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Mechanism of dopamine mediated inhibition of neuropeptide Y release from pheochromocytoma cells (PC12 cells)

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ABSTRACT

In rat pheochromocytoma (PC12) cells the dopamine D_2 receptor agonists apomorphine (APO) and *n*-propylnorapomorphine (NPA) produced a concentration dependent inhibition of K^+-evoked neuropeptide Y release (NPY-ir). The effect of APO was blocked by the dopamine D_2-receptor antagonist, eticlopride, but not the D_1/D_3 or the D_4/D_2 antagonists, SCH23390 or clozapine, respectively. The D_1/D_5 receptor agonist, SKF38393 or the D_3 agonists PD128907 and 7-OH DPAT had no effect. Selective N and L-type voltage gated Ca^{2+} channel blockers, ω-conotoxin GVIa (Ctx-GVIa) and nifedipine, respectively, produced a concentration dependent inhibition of NPY-ir release but were not additive with APO. The Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) II inhibitor KN-62 produced a concentration-dependent inhibition of NPY-ir release but the combination of KN-62 and APO produced no further inhibition. PMA-mediated protein kinase C stimulation significantly increased both basal and K^+-evoked release of NPY-ir, and in the presence of PMA APO had no inhibitory effect. The PKC antagonist, chelerythrine, inhibited K^+-evoked NPY-ir release but was not additive with APO. Neither forskolin-mediated adenylate cyclase activation and the active cAMP analog Sp-cAMPS, nor the adenylate cyclase inhibitor SQ 22536, and the competitive inhibitor of cAMP-dependent protein kinases Rp-cAMPS, had any significant effect on K^+-evoked NPY-ir release. This suggests the inhibitory effect of APO on K^+-evoked release of NPY-ir from PC12 cells is most likely mediated through activation of dopamine D_2 receptors leading to direct inhibition of N and L-type voltage gated Ca^{2+} channels, or indirect inhibition of PKC, both of which would reduce $[Ca^{2+}]_i$ and inactivate CaM kinase.

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1. Introduction

Neuropeptide Y (NPY) was isolated from porcine brain in 1982 [1] and is an N-terminal amidated 36 amino acid peptide. It is thought to be involved in a variety of physiological functions such as blood pressure regulation [2,3], memory processing and retention [4,5], and its most noticeable effect, that of stimulation of feeding [6,7]. It is a ubiquitous

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Abbreviations: NPY-ir, neuropeptide Y-immunoreactivity; Ctx-GVIa, ω-conotoxin GVIa

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neuropeptide both in the central and peripheral nervous system.

The synthesis, release and levels of NPY appear to be regulated by dopamine. For example, in the CNS blockade of dopaminergic receptors increased NPY immunoreactivity in various rat brain regions, suggesting inhibitory control of NPY levels [8–10]. The interrelationship between dopamine and NPY has also been demonstrated with the blockade of the dopaminergic receptors attenuating NPY synthesis and release [11]. Furthermore, dopamine can alter NPY gene expression both positively and negatively in specific brain regions [12–14]. Although these studies suggest that NPY can be regulated by dopamine there are few studies examining the pharmacological characterization or the mechanism by which such regulation occurs. We have observed that the dopamine agonist, apomorphine (APO), inhibits the K^+-evoked release of NPY-immunoreactivity compounds (NPY-ir) from nerve growth factor (NGF)-differentiated pheochromocytoma cells (PC12 cells) [15,16]. NGF-differentiated PC12 cells represent an immortalized homogenous cell line. We have previously demonstrated that the cell line synthesizes and releases both dopamine and NPY-ir [17–20], contains receptors to both substances [17,18] and contain a variety of ion channels, second messengers and G-proteins [19–23]. We believe, therefore that it can serve as a useful model system to investigate dopamine–NPY interactions. The purpose of the present study was to define the dopamine receptor subtype or subtypes mediating the inhibitory effect of NPY-ir release and, more importantly, to elucidate which signaling pathways mediate this effect.

