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Cardioprotective Effect of Histamine H_3-Receptor Activation: Pivotal Role of Gβγ-Dependent Inhibition of Voltage-Operated Ca^{2+} Channels

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Abstract

We previously showed that activation of $G_{i/o}$-coupled histamine H_3-receptors (H_3R) is cardioprotective since it attenuates excessive norepinephrine release from cardiac sympathetic nerves. This action is characterized by a marked decrease in intraneuronal Ca^{2+} ($[Ca^{2+}]_i$), as $G\alpha_i$ impairs the adenylyl cyclase-cAMP-PKA pathway, and this decreases Ca^{2+} influx via voltage-operated Ca^{2+} channels (VOCC). Yet, the $G_{i/o}$-derived βγ dimer could directly inhibit VOCC, and the subsequent reduction in Ca^{2+} influx would be responsible for the H_3R-mediated attenuation of transmitter exocytosis. Here, we tested this hypothesis in nerve-growth factor-differentiated rat pheochromocytoma cells (PC12) stably transfected with H_3R (PC12-H_3) and with the Gβγ scavenger β-ARK1-(495−689)-polypeptide (PC12-H_3/β-ARK1). Thus, we evaluated the effects of H_3R activation directly on: 1) Ca^{2+} current (I_{Ca}) using the whole-cell patch-clamp technique, and 2) K^+-induced exocytosis of endogenous dopamine. H_3R activation attenuated both peak I_{Ca} and dopamine exocytosis in PC12-H_3, but not in PC12-H_3/β-ARK1 cells. Moreover, a membrane permeable phosducin-like Gβγ scavenger also prevented the anti-exocytotic effect of H_3R activation. In contrast, the H_3R-induced attenuation of cAMP accumulation and dopamine exocytosis in response to forskolin were the same in both PC12-H_3 and PC12-H_3/β-ARK1 cells. Our findings reveal that while $G\alpha_i$ participates in the H_3-mediated anti-exocytotic effect when the adenylyl cyclase-cAMP-PKA pathway is stimulated, a direct Gβγ-induced inhibition of VOCC, resulting in an attenuation of I_{Ca} plays a pivotal role in the H_3R-mediated decrease in $[Ca^{2+}]_i$ and associated cardioprotective anti-exocytotic effects. The discovery of this H_3R signaling step may offer new therapeutic approaches to cardiovascular diseases characterized by hyperadrenergic activity.

Introduction

Sympathetic nerve endings in the mouse (Koyama et al., 2003), guinea pig (Endou et al., 1994) and human hearts (Imamura et al., 1995) express histamine H_3-receptors (H_3R). H_3R activation results in attenuation of excessive norepinephrine release in myocardial ischemia, a recognized cardioprotective effect, since it alleviates arrhythmic cardiac dysfunction (Levi and Smith, 2000).

We previously reported that imetit, a selective H_3R agonist (Garbarg et al., 1992), reduces norepinephrine exocytosis evoked by depolarization of cardiac sympathetic nerve endings (Imamura et al., 1994; Imamura et al., 1995), an action associated with a marked decrease in intraneuronal Ca^{2+} ($[Ca^{2+}]_i$) (Silver et al., 2002). It is conceivable that H_3R activation may

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decrease ($[Ca^{2+}]_i$) by inhibiting Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCC) in sympathetic nerve terminals. H_3R-mediated inhibition of N-type Ca^{2+} channel current has been claimed to occur in histaminergic neurons from the rat hypothalamus; an unverified claim, however, since selective H_3R antagonists were not shown to block this effect (Takeshita et al., 1998).

Although N-type Ca^{2+} channels are the dominant Ca^{2+} entry pathway triggering sympathetic transmitter release (Lipscombe et al., 1989; Zhu and Yakel, 1997), it is possible that entry of Ca^{2+} through L-type Ca^{2+} channels may also be important in norepinephrine exocytosis and be inhibited by H_3R activation. Indeed, we found that H_3R activation synergizes with both N- and L-type Ca^{2+} channel blockers to reduce K^+-induced norepinephrine release from cardiac synaptosomes (Seyedi et al., 2005).

H_3R-mediated attenuation of norepinephrine exocytosis from cardiac sympathetic nerves involves an H_3R-$G_{i/o}$ coupling, adenylyl cyclase inhibition by $G\alpha_i$, decreased cAMP formation and diminished PKA activity (Seyedi et al., 2005). Inasmuch as a decrease in PKA activity is likely to decrease phosphorylation of VOCC, which would lead to a decrease in voltage-activated calcium current (I_{Ca}), it is plausible that the H_3R-mediated decrease in norepinephrine exocytosis results from a decreased Ca^{2+} influx via VOCC due to diminished activity of the adenylyl cyclase-cAMP-PKA pathway.

On the other hand, Gβγ is known to decrease adenylyl cyclase activity (Taussig et al., 1993) and it is therefore possible that in addition to the attenuation of adenylyl cyclase by $G\alpha_i$ (Seyedi et al., 2005), Gβγ will also play a role in the H_3R-mediated decrease in cAMP. Yet, since the Gβγ dimer is known to directly inhibit VOCC (Ikeda, 1996; Herlitze et al., 1996), we questioned whether the H_3R-mediated attenuation of norepinephrine exocytosis may also result from an inhibition of Ca^{2+} influx by a Gβγ action.

Accordingly, the purpose of our investigation was to determine the role of a Gβγ-induced inhibition of VOCC in the H_3R-mediated attenuation of norepinephrine exocytosis. To accomplish this, we evaluated the effects of H_3R activation on I_{Ca} and endogenous transmitter exocytosis in nerve-growth factor (NGF)-differentiated rat pheochromocytoma cells (PC12) stably transfected with H_3R and the Gβγ scavenger β-ARK1-(495−689)-polypeptide (Koch et al., 1994; Dickenson and Hill, 1998). NGF-differentiated PC12 cells were chosen as a functional model of sympathetic neurotransmission because their phenotype closely resembles that of sympathetic neurons (Dichter et al., 1977; Taupenot, 2007). Our results demonstrate a pivotal involvement of the Gβγ subunit in H_3R-mediated attenuation of neuronal I_{Ca} and consequent neurotransmitter exocytosis.

Methods

Cell Culture

The rat pheochromocytoma PC12 cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS), 5% donor horse serum (DHS), 1% L-glutamine, and antibiotics at 37°C in 5% CO_2. The differentiating protocol involved plating PC12 cells on tissue culture plates coated with collagen (rat tail type-VII, Sigma) combined with exposure to low serum medium containing 1% FBS, 0.5% DHS, 1% L-glutamine, and antibiotics supplemented with 2.5S-NGF (Harlan Bioscience, Indianapolis, IN, USA). Culture medium and NGF were replenished every 2 days. PC12 cells were transfected with the human H_3R (donated by Dr. T. W. Lovenberg) and the β-ARK1 (495−689) minigene (obtained from Dr. R.J. Lefkowitz) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. PC12-H_3 and PC12-H_3/β-ARK1 cell lines were selected and

maintained in selection media containing 500 μg/ml G418 sulfate (Mediatech, Herndon, VA) and/or zeocin (Invitrogen) respectively.

