated inhibition of norepinephrine exocytosis from cardiac sympathetic nerves results sequentially from H3-receptor-Gi/Go coupling, inhibition of adenylyl cyclase activity, and decreased cAMP formation, leading to diminished PKA activity, and thus, decreased Ca2+ influx through voltage-operated Ca2+ channels.

Histamine H3-receptors (H3Rs) were first recognized as inhibitory autoreceptors on histamine-containing nerve terminals (Arrang et al., 1983) and have since been shown to regulate the release of several neurotransmitters in the central and peripheral nervous systems (Hill et al., 1997; Levi and Smith, 2000). We previously reported that imetit, a selective H3R agonist (Garbarg et al., 1992), attenuates norepinephrine exocytosis from cardiac sympathetic nerve terminals (Silver et al., 2002). Because ω-conotoxin (ω-CTX) and imetit each decreased [Ca2+]i and NE exocytosis (Silver et al., 2002), and since ω-CTX decreases [Ca2+]i by inhibiting Ca2+ influx through N-type Ca2+ channels (Sher et al., 1991), we speculated that, similar to ω-CTX, imetit-induced H3R activation might decrease [Ca2+]i by inhibiting Ca2+ influx through voltage-dependent Ca2+ channels in sympathetic nerve terminals (Silver et al., 2002). In fact, an H3R-mediated inhibition of N-type Ca2+ channel current has been claimed to occur in histaminergic neurons from the rat hypothalamus (Takeshita et al., 1998).

Cardiac H3Rs are possibly coupled to G/Go proteins, since we found that pertussis toxin, which inactivates G/Go (Bokoch et al., 1983; Codina et al., 1983), attenuates the associated with a marked decrease in intraneuronal Ca2+ ([Ca2+]i) (Silver et al., 2002). Because ω-conotoxin (ω-CTX) and imetit each decreased [Ca2+]i, and NE exocytosis (Silver et al., 2002), and since ω-CTX decreases [Ca2+]i by inhibiting Ca2+ influx through N-type Ca2+ channels (Sher et al., 1991), we speculated that, similar to ω-CTX, imetit-induced H3R activation might decrease [Ca2+]i by inhibiting Ca2+ influx through voltage-dependent Ca2+ channels in sympathetic nerve terminals (Silver et al., 2002). In fact, an H3R-mediated inhibition of N-type Ca2+ channel current has been claimed to occur in histaminergic neurons from the rat hypothalamus (Takeshita et al., 1998).

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H₃R-mediated inhibition of adrenergic responses in the heart (Endou et al., 1994). Furthermore, H₃R activation inhibits forskolin-stimulated cAMP formation in SKNMC neuroblastoma cells stably transfected with the human H₃R (Lovenberg et al., 1999). Because cAMP-dependent phosphorylation of N-type Ca²⁺ channels increases their activity (Ahlijanian et al., 1991; Hell et al., 1995; Catterall, 2000), a decreased phosphorylation due to inhibition of the cAMP/PKA pathway could conceivably be involved in the H₃R-mediated attenuation of N-type Ca²⁺ channel activity and NE exocytosis.

We have tested this hypothesis in human neuroblastoma cells transfected with human H₃R (SH-SY5Y-H₃) (Silver et al., 2002) and in sympathetic nerve endings (cardiac synaptosomes) expressing native H₃R (Seyedi et al., 1997). We report that the H₃R-mediated inhibition of NE exocytosis from cardiac sympathetic nerves results sequentially from H₃R-Gi/Gs coupling, inhibition of adenyl cyclase activity, and cAMP formation, leading to diminished PKA activity, decreased Ca²⁺ influx through voltage-operated Ca²⁺ channels (VOCC), and thus, attenuation of NE exocytosis.

Materials and Methods

Preparation of Cardiac Synaptosomes. Male Hartley guinea pigs (Charles River Laboratories, Inc., Wilmington, MA) weighing 250 to 300 g were anesthetized with CO₂ vapor and exsanguinated. The ribcage was rapidly opened and the heart dissected away. A cannula was inserted in the aorta and the heart was perfused for 5 min at constant pressure (40 cm of H₂O) in a Langendorff apparatus (Seyedi et al., 1997) with Ringer’s solution (containing 154 mM NaCl, 5.61 mM KCl, 2.16 mM CaCl₂, 5.95 mM NaHCO₃, and 5.55 mM glucose, pH 7.4) equilibrated with 100% O₂ at 37°C. This procedure ensured that no blood traces remained in the coronary circulation.