2. Materials and methods

2.1. Materials

Porcine NPY 1-36 was purchased from Peninsula Laboratories (Belmont, CA). Nifedipine, ω-conotoxin GVIa (Ctx-GVIa), pertussis toxin (PTX) were purchased from Sigma Chemicals (St. Louis, MO). NGF was obtained from Collaboration Biochemical Products (Bedford, MA). DMEM, fetal bovine serum and horse serum were from JRH Biosciences (Lenexa, KS). APO, NPA, SKF 38393, SCH 23390, 7-OH DPAT, PD 128907, Sp-adenosine 3′5 monophosphothioate (Sp-cAMPS), Rp-cAMPS, SQ 22536, PMA, forskolin, chelerythrine chloride and KN-62 were purchased from Research Biochemical International (Natick, MA). Polyclonal anti NPY antisera were gifts of Dr. Debora DiMaggio [18].

2.2. Cell culture

PC12 cells were a generous gift of Dr. Steven Sabol (NIH, Bethesda, MD). The cells were cultured in Falcon cultureware (175 cm^2 culture flask, Becton Dickenson, Lincoln Park, NJ) and were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 5% fetal bovine serum and 10% heat-inactivated (56 °C for 30 min) donor horse serum in a humidified atmosphere with 5% CO_2/95% O_2 at 37 °C. Cells were split and passaged at 1 week intervals by dissociation with calcium-free Hanks Balanced Salt solution (HBSS, JRH Biosciences) with a medium change every 2–3 days.

For NPY-ir release experiments, cells were grown directly on the substrate of six-well plates (treatment C, Mattek Corp, Ashland, MA) and initially seeded to obtain optimal neurite outgrowth for the cells after 5 days of treatment with nerve growth factor (NGF, 50 ng/ml). The media was aspirated and replenished every other day; NGF was added after each medium change.

2.3. NPY-ir experiments

NPY-immunoreactivity release study was determined using the protocol of Chen et al. [18]. At the end of the 5-day differentiation with NGF, the media was aspirated and cells placed in Krebs Ringer Bicarbonate (KRB, pH 7.4) and allowed to equilibrate for 15 min. The media was then replaced with 1.25 ml of KRB in the absence or presence of different drugs. The KRB consisted of (mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, CaCl 2.5, $NaHPO_4$ 1.0, $NaHCO_3$ 26.2 and glucose 6.0. The high potassium KRB (K^+-KRB) had a similar composition except for KCl and NaCl which was 50 and 71.5 mM, respectively. After a 15 min incubation period with KRB alone or KRB containing various drug combinations, the buffer was aspirated and stored. Total NPY-ir remaining in the cells was then extracted by the addition of 1 ml of 0.1N HCl to each well. The medium was kept on ice until assayed for NPY-ir content which was begun the same day as the experiment. In experiments in which the effect of antagonists was examined, these drugs were added to the KRB for 15 min prior to replacing with a high K^+-KRB containing antagonist and agonist. In experiments examining the effects of pertussis toxin (PTX) treatment PTX, 50 ng/ml, was added to the wells 18 h prior to conducting release experiments. All dopaminergic agents were added to appropriate buffer immediately before the experiment.

2.4. NPY-ir measurement from release buffer and cells

NPY-ir was determined directly in acid extracts of cell (total release) and release buffer (fractional release) by radioimmunoassay (RIA) using a specific rabbit antiserum that was raised in rabbit against porcine NPY [24]. RIA was performed using a 5-day disequilibrium method using the protocol of Chen et al. [18]. Duplicate samples (0.4 ml for release buffer, 0.05 ml for cell extracts supernatant) were incubated with NPY antisera in Tris (50 mM, pH 7.8) at 4 °C. Twenty-four hours later, ^{125}I NPY was added to each tube. Following a 72 h (at 4 °C) incubation period, antibody bound ^{125}I NPY was separated from free ^{125}I NPY by centrifugation after addition of a second antibody (goat anti-rabbit serum) and polyethylene glycol. The radioactivity measured in a Packard gamma counter. Triplicates were used in standard curves with a sensitivity range of 1–100 fmol. The amounts of NPY released was calculated on the basis of (fractional release/[fractional release + total release]). Appropriate controls were carried out to account for the addition of KRB and 0.1N HCl extraction solutions. For instance, 0.4 ml normal and high K^+-KRB solutions were added to the RIA to see if these gave false readings for NPY-ir, this was not observed normal and high K^+-KRB solutions. The addition of 50 μl 0.1N HCl did not significantly affect the sensitivity of the RIA.