Reverse-Transcriptase PCR

Total RNA was isolated from PC12 and PC12-H3 cells using the TRIzol RNA purification kit (Invitrogen). cDNAs were synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) and random primers as described by the manufacturer. PCR was used to detect the H_3 mRNA expression with cDNA from either PC12 or PC12-H3 as templates, and with P1: 5'-CTCTGCAAGCTGTGGCTGGTGGTAGACTACCTACTGTGTG-3' and P2: 5'-CTTCTTGTCCCGCGACAGCCGAAAGCGCTGGGTGATGCTT-3' as primers (Invitrogen). PCRs were performed under conditions of 94°C, 40 s; 65°C, 40 s; 72°C, 2 min for 40 cycles. As control, total HeLa RNA was used as the template for amplification of a 353-bp segment of β-actin mRNA in a parallel PCR reaction. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Western blotting

Cell lysates (20 μg) prepared from PC12-H3 and PC12-H3/β-ARK1 cells were separated by SDS/PAGE 10−20% acrylamide gel and transferred to a PVDF membrane (Millipore, Billerica, MA). Following transfer, the membrane was washed with Tris-buffered saline (TBS) and blocked for 2 h at room temperature in blocking buffer (TBS containing 0.1% Tween 20, 5% (w/v) non-fat dry milk). Blots were then incubated overnight at 4°C with anti-β-ARK1 primary antibody (Epitomics, Burlingame, CA) at 1:500 dilution in 5% (w/v) bovine serum albumin (BSA) dissolved in TBS-Tween 20 (0.1%). The primary antibody was removed and the blot extensively washed with TBS/Tween 20. Blots were then incubated for 2 h at room temperature with horseradish peroxidase-coupled anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) at a 1:3000 dilution in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Pierce, Rockford, IL) followed by exposure to X-ray film (Biomax MR; Eastman Kodak, Rochester, NY).

cAMP measurement

Cellular cAMP accumulation was measured in PC12-H_3 and PC12-H_3/β-ARK1 cells seeded in 96-well plates and differentiated with NGF (100 ng/mL) for 5−7 days. After a 20-min treatment with the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (2 mM), cells were incubated for 5 min with or without the H_3R agonist imetit (100 nM), either alone or in combination with the H_3R antagonist CBP (50 nM). Cells were incubated with CBP for 5 min before the addition of imetit. Intracellular cAMP levels were then enhanced with forskolin (10 μM) for 20 min. The incubation buffer was immediately aspirated, cells were lysed and intracellular cAMP levels determined using a cAMP Biotrak EIA kit (Amersham Biosciences Inc., Piscataway, NJ) following the manufacturer's protocol. All drugs were constituted in HEPES-buffered Na^+ Ringer's solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4).

Dopamine assay

PC12-H_3 and PC12-H_3/β-ARK1 cells cultured in 12-well plates and differentiated with NGF (100 ng/ml) for 5−7 days were incubated with drugs for 5 min. When CBP or the anti-βγ peptide was used, cells were incubated with these compounds for 5 min before incubation with imetit. For PTX treatment, cells were incubated for 24 h with PTX (200 ng/ml) prior to assay. Dopamine exocytosis was elicited by incubating samples for 5 min with K^+ 100 mM (osmolarity was maintained constant by adjusting the NaCl concentration), phorbol 12-myristate 13-acetate (PMA, 300 nM) or for 20 min with forskolin (10 μM). At the end of the

incubation period, aliquots of the supernatant and cell lysates (after a 30-min treatment with 0.3% Triton X-100) were taken from each well and analyzed for dopamine content by high-performance liquid chromatography with electrochemical detection as previously described (Seyedi et al., 2005) with a 6.0-min retention time.

Electrophysiology

Whole-cell voltage-clamp studies were performed on PC12 cells. The cells were transferred to 22 × 22 mm glass coverslips coated with poly-L-lysine and collagen and differentiated with NGF (50 ng/ml) for 3−7 days before use in voltage-clamp experiments. Bath solution was (in mM): 20 $BaCl_2$, 125 N-methyl-D-glucamine, 10 HEPES (pH 7.5). Pipettes had resistances of 6−9 MΩ when filled with intracellular solution containing (in mM): 100 N-methyl-D-glucamine, 20 TEACl, 10 EGTA, 2 $MgCl_2$, 10 glucose, 2 Na_2ATP, 10 HEPES (pH 7.35). Fragments of coverslip were transferred to a recording chamber and the medium rinsed off with bath solution. Following adoption of whole-cell configuration, currents were recorded either in the absence (baseline) or presence of drugs. Cells were held at −40 mV and subjected to 200 ms test pulses from −40 mV to +40 mV in 10 mV increments every 30 s. Recordings were performed at 22°C to 25°C using an IX50 inverted microscope (Olympus, Tokyo, Japan), a Multiclamp 700A Amplifier, a Digidata 1300 Analog/Digital converter and pClamp9 software (Axon Instruments, Foster City, CA). Recordings were performed at 10 kHz sampling frequency, with no filtering prior to analysis, and were subsequently low-pass Bessel filtered for presentation purposes. All recordings with leak currents >20 pA or an access resistance >25 MΩ were discarded.

Current-voltage relationships were obtained by measuring the peak current during depolarizing pulses. Data analysis was performed using Clampfit 9 (Axon Instruments). Current density was calculated by dividing the current (pA) recorded from each individual cell by the capacitance (pF) of that cell. I_{Ca} activation was described by fitting a single exponential function to the current trace from the beginning of the pulse to the point where a steady-state level was reached so as to obtain the time constant of activation (τ_{act}).

Reagents

CBP, imetit, IBMX, NGF, ω-CTX, PMA and PTX were prepared in aqueous solution while DMSO was the vehicle for IBMX, forskolin and nifedipine. The anti-βγ peptide was obtained from Anaspec (San Jose, CA), imetit from Tocris (Ellisville, MO) and PMA from LC Laboratories (Woburn, MA). All other chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO).

Statistics

cAMP levels are expressed as mean absolute values ± SEM. Dopamine release values are expressed as mean percent increases above basal level ± SEM. Current density is expressed as mean ± SEM, with *n* specifying the number of independent experiments. Statistical significance was assessed by Student's *t* test or one-way ANOVA followed by post hoc testing (Dunnett's test) as indicated in the appropriate figure legend. Significance was asserted if $p < 0.05$.

Results

We first ascertained by RT-PCR the stable expression of human H_3R in PC12 cells. A segment of ~700 bp was amplified demonstrating the presence of H_3R in PC12-H_3 cells; this band was absent in non-transfected PC12 cells (Fig. 1A). The transfected H_3R were $G_{i/o}$-coupled as demonstrated by the loss of their anti-exocytotic activity after pretreatment with PTX (100 ng/ml; Fig. 2C and 4). Stable expression of β-ARK1-(495−689)-polypeptide in PC12 cells was

determined by Western blotting. A band of ~27 kDa confirmed the expression of β-ARK1 in PC12-H3/β-ARK1 cells, but not in PC12-H_3 cells (Fig. 1B).

We next verified the functionality of transfected H_3R. Inasmuch as H_3R are known to be negatively coupled to adenylyl cyclase via $G\alpha_i$ (Lovenberg et al., 1999), we determined that H_3R activation did indeed reduce the formation of cAMP in PC12-H_3 cells in response to forskolin. In the presence of the selective agonist imetit (100 nM) (Garbarg et al., 1992) a ~20-fold forskolin-induced (10 μM) increase in intracellular cAMP concentration was attenuated by 70% (Fig. 2A). Clobenpropit (50 nM), a selective H_3R antagonist (Van der Goot et al., 1992), reduced the effect of imetit by ~60% (Fig. 2A). This confirmed that the $G_{i/o}$ signaling cascade associated with transfected H_3R was functional.