Hearts were then freed from fat and connective tissue and minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.4. Synaptosomes were isolated as described previously (Imamura et al., 1995; Seyedi et al., 1997), with the following modifications. Minced tissue was digested with 40 to 75 mg of collagenase (type II, Worthington Biochemicals; Freehold, NJ) per 10 ml of HEPES-buffered saline solution (HBS; containing 50 mM HEPES, pH 7.4, 144 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 1 mM pargyline hydrochloride to prevent enzymatic destruction of synaptosomal NE) per gram wet heart weight for 1 h at 37°C. After low-speed centrifugation (10 min at 1200 g, 4°C), the resulting pellet was suspended in 2 ml of HBS for 10 min. The pellet, which contained cellular debris, was discarded, and the supernatants from the last two spins were combined and equally subdivided into 10 to 12 tubes. Each tube was centrifuged at 20,000 g for 20 min, 4°C. Each pellet containing cardiac synaptosomes was resuspended in HBS to a final volume of 500 μl and incubated with KCl (10–100 mM) or forskolin (0.1–10 μM) 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 release) and the others were treated with high K⁺ or forskolin, high K⁺ or forskolin plus drugs, or with drugs alone. When high K⁺ was used, osmolarity was maintained constant by adjusting the NaCl concentration. Treated samples were incubated with a given agent for 10 min and then with high K⁺ for 5 min or forskolin for 20 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. At the end of the incubation period, each sample was centrifuged again at 20,000g for 20 min, 4°C. The supernatant was assayed for NE content by high-pressure liquid chromatography with electrochemical detection (Seyedi et al., 1997).

The pellet was assayed for protein content, by a modified Lowry procedure (Markwell et al., 1978).

Treatment of Synaptosomes with Pertussis Toxin (PTX). When PTX was used, isolated synaptosomes were incubated with PTX for 60 min, and then synaptosomes were washed free of PTX as follows. Tubes containing synaptosomes and 0.3 μg/ml PTX were centrifuged at 20,000g for 5 min, 4°C; the supernatant was discarded and the pellet was suspended in 2 ml of HBS for 10 min. The pellet was centrifuged at 20,000g for 5 min, 4°C, and the supernatant was discarded. The resulting pellet was resuspended in 1 ml of HBS and incubated with KCl (30 and 100 mM) or forskolin (10 μM) in the presence or absence of pharmacological agents for a total of 20 to 25 min in a water bath at 37°C, and then centrifuged at 20,000g for 20 min, 4°C. The supernatant and pellet were assayed for NE and protein content, as mentioned above, respectively.

SH-SY5Y-H₃ Cells. A human neuroblastoma cell line stably transfected with the H₃R (SH-SY5Y-H₃) was kindly supplied by Dr. T. Lovenberg (Silver et al., 2002). 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 gentamicin, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C, 5% CO₂. Cells were grown to confluence in 6-, 24-, or 96-well plates for PKA activity, [³H]NE release experiments, and cAMP assay, respectively, or for 4 to 5 days on 22-mm² standard glass coverslips (no. 1) for (Ca²⁺)i measurements. Cell culture media and supplements were purchased from Mediatech (Herndon, VA).

[³H]NE Release Assay. The [³H]NE release method was adapted from that described by Murphy et al. (1991). The culture medium was removed and cells were washed once with HEPES-buffered Na⁺ Ringer’s solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4), and then incubated in 230 μl/well of Na⁺ Ringer’s buffer containing 50 nM [³H]NE for 60 min at 37°C. This was followed by three washes with 450 μl/well Na⁺ Ringer’s buffer (containing 1 μM desipramine). Release buffer (Na⁺ Ringer’s buffer with 100 mM K⁺, adjusted to maintain osmolality) was then added to each well (450 μl/well) for 5 min at room temperature. A 300-μl aliquot of the supernatant was taken from each well for counting, and the remaining solution was discarded. Then 0.3% Triton X-100 was added to the cells (450 μl/well) for 30 min, and 300 μl of lysisate was taken for counting. Samples taken for counting were each added to 4 ml of Bio-Safe II scintillation cocktail and counted on a Beckman Coulter LS6800 scintillation counter. For drug experiments, after the three washes cells were incubated in 450 μl of Na⁺ Ringer’s buffer containing the given drug for 5 min at 37°C, followed by release as described above. [³H]NE release was expressed as a percentage of the total [³H]NE content.

cAMP Assay. SH-SY5Y-H₃ cells were grown to confluence in 96-well plates. After a 20-min treatment with the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2 mM), the cells were incubated for 5 min with or without the H₃R agonist imetit (100 nM), either alone or in combination with the H₃R antagonist clobenpropit (CBP; 25 nM). Cells were incubated with CBP for 5 min before the addition of imetit. Intracellular cAMP levels were then stimulated by either forskolin (10 μM) for 20 min, or high K⁺ (100 mM) for 5 min. The cells were immediately aspirated and the intracellular cAMP levels determined using a cAMP Biotrak ELA kit (Amersham Biosciences Inc., Piscataway, NJ) following the manufacturer’s protocol.