Inasmuch as some forms of adenylyl cyclase are inhibited by the Gβγ dimer (Tang and Gilman, 1991), we questioned whether the decrease in intracellular cAMP concentration in the presence of imetit was due to a Gβγ-induced diminution of cAMP-PKA activity. To evaluate this possibility, we determined cAMP accumulation and endogenous dopamine exocytosis (Chen and Westfall, 1994) in response to forskolin, in control and H_3R-activated conditions, in PC12-H_3 cells stably transfected with the Gβγ scavenger β-ARK1 polypeptide (Koch et al., 1994) and compared them to those measured in PC12-H_3 cells. In PC12-H_3/β-ARK1 cells forskolin (10 μM) elicited a ~15-fold increase in intracellular cAMP concentration, which was comparable to that observed in PC12-H_3 cells (compare panels A and B in Fig. 2). In the presence of imetit (100 nM) the increase in cAMP formation was attenuated by ~70%; clobenpropit (50 nM) reduced the effect of imetit by ~60% (Fig. 2B). Thus, H_3R activation produced effects of similar magnitude in PC12-H_3 and PC12-H_3/β-ARK1 cells (compare panels A and B in Fig. 2), indicating that the Gβγ complex does not have inhibitory effects on the form of adenylyl cyclase present in PC12 cells. Furthermore, forskolin (10 μM) elicited a ~2- and ~3-fold increase in dopamine release in PC12-H3 and PC12-H3/β-ARK1 cells, respectively (Fig. 2C and D). H_3R activation with imetit also significantly attenuated dopamine exocytosis (by ~60%) in both PC12-H3 and PC12-H3/β-ARK1 cells; this effect was inhibited by H_3R blockade with clobenpropit (Fig. 2C and D). Thus, in differentiated PC12 cells stimulated with forskolin, the H_3R-mediated decrease in cAMP and associated anti-exocytotic effect derive mostly from an inhibition of adenylyl cyclase by $G\alpha_i$ and not by Gβγ.

We next measured peak I_{Ca} in PC12-H_3 cells using the conventional whole-cell patch-clamp technique (Fig. 3). The I_{Ca} activated at voltages equal or positive to −20 mV and reached a peak at +10 mV. Peak I_{Ca} was markedly reduced by incubation with ω-conotoxin GVIA (ω-CTX; 100 nM) or nifedipine (5 μM), indicating that both N- and L-type Ca^{2+} channels contribute to I_{Ca} in these cells (Fig. 3A and E). Indeed, combined treatment of the cells with nifedipine and ω-CTX blocked 90% of the I_{Ca} (Fig. 3A). When PC12-H_3 cells were incubated with imetit (100 nM), peak I_{Ca} was significantly attenuated; this response was antagonized by clobenpropit (50 nM) (Fig. 3B, C and E). Stimulation of adenylyl cyclase with forskolin (10 μM) enhanced peak I_{Ca} by ~25%; again, this current was markedly reduced by imetit, and clobenpropit antagonized the effect of imetit (Fig. 3D and E).

We next depolarized PC12-H_3 cells with K^+ (100 mM) and determined the exocytosis of endogenous dopamine. Depolarization with K^+ elicited a ~2.5-fold increase in dopamine release, a response curtailed by ~70% by the L-type Ca^{2+} channel antagonist nifedipine (5 μM), but not by the N-type channel antagonist ω-CTX (100 nM)(Fig. 4). H_3R activation with imetit also significantly attenuated dopamine exocytosis (~30%); this effect was abolished by H_3R blockade with clobenpropit, a further confirmation of the functionality of transfected H_3R (see Fig. 2A). Thus, H_3R activation attenuated both peak I_{Ca} and dopamine exocytosis, suggesting that H_3R-mediated anti-exocytotic effects could result from a reduced Ca^{2+} influx via VOCC.

Since H_3R are $G_{i/o}$-coupled and Gβγ subunits are known to directly inhibit VOCC function (Ikeda, 1996; Herlitze et al., 1996), we next assessed whether the Gβγ dimer plays a role in the decrease in I_{Ca} and associated anti-exocytotic effect observed with H_3R activation. Peak I_{Ca} in PC12-H_3/β-ARK1 cells did not differ from that recorded in PC12-H_3 cells (Fig. 5A), suggesting that Gβγ was not constitutively active in these cells. In contrast to what was observed in PC12-H_3 cells, imetit failed to reduce peak I_{Ca} in PC12-H_3/β-ARK1 cells, indicating that the Gβγ dimer plays a role in the H_3R-mediated reduction of I_{Ca} (compare Figs. 3B and 5A). Furthermore, the slowing of current activation, as demonstrated by an increase in τ_{act} in the presence of imetit, supports a direct Gβγ-induced inhibition of VOCC (Fig. 3C) (Stephens et al., 1998). Additionally, whereas the anti-exocytotic effect of nifedipine was preserved in PC12-H_3/β-ARK1 cells, imetit failed to affect dopamine exocytosis (Fig. 5B), nor did imetit modify dopamine exocytosis in PC12-H_3 cells incubated with a membrane-permeable phosducin-like anti-βγ peptide (1μM) (Chang et al., 2000) (Fig. 4). Collectively, these findings indicate that the H_3R-mediated anti-exocytotic effect derives from the Gβγ-dependent inhibition of I_{Ca} and not from a βγ-induced inhibition of adenylyl cyclase.

Activation of protein kinase C (PKC) upon application of phorbol esters is known to increase I_{Ca} (Zamponi et al., 1997), an action negatively modulated by Gβγ subunits (Ikeda, 1996; Herlitze et al., 1996). Accordingly, we next assessed whether H_3R stimulation would attenuate PKC-activated I_{Ca} and associated increase in dopamine exocytosis via a Gβγ-mediated effect. Incubation of PC12-H_3 cells with phorbol 12-myristate 13-acetate (300 nM; PMA) enhanced peak I_{Ca} by ~30% (compare Fig. 5A and Fig. 6A). In the presence of imetit (100 nM), PMA-stimulated I_{Ca} was reduced by ~45% and this response was antagonized by clobenpropit (50 nM)(Fig. 6A). PMA also elicited a concentration-dependent increase in dopamine release: in the 10 nM-1 μM PMA concentration range, dopamine release was increased ~2−3-fold above control in both PC12-H_3 and PC12-H_3/β-ARK1 cells (Fig. 7A and B). In the presence of imetit (100 nM) the concentration-response curve for PMA was shifted to the right by two orders of magnitude in PC12-H_3 cells (Fig. 7A). Incubation with clobenpropit (50 nM) antagonized the effect of imetit (Fig. 7C).

Application of PMA (300 nM) to PC12-H_3/β-ARK1 cells enhanced peak I_{Ca} by ~30% (Fig. 6B). Notably, activation of H_3R with imetit in PC12-H_3/β-ARK1 cells failed to attenuate the PMA-stimulated I_{Ca} (Fig. 6B). Moreover, in PC12-H_3/β-ARK1 cells, H_3R activation did not modify PMA-induced dopamine exocytosis (Fig. 7B). Indeed, the PMA concentration-response curve for the promotion of dopamine exocytosis was the same whether in the presence or absence of imetit (Fig. 7B), and the magnitude of the exocytotic response to the 300 nM concentration of PMA did not differ from that recorded in the presence of imetit, either alone or together with clobenpropit (Fig. 7D). Thus, stimulation of PKC in PC12-H_3 and PC12-H_3/β-ARK1 cells confirmed that a Gβγ-dependent inhibition of I_{Ca} plays a pivotal role in the anti-exocytotic effect of H_3R activation.

Discussion

Our findings indicate that Gβγ subunits, liberated from Gαβγ trimers upon H_3R activation attenuate neuronal I_{Ca} and consequent neurotransmitter exocytosis. This establishes a novel mechanism of H_3R transduction capable of preventing excessive norepinephrine release, a recognized cardioprotective action (Levi et al., 2007).

In our investigation we utilized the PC12 rat pheochromocytoma cell line, which acquires a sympathetic nerve phenotype when treated with NGF (Dichter et al., 1977; Taupenot, 2007). Having stably transfected PC12 cells with human H_3R, a PTX-sensitive $G_{i/o}$-coupled receptor (see Figs. 2 and 4), we set out to demonstrate - using the whole-cell patch clamp technique - the presence of an I_{Ca} likely responsible for the exocytosis of dopamine, the endogenous

transmitter in these cells (Chen and Westfall, 1994). Indeed, by stepping to different test potentials in the −40 to +40 mV range, we identified a current in PC12-H_3 cells with typical I_{Ca} characteristics (i.e., shape of the I/V curve, its persistence in the absence of Na^+ in the bathing solution and its sensitivity to 0.1 mM $CdCl_2$) which was also inhibited by the L- and N-type Ca^{2+} channel blockers nifedipine and ω-CTX, respectively. Moreover, this I_{Ca} was amplified by stimulation of adenylyl cyclase with forskolin and by PKC activation with a phorbol ester, responses which are typical of neuronal Ca^{2+} channel currents (Bean et al., 1984; Yang and Tsien, 1993). Notably, H_3R activation with the selective agonist imetit (Garbarg et al., 1992) markedly reduced the I_{Ca} in control and both adenylyl cyclase- or PKC-stimulated conditions, an action inhibited by H_3R blockade with the selective antagonist clobenpropit (Van der Goot et al., 1992). These findings reveal that the decrease in intracellular Ca^{2+} which we had previously described to occur in response to H_3R activation (Silver et al., 2002; Seyedi et al., 2005) originates from a reduction in Ca^{2+} influx via VOCC.

To determine whether the H_3R-mediated decrease in I_{Ca} is due to inhibition of VOCC by the $G_{i/o}$-derived Gβγ dimer, we transfected PC12-H_3 cells with the Gβγ scavenger β-ARK1 (Koch et al., 1994). H_3R activation in PC12-H_3/β-ARK1 cells failed to inhibit I_{Ca}, unequivocally demonstrating the pivotal role played by the Gβγ dimer in the H_3R-mediated inhibition of VOCC. Clearly, the Gβγ dimer-induced VOCC inhibition is also of major importance for the anti-exocytotic effects of H_3R activation. Indeed, we found that the imetit-induced inhibition of dopamine exocytosis in PC12-H_3 cells was prevented not only by a membrane-permeable phosducin-like anti-βγ peptide (Chang et al., 2000), but also in cells transfected with the Gβγ scavenger β-ARK1. Moreover, the Gβγ scavenger prevented the H_3R-mediated attenuation of dopamine exocytosis elicited by PKC activation with the phorbol ester PMA.

Interestingly, whereas nifedipine and ω-CTX each inhibited I_{Ca}, only nifedipine attenuated dopamine release. Thus, although both L- and N-type Ca^{2+} channels are present in PC12-H_3 cells, only the L-type appears to be involved in dopamine exocytosis. In fact, calcium entering through the L-type channel is the likely predominant stimulus for dopamine release in PC12 cells (Avidor et al., 1994; Kanwal et al., 1997). Furthermore, although the Gβγ dimer is generally viewed as an N-type Ca^{2+} channel inhibitor (Ikeda, 1996; Herlitze et al., 1996; Catterall, 2000), a Gβγ-induced inhibition of the L-type Ca^{2+} channel has also been recognized (Ivanina et al., 2000). In contrast to dopamine release in PC12 cells, both L- and N-type Ca^{2+} channels participate in neurotransmitter release from cardiac sympathetic nerve endings. Indeed, we had found that the H_3R agonist imetit synergizes with either nifedipine or ω-CTX to attenuate norepinephrine exocytosis from cardiac synaptosomes (Seyedi et al., 2005).

Expression of the β-ARK1 polypeptide failed to modify the H_3R-mediated attenuation of the forskolin-induced increase in intracellular cAMP and associated dopamine exocytosis (see Fig. 2). Although a βγ-induced inhibition of adenylyl cyclase has been described in insect ovarian cells (Taussig et al., 1993), our findings clearly indicate that the Gβγ dimer liberated upon H_3R activation is not responsible for the reduction of adenylyl cyclase activity in differentiated PC12-H_3 or PC12-H_3/β-ARK1 cells. Therefore, a direct βγ-induced inhibition of I_{Ca} at the VOCC level is likely to play a key role in the H_3R-mediated anti-exocytotic effect. The Gβγ dimer is also liberated upon PGE_2-induced activation of EP_3R, another $G_{i/o}$-coupled receptor with anti-exocytotic properties. PGE_2 formation is the final step of the MAPKinase signaling cascade initiated by H_3R activation, culminating in the attenuation of norepinephrine exocytosis from cardiac sympathetic nerve endings (Levi et al., 2007). This substantiates the present findings on the pivotal role of the Gβγ dimer as an inhibitor of I_{Ca} and consequent exocytosis.

We had previously reported that the anti-exocytotic effect of H_3R activation is associated with a decrease in intraneuronal Ca^{2+} concentration in SH-SY5Y human neuroblastoma cells stably

transfected with H_3R (Silver et al., 2002). We hypothesized that the decrease in $[Ca^{2+}]_i$ was due to a $G\alpha_i$-induced inhibition of adenylyl cyclase, ultimately resulting in a decreased PKA-induced phosphorylation of VOCC (Seyedi et al., 2005). The novelty of the present findings is that a direct $G\beta\gamma$-induced inhibition of VOCC, resulting in an attenuation of I_{Ca}, plays a pivotal role in the H_3R-mediated decrease in $[Ca^{2+}]_i$ and associated anti-exocytotic effects. The identification of this final step in the H_3R transduction cascade has broad implications in the development of new therapeutic strategies in cardiovascular diseases characterized by hyperadrenergic activity, such as myocardial ischemia and congestive heart failure.

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The first two authors, Christopher Morrey and Rima Estephan contributed equally to this research.

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Non-standard abbreviations

β-ARK1, β-adrenergic receptor kinase 1
$[Ca^{2+}]_i$, intraneuronal Ca^{2+}
H_3R, histamine H_3-receptors
I_{Ca}, Ca^+ current
NGF, nerve-growth factor
RT-PCR, reverse-transcriptase polymerase chain reaction
PKA, protein kinase A
PKC, protein kinase C
PMA, phorbol 12-myristate 13-acetate
PTX, pertussis toxin
τ_{act}, time constant of activation
VOCC, voltage-operated Ca^{2+}-channels
ω-CTX, ω-conotoxin GVIA.