Treatment of SH-SY5Y-H₃ Cells with PTX. SH-SY5Y-H₃ cells were grown to confluence in six-well plates. Cells were incubated in PTX (200 ng/ml) for 24 h before experimentation.

Detection of PKA Activation. When SH-SY5Y-H₃ cells were confluent, cells were washed with serum-free medium and then maintained in Eagle’s minimal essential medium/Ham’s F-12 with 0.1% bovine serum albumin for 48 h. PKA phosphorylation (i.e., an indication of PKA activation) was elicited by incubating SH-SY5Y-H₃ cells with forskolin (10 μM) or K⁺ (100 mM) for 5 min, in the absence of drugs.
or presence of imetit (100 nM), either with or without CBP (25 nM). SH-SY5Y- H3 cells were lysed (lysis buffer composition: 1% Triton X-100, 0.5% deoxycholic acid, 50 mM Tris-HCl, 0.1% SDS, 1 mM EDTA, 50 mM NaCl). Samples of lysate (10 μg/lane) were prepared with 5× Tris-glycine SDS sample buffer and boiled for 5 min before separation on 8% Tris-glycine SDS-polyacrylamide minigels (Gradi- pore, French's Forest, NSW, Australia). Electrophoresis was carried out at 200 V, 40 mAigel for 1 h. Gels were soaked in transfer buffer (25 mM Tris-base, 0.2 M glycine, and 10% methanol, pH 8.5) and electrotransferred to polyvinylidine difluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA) for 90 min at 200 V, 100 mA, room temperature. Membranes were blocked for at least 2 h in blocking buffer [Tris-buffered saline (TBS) containing 0.1% Tween 20, 5% (w/v) nonfat dry milk]. p-PKA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a primary antibody. It was incubated with the PVDF membrane overnight at 4°C, diluted appropriately in primary antibody dilution buffer (TBS containing 0.1% Tween 20, 5% bovine serum albumin). The PVDF membrane was washed three times with TBS and then horseradish peroxidase-coupled anti-rabbit IgG (Cell Signaling Technology Inc., Beverly, MA) was added at a 1:2000 dilution in blocking buffer for 1 h. After three further TBS washes, the protein of interest was detected using enhanced chemiluminescence (LumiGLO; Cell Signaling Technology Inc.) and exposure to X-ray film (Biomax MR; Eastman Kodak, Rochester, NY). In the immunoblot, phosphorylated PKA was visualized, as expected, as a single band at 54 kDa (Tasken et al., 1995). Bands were analyzed by densitometry using NIH Image, version 1.61.

[Ca2+]i Measurements. SH-SY5Y- H3 cells grown on coverslips were loaded with the membrane-permeant form of the [Ca2+]i indicator Fura-2 (10 μM) for 40 min at 37°C. After loading with the dye, the cells were rinsed with Na+ Ringer’s solution. The coverslip was attached to the bottom of a flow-through superfusion chamber and mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot). The cells in the chamber were superfused and maintained at 37°C as described previously (Cardone et al., 1996). Cells were first visualized under transmitted light with a Nikon CF Fluor (40×/1.3 numerical aperture oil immersion objective) before starting fluorescence measurements. Cells were depolarized with a high-K+ solution (based on the Na+ Ringer’s composition described above with 100 mM KCl replacing 100 mM NaCl). Calibration of the emitted Fura-2 signal from each cell in the field was carried out in the presence of the Ca2+ ionophore ionomycin (10 μM) in the presence of HEPES buffer containing either 2.6 mM Ca2+ or 10 mM EGTA titrated to pH 7.4. [Ca2+]i, levels were calculated as described previously (Grynkiewicz et al., 1985). Cells in the experimental field of view were analyzed singularly and independently from their neighbors.

Reagents. Ionomycin was prepared in dimethyl sulfoxide to a concentration of 10 mM; it was subsequently diluted as mentioned above to a 10 μM solution. Individual vials (50 μg) of the acetyoxymethyl derivative of Fura-2 were stored dry at 0°C and reconstituted in dimethyl sulfoxide, at a concentration of 5 mM, for each experiment.

Equipment. The basic components of the experimental apparatus have been described previously (Cardone et al., 1996; Silver et al., 2001). The imaging workstation was controlled using the Metafluor software package (Universal Imaging Corporation, Downingtown, PA). Quantitative image pairs at 340- and 380-nm excitation with emission at 510 nm were obtained either every 15 s before K+ depolarization or every second immediately preceding and during depolarization. The fluorescence excitation was shuttered off except during the brief periods required to record an image. To check for interference from intrinsic autofluorescence and background, images were obtained on cells by using the same exposure time and filter combination used for the experiments, and found to be minor compared with the fluorescence signal.