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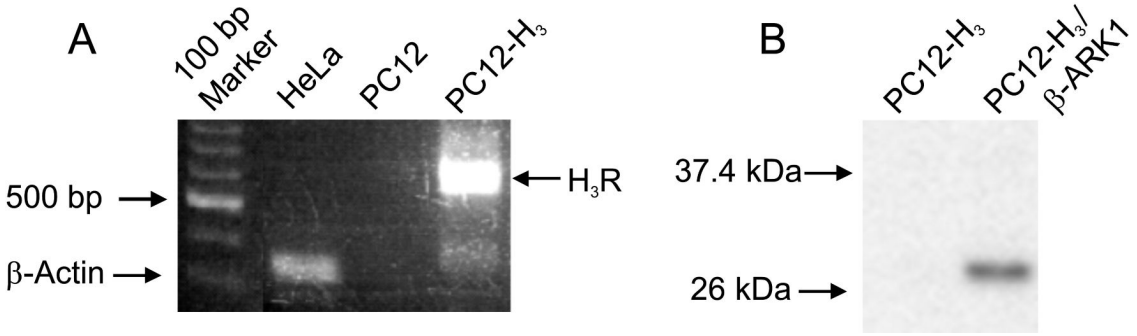

Figure 1.

Detection of H_3R and over-expression of β-ARK1(495−689) minigene in PC12-H_3 cells. (A) Reverse-transcriptase PCR. cDNA was synthesized from total RNA prepared from PC12 and PC12-H_3 cells and used as template in a PCR reaction. The PCR products were run on a 1.5 % agarose gel and detected under UV light. As controls, β-Actin primers were used in a parallel PCR reaction for amplification of a 353-bp segment. (B) Western blot analysis of β-ARK1(495 −689) minigene. Cell lysates (20 μg) isolated from PC12-H_3 and PC12-H_3/β-ARK1 cells were resolved by SDS-PAGE and transferred to PVDF membrane. Blotting of the membrane with anti-β-ARK1 monoclonal antibody (1:500) revealed a ~27 kDa specific band. Positions of standard molecular-mass markers of 26 and 37.4 kDa are shown on the left.

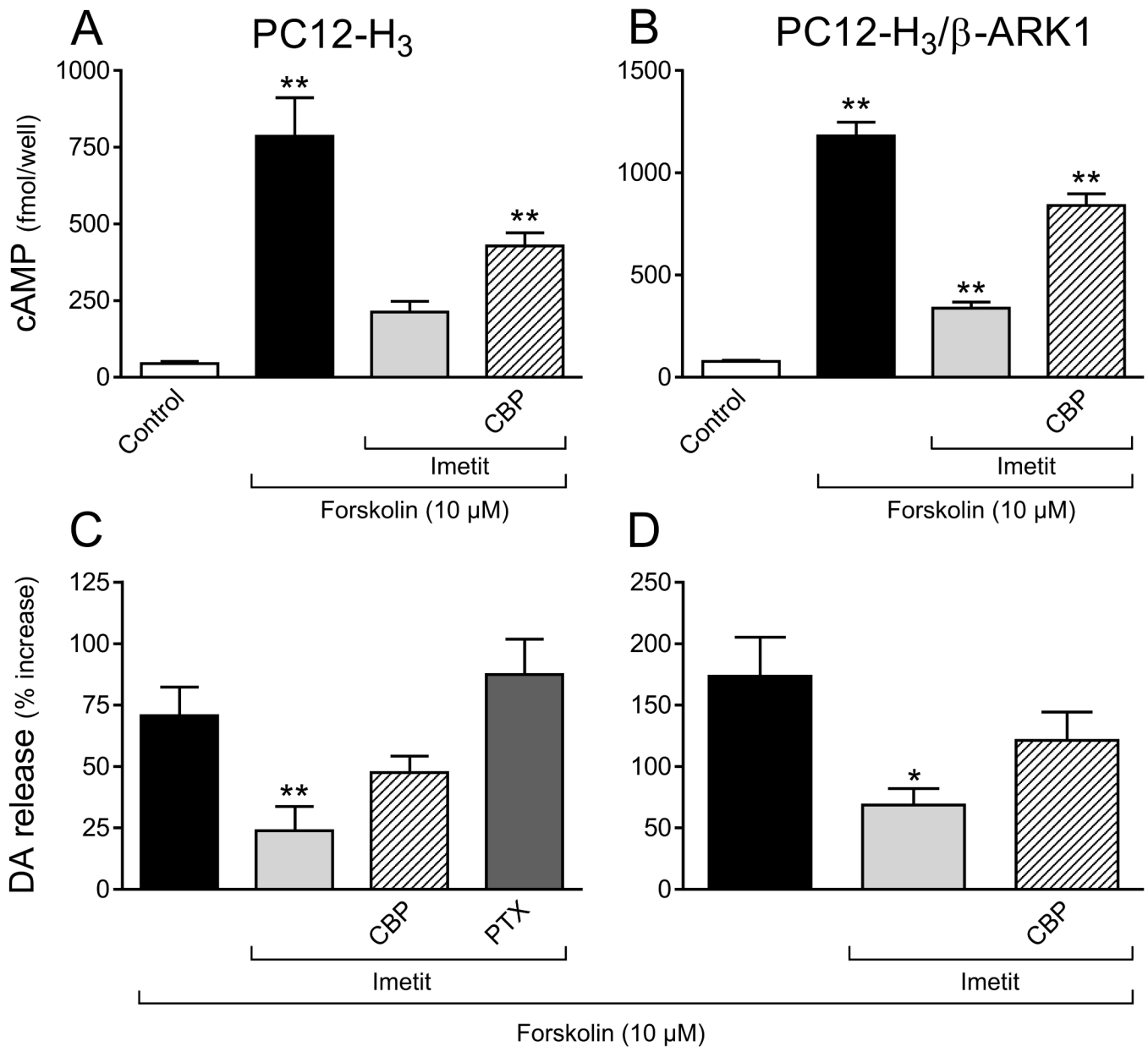

Figure 2.

H_3R activation reduces intracellular cAMP accumulation and dopamine exocytosis elicited by forskolin (10 μM) in NGF-differentiated rat pheocromocytoma cells stably transfected with human H_3R (PC12-H_3) and in PC12 cells transfected with both H_3R and the Gβγ scavenger β-ARK1 (PC12-H_3/β-ARK1). Panels A and B: intracellular cAMP accumulation (absolute values); panels C and D: dopamine release, in the absence or presence of the H_3R agonist imetit (100 nM), either alone or in combination with the H_3R antagonist clobenpropit (CBP, 50 nM), or after pre-treatment with PTX (200 ng/ml for 24 h). Dopamine release is expressed in percent increase above basal level. Bars are means ± SEM (n = 15−19 for A and B and n = 10−15 for C and D). Significantly different from control or forskolin (*$p < 0.05$ and **$p < 0.01$ by ANOVA followed by post hoc Dunnett's test).

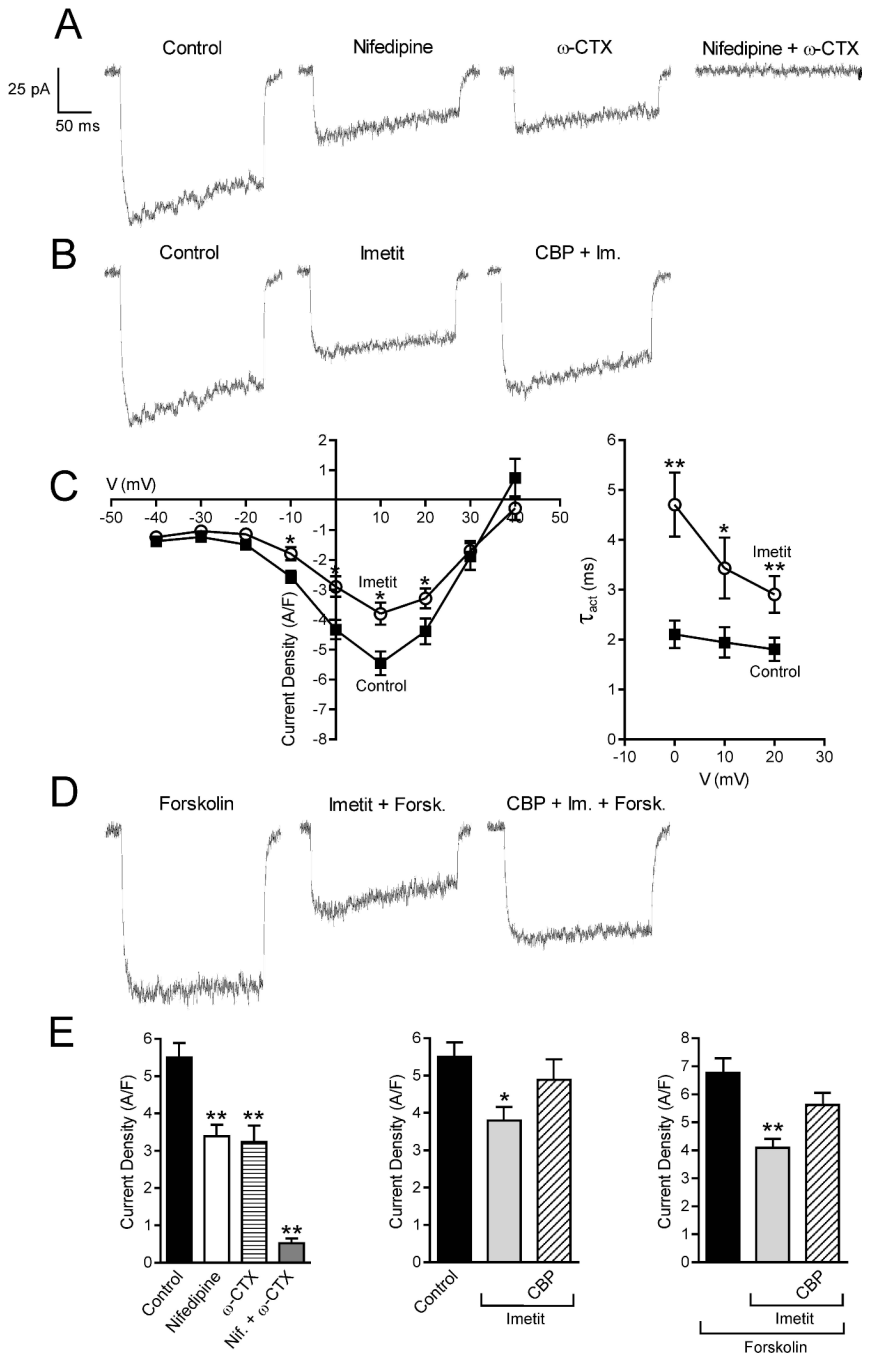

Figure 3.

Calcium current traces and peak current density recorded in NGF-differentiated PC12-H_3 cells by stepping from the holding potential of −40 mV to the test potential of +10 mV. Panel A and E (Left): calcium current is inhibited by selective blockers of N- and L-type Ca^{2+} channels, i.e., ω-CTX (100 nM) and nifedipine (5 μM), both alone (n = 20) and in combination (n = 5). Panel B and E (Center) H_3R activation with imetit (100 nM) attenuates calcium current, an effect prevented by the H_3R antagonist clobenpropit (CBP; 50 nM). Panel C (Left): I-V curve in the absence and presence of imetit (100 nM) (means ± SEM; n = 20; *p < 0.05, from control by Student's t test). Panel C (Right): τ_{act}-voltage relationship. Imetit produced a slowing of current activation indicated by an increase in τ_{act} (means ± SEM; n = 12; *p < 0.05, **p < 0.01

from control by Student's *t* test). Panel D: Stimulation of adenylyl cyclase with forskolin (10 μM) increases calcium current. Incubation with imetit markedly reduces the forskolin-stimulated calcium current; CBP antagonizes this effect. Bars represent means (± SEM; $n = 20$; *$p < 0.05$, **$p < 0.01$ from control or forskolin by ANOVA followed by post hoc Dunnett's test).

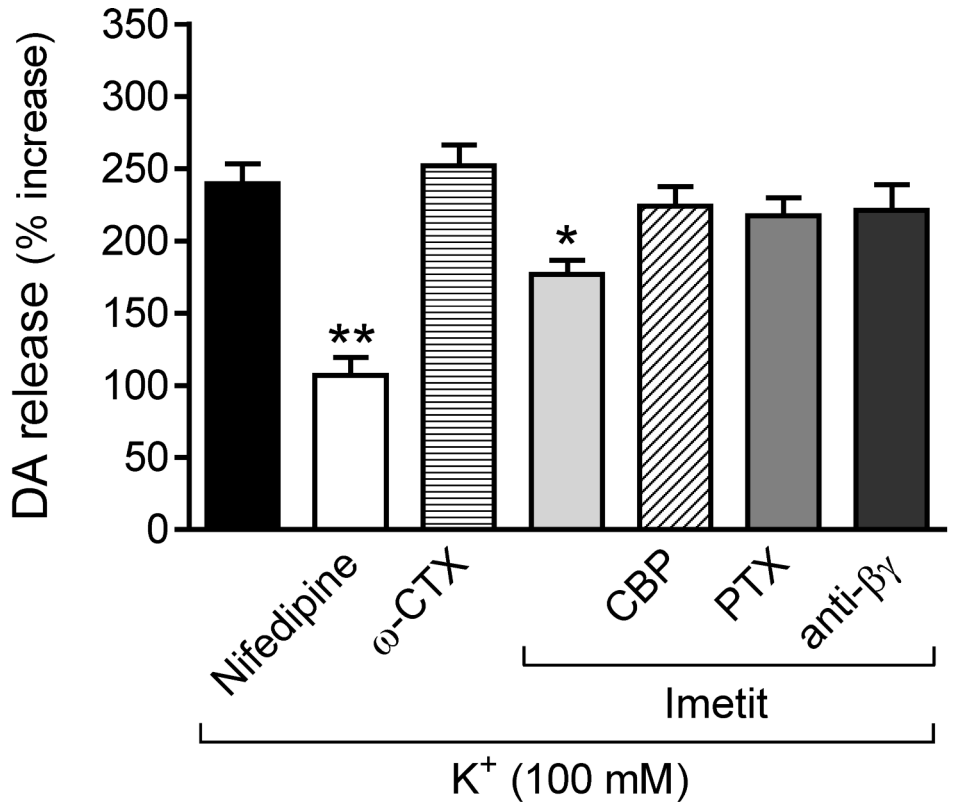

Figure 4.