Drugs and Chemicals. Desipramine hydrochloride (DMI), atro- pine sulfate, imetit dihydrobromide, clenodipropit dihydrobromide (CBP), PTX, carbamyl choline chloride (carbachol), N6-cyclopentyl adenosine (CPA), 3-isobutyl-1-methylxanthine, forskolin, o-fi nedpine, ionomycin, pargyline, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). 8-Bromo-cAMP (8-bromoadenosine-3’,5’-cyclic monophosphate Na), 2’,5’-dideoxyxadenosine 9-(2’,5’-dideoxy-erythro-pento furanosyl) adenine (F site ligand), and myristoylated PKI(14–22) amide (PKA inhibitor) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). 8-Cyclopentyl-1,3-Dipropylxanthine (DPCPX) was purchased from Sigma/RBI (Natick, MA). [3H]NE (28.0 Ci/mmol) was purchased from Amersham Biosciences, Inc. Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Forskolin, DPCPX, nifedipine, and o-CTX were dissolved in dimethyl sulfoxide. Further dilutions were made with HBS buffer; at the concentration used, dimethyl sulfoxide did not affect NE release.

Statistics. NE release values are expressed as mean percentage of increases above basal NE release ± S.E.M. cAMP levels are expressed as mean absolute values ± S.E.M. PKA phosphorylation is expressed as mean percentage of change from the effect of forskolin (10 μM). K+–induced peak [Ca2+]i levels are expressed as mean values (nanomolar) ± S.E.M. Decreases in K+–induced peak [Ca2+]i, are expressed as percentage of inhibition ± S.E.M. Statistical analysis was performed by unpaired t test or by one-way ANOVA followed by post hoc testing (Dunnett’s test) as indicated in the legend to each figure. A P value of <0.05 was considered statistically significant.

Results.

Activation of H3R in Cardiac Synaptosomes Attenuates NE Exocytosis Elicited by K+ and Forskolin. As shown in Fig. 1A, exposure of sympathetic nerve endings isolated from guinea pig hearts (cardiac synaptosomes) to increasing extracellular K+ concentrations (10–100 mM) resulted in an ~12–33% increase in endogenous NE release. In the presence of the selective H3R agonist imetit (100 nM) the K+–induced NE exocytosis was markedly attenuated. Imetit-
induced attenuation of NE exocytosis was abolished when synaptosomes were preincubated with the selective H₃R antagonist CBP (25 nM) (Van der Goot et al., 1992) (Fig. 1A).

Since NE exocytosis can also be elicited when the intraneuronal cAMP level is increased (Markstein et al., 1984; May et al., 1995), we incubated cardiac synaptosomes with increasing concentrations of the adenylyl cyclase activator forskolin (0.1–10 μM). This resulted in an ~10 to 35% increase in endogenous NE release (Fig. 1B). In the presence of imetit (100 nM) the forskolin-induced NE release was markedly attenuated. CBP (25 nM) abolished the effect of imetit.

The H₃R-Induced Attenuation of NE Exocytosis in Cardiac Synaptosomes Is Mediated by a Gₛ/Gₛₛ-Coupled Decrease in Adenylyl Cyclase Activity. These findings revealed that activation of H₃R attenuates NE exocytosis whether it is elicited by membrane depolarization with K⁺ or by stimulation of adenylyl cyclase with forskolin. As shown in Fig. 2, NE exocytosis from cardiac synaptosomes was enhanced not only by forskolin but also by administration of the permeant form of cAMP, 8-bromo-cAMP (1 mM) (Meyer and Miller, 1974). Similar to imetit, the adenylyl cyclase inhibitor P-site ligand (Desaubry et al., 1996) markedly attenuated the forskolin-induced NE release from cardiac synaptosomes (Fig. 2). In contrast, when NE release was induced by 8-bromo-cAMP, neither imetit nor the P-site ligand had any effect (Fig. 2). This suggested that the release of NE elicited by forskolin results from an increase in intraneuronal cAMP and that a reduction in adenylyl cyclase activity is the pivotal mechanism by which imetit attenuates NE exocytosis.

Since inhibition of adenylyl cyclase could be due to coupling of H₃R to Gₛ/Gₛₛ protein (Lovenberg et al., 1999), we preincubated cardiac synaptosomes with the inhibitor of Gₛ/Gₛ PTX. As shown in Fig. 3A, in cardiac synaptosomes preincubated with PTX, imetit failed to attenuate NE exocytosis elicited by depolarization with K⁺. PTX pretreatment also prevented the imetit-induced attenuation of NE release elicited by forskolin (Fig. 3B). As a positive control of a Gₛ/Gₛₛ involvement in the imetit-induced attenuation of NE exocytosis, we activated muscarinic (i.e., M₂R and M₄R) and adenosine A₁-receptors, which are both known to be coupled to Gₛ/Gₛ (Caulfield and Birdsall, 1998; Fredholm et al., 2001). As shown in Fig. 4, the forskolin-induced NE release was greatly attenuated by the muscarinic agonist carbachol (100