H_3R activation attenuates endogenous dopamine exocytosis elicited by depolarization with K^+ (100 mM) in NGF-differentiated rat pheocromocytoma cells transfected with human H_3R (PC12-H_3). Dopamine exocytosis is inhibited by nifedipine (5 μM), an L-type calcium channel blocker, but not by ω-CTX (100 nM), an N-type calcium channel blocker. The anti-exocytotic effect of imetit (100 nM) is prevented by the H_3R antagonist clobenpropit (CBP; 50 nM), a membrane-permeable phosducin-like anti-βγ peptide (1 μM), and by pre-treatment with PTX (200 ng/ml for 24 hr). Bars are means ± SEM of percent increases in dopamine release above control (n = 18−21). Significantly different from K^+ alone (*p < 0.05 and **p < 0.01 by ANOVA followed by post hoc Dunnett's test).

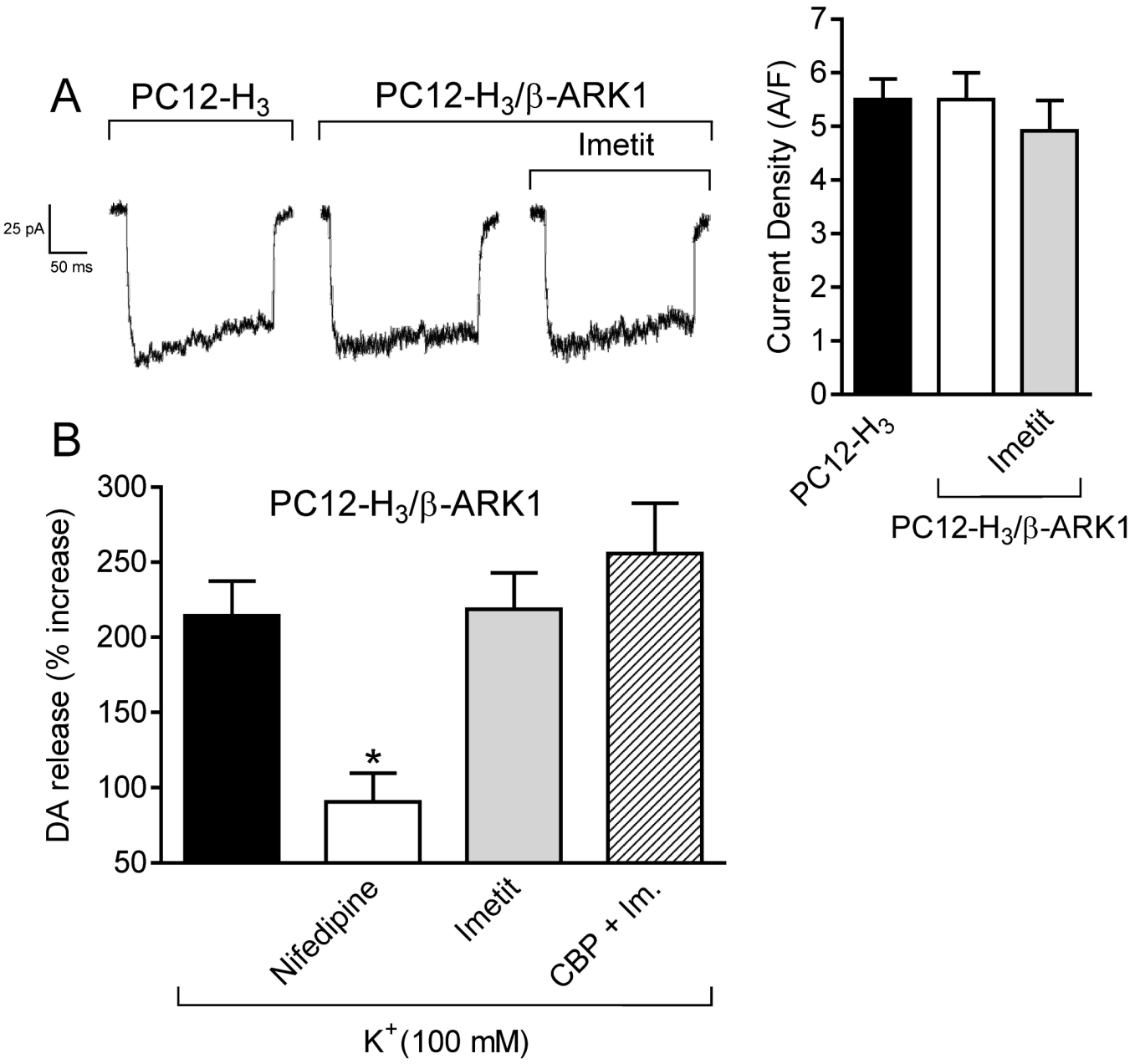

Figure 5.

Panel A: Calcium current traces and peak current density, recorded in NGF-differentiated PC12-H_3 by stepping from the holding potential of −40 mV to the test potential of +10 mV, do not differ from calcium currents evoked in PC12-H_3 cells transfected with the Gβγ scavenger β-ARK1 (PC12-H_3/β-ARK1). Notably, H_3R activation with imetit (100 nM) fails to reduce peak calcium current in PC12-H_3/β-ARK1 cells. Bars represent means (± SEM; n = 20). Panel B: endogenous dopamine release was measured in PC12-H_3/β-ARK1 cells in response to K^+ (100 mM). Dopamine exocytosis was inhibited by nifedipine (5 μM), but not by imetit (100 nM), either alone or in combination with clobenpropit (CBP, 50 nM). Bars are means ± SEM of percent increases in dopamine release above control (n = 20−25). Significantly different from K^+ alone (*p < 0.05 by ANOVA followed by post hoc Dunnett's test).