![Fig. 2. H₃R activation inhibits NE release elicited by adenylyl cyclase stimulation but not by 8-bromo-cAMP administration to cardiac sympathetic nerve endings. Imetit (100 nM) and the adenylyl cyclase inhibitor P-site ligand (100 μM) markedly attenuated the release of endogenous NE elicited by forskolin in guinea pig heart synaptosomes. Note that neither imetit nor P-site ligand modifies NE release when elicited by 8-bromo-cAMP (8-B cAMP; 1 mM). Columns represent mean values (±S.E.M.; n = 4–13). Basal NE release was 1.54 ± 0.06 pmol/mg protein (n = 13). *P < 0.05 versus forskolin-evoked NE release, by ANOVA followed by post hoc Dunnett’s test.

![Fig. 3. Pretreatment with PTX abolishes the H₃R-mediated attenuation of NE exocytosis in K⁺-depolarized guinea pig heart sympathetic nerve endings as well as the attenuation of forskolin-induced NE exocytosis elicited by activation of muscarinic, adenosine A₁- and H₃-receptors. A, effect of forskolin results from an increase in intraneuronal cAMP and that a reduction in adenylyl cyclase activity is the pivotal mechanism by which imetit attenuates NE exocytosis. Basal NE release was 1.62 ± 0.02 pmol/mg protein (n = 12). Basal NE release was 1.53 ± 0.02 pmol/mg protein, *P < 0.05 versus corresponding K⁺-evoked NE release, by ANOVA followed by post hoc Dunnett’s test. B, release of NE from cardiac synaptosomes upon stimulation of adenylyl cyclase with forskolin (10 mM). The effect of forskolin is inhibited by activation of muscarinic, adenosine A₁- and H₃-receptors with carbachol (C; 100 nM), CPA (100 nM), and imetit (Im.; 100 nM), respectively. The effects of carbachol, CPA, and imetit are abolished when synaptosomes were pretreated with PTX (0.3 μg/ml). Columns represent mean values (±S.E.M.; n = 12). Basal NE release was 1.62 ± 0.05 pmol/mg protein (n = 15). *P < 0.05 versus forskolin-evoked NE release, by ANOVA followed by post hoc Dunnett’s test.]

![Fig. 4. Activation of muscarinic and adenosine A₁R inhibits NE release from guinea pig heart sympathetic nerve endings elicited by stimulation of adenylyl cyclase with forskolin. The effect of forskolin is inhibited by carbachol (100 nM; blocked by atropine 100 nM) and by CPA (100 nM; blocked by DPCPX 300 nM). Points are mean values (±S.E.M.; n = 4 for both A and B). Basal NE release was 1.28 ± 0.025 pmol/mg protein for A and 1.47 ± 0.08 for B. *P < 0.05 from corresponding control NE level by unpaired t test.]

PKA Inhibition Mediates H₃R Attenuation of NE Exocytosis 275
nM; Fig. 4A) and by the selective adenosine A₁-receptor agonist CPA (100 nM; Fig. 4B). The effects of carbachol and CPA were prevented by the respective muscarinic- and A₁-receptor antagonist atropine (100 nM) and DPCPX (300 nM) (Fig. 4, A and B). Similar to imetit, the carbachol- and CPA-induced attenuation of NE release was abolished by PTX pretreatment (Fig. 3B).

The H₃R-Mediated Attenuation of NE Exocytosis in SH-SY5Y-H₃ Cells Is Associated with a Decrease in Intracellular cAMP. Since these findings implied an H₃R-mediated, Gₛ/Gap-coupled, decrease in adenyl cyclase activity, we next assessed whether this would result in a decrease in intracellular cAMP levels. For this, we used the H₃R-transfected neuroblastoma cell line SH-SY5Y (SH-SY5Y-H₃) (Silver et al., 2002). As shown in Fig. 5A, forskolin (0.1–10 μM), elicited an ~10–35% increase in [³H]NE release. At 10 μM, forskolin also caused a large increase in cAMP (Fig. 5D). Imetit (100 nM) markedly attenuated the increase in cAMP and associated NE release in response to 10 μM forskolin (Fig. 5, B and D). Both effects were inhibited by the H₃R-antagonist CBP (25 nM) (Fig. 5, B and D). Similarly, imetit antagonized the increase in cAMP and associated NE release elicited by K⁺ depolarization; CBP blocked both of these effects (Fig. 5, C and E).

The H₃R-Mediated Attenuation of NE Exocytosis in SH-SY5Y-H₃ Cells Leads to a Reduction in [Ca²⁺], and an Attenuation of NE Exocytosis. We next questioned whether the H₃R-mediated decrease in PKA activity would lead to a reduction in [Ca²⁺] and, thus, NE exocytosis. For this, we measured peak [Ca²⁺], in SH-SY5Y-H₃ cells depolarized with K⁺, in the absence and presence of imetit (100 nM), either alone or in combination with PKI(14–22) (0.2–30 nM). The effect of PKI(14–22) was similar to that of imetit (Fig. 7, C and D). When subthreshold concentrations of PKI(14–22) (0.2 nM) and imetit (0.2–3 nM) were used in combination, a marked synergistic effect was observed (Fig. 7, E and F). Collectively, these findings suggested that a decrease in PKA activity is likely to be involved in the H₃R-mediated attenuation of NE exocytosis.

A Decrease in Ca²⁺ Influx through N- and L-Type Ca²⁺ Channels Is Involved in the H₃R-Mediated Attenuation of NE Exocytosis in Cardiac Synaptosomes. The finding that H₃R activation and PKA inhibition acted synergistically to reduce peak [Ca²⁺], in response to K⁺ implied that a decreased phosphorylation of VOCC and thus, Ca²⁺ influx, may be responsible for the H₃R-mediated reduction in peak [Ca²⁺]. We thus assessed the role of VOCC in the K⁺ and forskolin-induced NE exocytosis in cardiac synapto-
markedly diminished by the selective N-type and L-type Ca\(^{2+}\) channel inhibitors somes. As shown in Fig. 9, the K\(^+\)-induced NE release was also markedly diminished by the selective N-type and L-type Ca\(^{2+}\) channel inhibitors ω-CTX (100 nM) and nifedipine (5 μM), both alone and in combination (Fig. 9A). The forskolin-induced NE release was also markedly diminished by ω-CTX (100 nM) and nifedipine (5 μM), both alone and in combination (Fig. 9B). These findings revealed not only a similarity between K\(^+\)-depolarization and forskolin administration but also a resemblance of H\(_3\)R activation to N- and L-type Ca\(^{2+}\) channel inhibition. As this resemblance suggested a similarity of mechanisms of action, we next investigated the effects of imetit in combination with either Ca\(^{2+}\) channel blocker. As shown in Fig. 10, A and B, ω-CTX and nifedipine each inhibited as a function of their concentration NE exocytosis elicited by K\(^+\)-depolarization in SH-SY5Y-H\(_3\) cells. 

**Fig. 6.** H\(_3\)R activation inhibits the phosphorylation of PKA (i.e., PKA activation) elicited by forskolin or K\(^+\) in SH-SY5Y-H\(_3\) cells. Upper strips, representative immunoblots of SH-SY5Y-H\(_3\) cell lysates probed with anti-phosphorylated PKA antibody (see Materials and Methods for details; 10 μg of protein/well). Columns (means ± S.E.M. of 6 and 12 experiments for forskolin and K\(^+\), respectively) represent the level of PKA phosphorylation, expressed as percentage of maximal response to 10 μM forskolin or 100 nM K\(^+\), in the absence or presence of imetit (100 nM), either alone or in combination with CBP (25 nM). *, significantly different from control (P < 0.01 by ANOVA followed by post hoc Dunnet't test).

**Discussion**

We had reported that activation of presynaptic H\(_3\)R inhibits the exocytotic release of NE elicited by depolarization of

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*Fig. 7.** H\(_3\)R activation and PKA inhibition act synergistically to attenuate the release of endogenous NE elicited by K\(^+\)-depolarization or forskolin in sympathetic nerve endings from guinea pig heart. A and B, concentration-response curves for the attenuating effect of the PKA inhibitor PKI(14–22). C and D, concentration-response curves for the inhibitory effect of imetit. E and F, inhibition of NE release from cardiac synaptosomes by subthreshold concentrations of imetit and PKI(14–22), administered either alone or in combination; note that a significant attenuation of NE release occurs when imetit and PKI(14–22) are combined [*+, significantly different from the sum of imetit + PKI(14–22), P < 0.01 by unpaired t test]. Points and columns are means ± S.E.M. (n = 12, 6–12, 4, 6–12, 4–16, and 6 for A to F, respectively. Basal NE level was 1.38 ± 0.13 pmol/mg protein (n = 62).

*Fig. 8.** H\(_3\)R activation and PKA inhibition act synergistically to attenuate the peak [Ca\(^{2+}\)]\(_i\), elicited by K\(^+\)-depolarization in SH-SY5Y-H\(_3\) cells. A, peak [Ca\(^{2+}\)]\(_i\), levels elicited by depolarization with 100 mM K\(^+\), in the absence (C) or presence of imetit (3 and 100 nM) and PKI(14–22) (0.02 and 20 nM), either alone or in combination (0.02 nM PKI(14–22) + 3 nM imetit). Basal [Ca\(^{2+}\)]\(_i\), level before K\(^+\)-depolarization was 105 ± 32 nM (n = 1057 cells). B, percentage of inhibition of K\(^+\)-induced peak [Ca\(^{2+}\)]\(_i\), demonstrating synergism of action between imetit and PKI(14–22) (same data as in A). Columns represent means ± S.E.M. of 325 cells (Control), 72 and 205 cells (for 3 and 100 nM imetit, respectively), 207 and 142 cells (for 0.02 and 20 nM PKI(14–22), respectively), and 106 cells (for 0.02 and 20 nM PKI(14–22) + 3 nM imetit). †, P < 0.01, from control; ††, P < 0.01, from 3 nM imetit; †††, P < 0.001, from the sum of imetit + PKI(14–22) by unpaired t test.