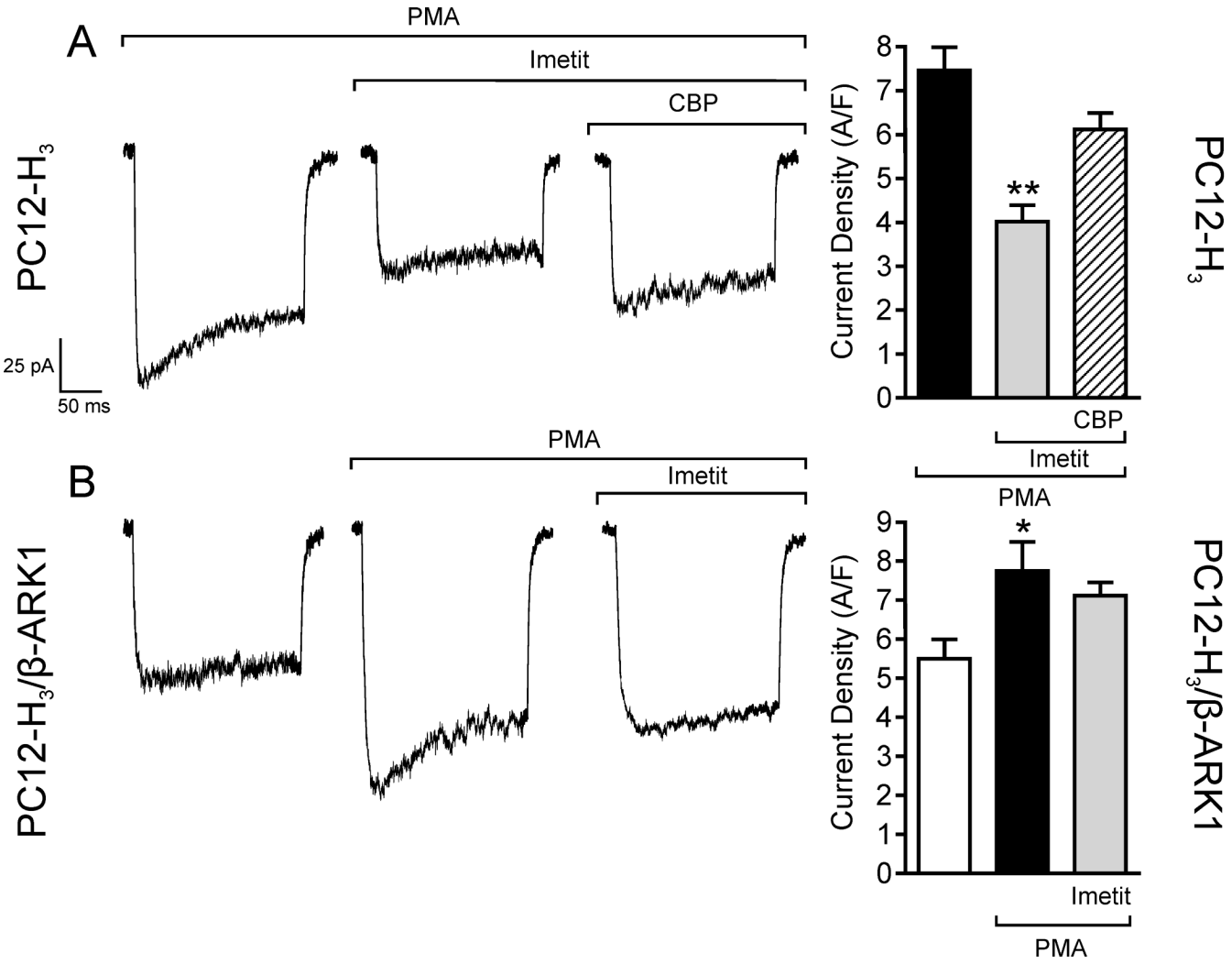

Figure 6.

Activation of PKC with the phorbol ester PMA (300 nM) increases peak calcium current and current density in both PC12-H_3 (Panel A) and PC12-H_3/β-ARK1 cells (Panel B). In PC12-H_3 cells, incubation with imetit (100 nM) significantly reduces peak calcium current, an effect inhibited by CBP (50 nM). Notably, the imetit-induced calcium current reduction in PC12-H_3 cells is not present in PC12-H_3/β-ARK1 cells. Bars represent means (± SEM; $n = 20$; *$p < 0.05$ and **$p < 0.01$ by ANOVA followed by post hoc Dunnett's test).

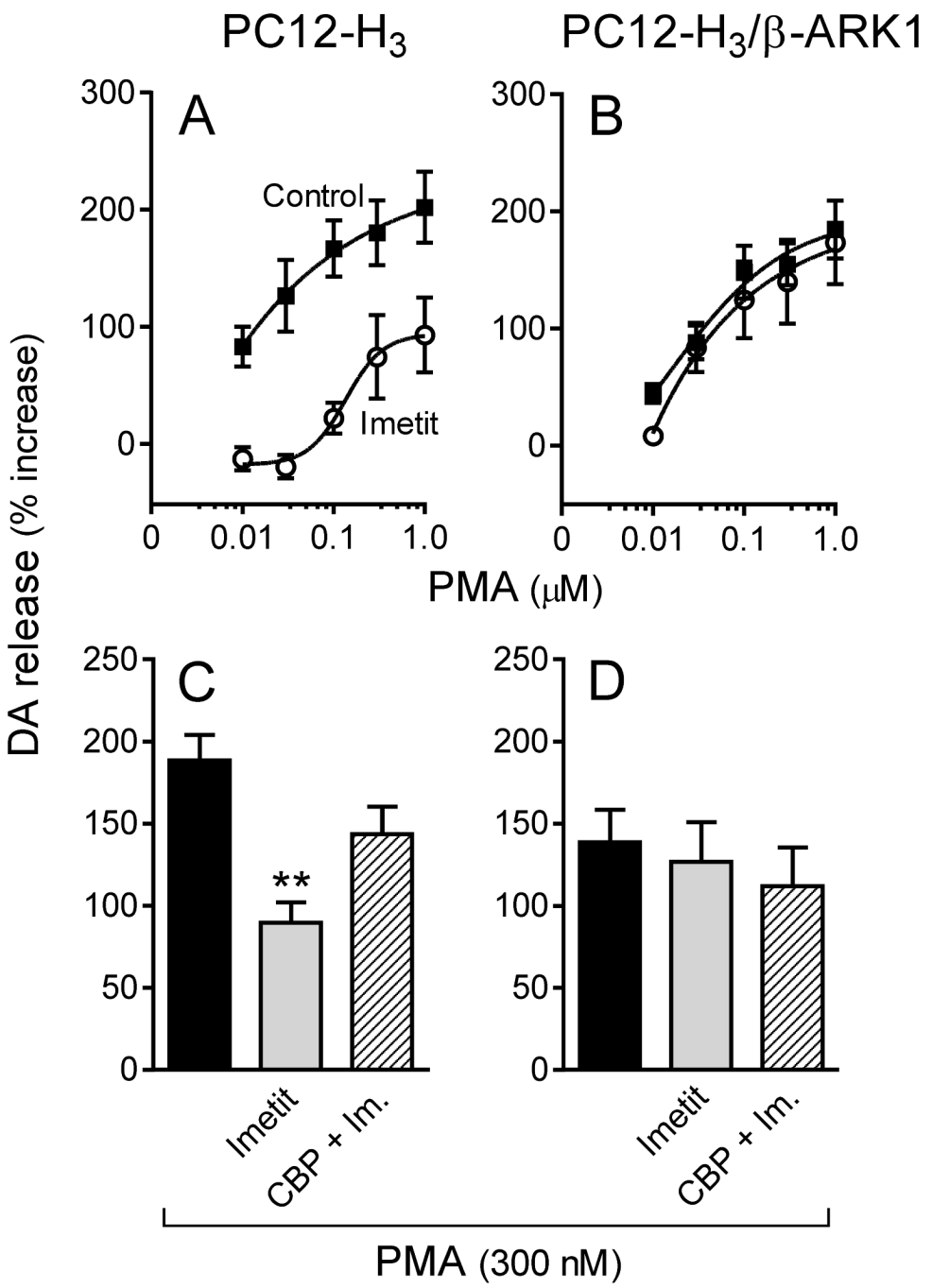

Figure 7.

Activation of H_3R attenuates endogenous dopamine exocytosis elicited by PKC activation with PMA (300 nM) in PC12-H_3 cells but not in PC12-H_3/β-ARK1 cells. Panels A and B: concentration-response curves for PMA-induced dopamine release in PC12-H_3 and PC12-H_3/β-ARK1 cells, respectively. Activation of H_3R with imetit (100 nM) significantly reduced PMA-induced dopamine release in PC12-H_3 cells (A), an effect that was antagonized by CBP (50 nM; panel C). In PC12-H_3/β-ARK1 cells (B), H_3R activation with imetit (100 nM) failed to affect the PMA-induced dopamine release. Panels C and D represent dopamine release elicited by activation of PKC with PMA (300 nM) in the absence or presence of imetit (100 nM), either alone or in combination with clobenpropit (CBP, 50 nM). Bars are means ± SEM

($n = 10$ for C and $n = 15$ for D). Significantly different from PMA alone (**$p < 0.01$ by ANOVA followed by post hoc Dunnett's test). Dopamine release is expressed as percent increase above basal level.