**Fig. 9.** K\(^+\)-induced NE release was also markedly diminished by ω-CTX (100 nM) and nifedipine (5 μM), both alone and in combination (Fig. 9A). The forskolin-induced NE release was also markedly diminished by ω-CTX (100 nM) and nifedipine (5 μM), both alone and in combination (Fig. 9B). These findings revealed not only a similarity between K\(^+\)-depolarization and forskolin administration but also a resemblance of H\(_3\)R activation to N- and L-type Ca\(^{2+}\) channel inhibition. As this resemblance suggested a similarity of mechanisms of action, we next investigated the effects of imetit in combination with either Ca\(^{2+}\) channel blocker. As shown in Fig. 10, A and B, ω-CTX and nifedipine each inhibited as a function of its concentration NE exocytosis elicited by K\(^+\)-depolarization of cardiac synaptosomes. When a subthreshold concentration of imetit (3 nM) was used in combination with a subthreshold concentration of either ω-CTX (0.1 nM) or nifedipine (0.1 μM), a marked synergistic effect was observed (Fig. 10C). These findings suggested that a decrease in Ca\(^{2+}\) influx through N- and L-type Ca\(^{2+}\) channels is likely to be involved in the H\(_3\)R-mediated attenuation of NE exocytosis.
cardiac sympathetic nerve endings (cardiac synaptosomes) (Seyedi et al., 1997), atrial tissue (Imamura et al., 1995), and intact heart (Imamura et al., 1994). While exploring possible mechanisms of this modulatory action, we found that H3R activation is associated with a marked decrease in [Ca\(^{2+}\)]i and NE exocytosis. Indeed, a decreased influx, [Ca\(^{2+}\)]i and NE exocytosis. Since NE exocytosis can be elicited when intraneuronal cAMP is enhanced (Markstein et al., 1984; May et al., 1995), and an increase in extracellular K+ is known to elevate intraneuronal cAMP (Cooper et al., 1998), we compared NE exocytosis initiated by K+-induced depolarization with the exocytosis elicited by adenylyl cyclase stimulation with forskolin. As a target, we used cardiac adrenergic nerve terminals isolated from the guinea pig heart (i.e., synaptosomes) (Seyedi et al., 1997) and SH-SYSY-H3 neuroblastoma cells (Silver et al., 2002), endowed with native and stably transfected H3R, respectively. We found that H3R activation attenuated NE exocytosis independently of whether it was elicited by neuronal depolarization or adenylyl cyclase stimulation. Although this indicated an effect on a common downstream signal, most likely cAMP, we found that imetit effectively attenuated NE exocytosis initiated by forskolin, but not that initiated by the administration of the permeant form of cAMP, 8-bromo-cAMP. This clearly indicated that adenylyl cyclase, and not cAMP, is the pivotal initial site of H3R-induced attenuation of NE exocytosis. Indeed, a decreased generation of cAMP by forskolin had already been observed in SKNMC-H3 neuroblastoma cells as a result of H3R stimulation (Lovenberg et al., 1999). The next question was how H3R activation results in a diminished adenylyl cyclase activity. Preliminary evidence from our laboratory had suggested that G\(_i\)/G\(_o\) may be involved in the H3R-mediated attenuation of adrenergic inotropic responses in the heart (Endou et al., 1994). Other investigators had also suggested a G\(_i\)/G\(_o\) involvement in the H3R-mediated inhibition of NE release from intestinal sympathetic nerves (Blandizzi et al., 2000). We therefore determined whether the H3R-mediated inhibition of NE exocytosis elicited by K+ or forskolin is attenuated by PTX, a
toxin that inactivates G_i/G_o via ADP-ribosylation (Bokoch et al., 1983; Codina et al., 1983). Inasmuch as muscarinic and adenosine A1-receptors are coupled to G_i/G_o (Caulfield and Birdsal, 1998; Fredholm et al., 2001), we activated these receptors on cardiac synaptosomes (with carbachol and CPA, respectively), we verified that their activation would attenuate the forskolin-induced NE exocytosis, and then, as a positive control, ensured that pretreatment with PTX would prevent the attenuation of NE exocytosis by carbachol and CPA. Indeed, PTX pretreatment abolished equally well the inhibitory effects of carbachol, CPA, and imetit. In fact, PTX abolished also the imetit-induced attenuation of NE exocytosis elicited by depolarization with K^+.

Accordingly, our data compellingly demonstrate that H3R are negatively coupled to adenylcyclase via G_i/G_o.

Given this H3R-mediated, G_i/G_o-coupled decrease in adenylcyclase activity, we next assessed whether H3R activation would lower intracellular cAMP levels. For this, we used the SH-SY5Y-H3 neuroblastoma cell line, an optimal model of sympathetic nerve endings (Silver et al., 2002), better suited than cardiac synaptosomes for the measurement of cyclic nucleotides. We found that NE exocytosis from SH-SY5Y-H3 cells, elicited by either K^+ or forskolin, was associated with an increase in cAMP. Notably, H3R activation inhibited NE exocytosis as well as the increase in cAMP, suggesting that the H3R-induced attenuation of NE exocytosis is mediated by a decrease in cAMP. Inasmuch as a fall in cAMP would be expected to result in a decreased cAMP-dependent phosphorylation, we next assessed whether H3R activation is associated with a decrease in PKA activity. Since PKA phosphorylation is a measure of PKA activity (Erlichman et al., 1974), we determined levels of PKA phosphorylation by Western blot analysis in SH-SY5Y-H3 cells. We found that H3R activation significantly reduced the stimulation of PKA activity in response to either forskolin or K^+.

Moreover, we found that the selective H3R agonist imetit (Garbarg et al., 1992) and the specific PKA inhibitor PKI(14–22) (Glass et al., 1989) acted synergistically to inhibit NE exocytosis from cardiac synaptosomes, independently of whether NE exocytosis was elicited by K^+ or forskolin. Notably, 8-bromo-cAMP is a potent stimulator of PKA (Hei et al., 2001). We found that imetit, like the P-site ligand, inhibited the forskolin-induced NE exocytosis but not that elicited by 8-bromo-cAMP (see Fig. 2). Thus, all of our evidence indicates that the reduction in adenylcyclase activity which is the hallmark of H3R activation (Lovenberg et al., 1999) leads to a decrease in cAMP and PKA activity in cardiac sympathetic nerve terminals.

cAMP-dependent phosphorylation of VOCC increases their activity (Ahlijanian et al., 1991; Hel et al., 1995; Catterall, 2000); thus, a decreased phosphorylation due to inhibition of the cAMP/protein kinase A pathway could conceivably be involved in the H3R-mediated attenuation of Ca2^+- influx, [Ca2^+]_i, and NE exocytosis. Indeed, we found that H3R activation and PKA inhibition each reduced peak [Ca2^+]_i in response to K^+-depolarization in SH-SY5Y-H3 cells; moreover, imetit and PKI(14–22) acted synergistically to diminish peak [Ca2^+]_i. This implied that a decreased phosphorylation of VOCC and thus, Ca2^+- influx, may be responsible for the H3R-mediated reduction in [Ca2^+]_i. In fact, we observed that ω-CTX (Sher et al., 1991) and nifedipine (Vater et al., 1972), both alone and in combination, markedly diminished NE exocytosis elicited by K^+ and forskolin in cardiac synaptosomes. These findings revealed that both N- and L-type Ca2^+ channels are involved in the exocytotic release of NE from cardiac sympathetic nerve endings. Furthermore, given the similarity between H3R activation and VOCC inhibition (compare Figs. 1 and 9), our findings implied that the H3R-induced decrease in NE exocytosis is ultimately due to an inhibition of Ca2^+- influx. In support of this concept, we found that imetit acted synergistically with each of the N- and L-type Ca2^+ channel blockers, ω-CTX and nifedipine, in attenuating NE exocytosis.

Collectively, our findings suggest that the H3R-mediated attenuation of NE exocytosis from cardiac sympathetic nerves involves an H3R-G_i/G_o coupling, adenyl cyclase inhibition by G_i, decreased cAMP formation, diminished PKA activity, decreased Ca2^+- influx through VOCCs, culminating in a decreased [Ca2^+]_i, transient and thus, in an impaired exocytosis. The possibility should also be considered that the H3R-mediated attenuation of NE exocytosis may result in part from an inhibition of Ca2^+- influx by direct coupling of the G_i subunit to VOCC (Herlitze et al., 1996; Ikeda, 1996; Catterall, 2000) or to a direct interaction between G_i and the exocytotic fusion machinery at the presynaptic terminal downstream of Ca2^+- entry (Blackmer et al., 2001).

In conclusion, the elucidation of transductional mechanisms implicated in the H3R-induced modulation of NE exocytosis in cardiac sympathetic nerve terminals will help our understanding of neurotransmitter release in hyperadrenergic states characterized by enhanced NE exocytosis, such as myocardial ischemia, hypertension, and congestive heart failure.

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References


Histamine H3-receptors: A new frontier in myocardial ischemia.


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