2.5. Statistical analysis

Experiments were done in five to seven separate wells for each data point. NPY-ir release data were calculated as fractional release which is the NPY-ir appearing in the release buffer divided by the total NPY-ir from each well including buffer and cell NPY-ir content. Data values were expressed as mean ± S.E.M. Analysis of variance was used and followed by Neuman–Keuls or Duncans test to analyze the difference between the control and experimental groups. Where indicated in the text, analysis of variance (ANOVA) was also carried out to analyze the difference between and within control and experimental groups.

3. Results

3.1. Effects of dopamine agonists on K^+-evoked release of NPY-ir

The percent fractional release of NPY-ir in a normal buffer, or in the presence of a high K^+-KRB buffer, in the absence or presence of increasing concentrations of APO or NPA was examined (Fig. 1). Neither APO nor NPA altered the basal release of NPY-ir (data not shown), but both produced a concentration dependent inhibition of the K^+-evoked release of NPY-ir.

To further investigate the dopamine receptor subtypes that mediate the APO- and NPA concentration dependent inhibition of the K^+-evoked NPY-ir release several dopamine agonists were examined. SKF 38393 which has high affinity for D_1/D_5 (D_1-like) receptors but low affinity for D_2-like receptors; PD 128907 which has a much higher affinity for D_3 compared to D_2 and D_4 receptors [25]; and 7-OH DPAT which also shows considerable specificity for D_3 receptor subtype. Compared to the K^+-evoked stimulus of NPY-ir release none of the dopamine agonists SKF, PD 128907 or DPAT had any effect on basal or K^+ evoked NPY-ir release. The D_1/D_5 agonist SKF 38393 at concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, altered the fractional release of NPY 17.2 ± 2%, 19.5 ± 2.5%, 19.2 ± 1.5%, and 17.8 ± 1%, respectively, compared to K^+ evoked NPY-ir release (18.5 ± 1.0%). The preferential D_3 agonist 7-OH DPAT (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) also changed the fractional release of NPY 17.2 ± 2%, 17.2 ± 3%, 18.9 ± 2%, and 16.0 ± 3%, respectively, compared to K^+ evoked NPY-ir release. Similarly, PD 128907 (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) modified the K^+ evoked fractional release of NPY-ir 15.5 ± 1.5%, 15 ± 3.5%, 14.5 ± 2%, and 15.8 ± 2.8%, respectively, compared to KCl alone. When the results were analyzed by two-way ANOVA they neither statistically stimulated nor inhibited the release of NPY from differentiated PC12 cells over a wide concentration range (10^{-7} to 10^{-4} M). These results suggest that activation of D_2 receptors, but not D_1, D_3 or D_4 receptors, are involved in the dopaminergic-induced inhibition of NPY-ir release.

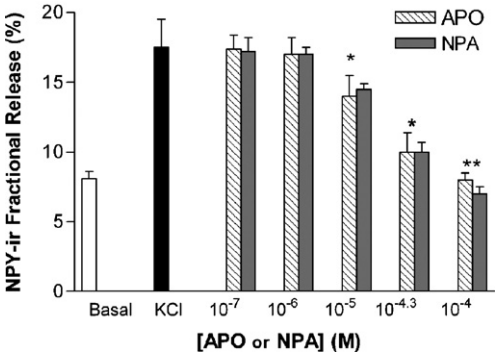

Fig. 1 – The effect of apomorphine and *n*-propylnorapomorphine on NPY release. The percent fractional release of NPY-ir under basal conditions or in the presence of a high potassium buffer (50 mM) in the absence (KCl; solid black bar) or presence of various concentrations of apomorphine (APO; 10^{-7} to 10^{-4} M; hatched bars) or *n*-propylnorapomorphine (NPA; 10^{-7} to 10^{-4} M; solid gray bars) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar represents the mean ± S.E.M. of 5–7 observations. Student–Neuman–Keuls and the Duncan multiple-range test were used in conjunction with ANOVA to determine the significance $^{*}p < 0.05$; $^{**}p < 0.01$ vs. K^+-evoked NPY release.

3.2. Effect of dopamine receptor antagonists on apomorphine induced inhibition of NPY release

To further characterize the receptor subtypes responsible for dopamine agonist-induced inhibition of NPY release from PC12 cells, we examined the effects of several specific dopamine receptor antagonists. Concentration effect curves were carried out with agents that have specificity for D_1/D_5, D_2 and D_4/D_2 dopamine receptors. As depicted in Fig. 2, the inhibitory effect of APO (10^{-5} M) on the K^+-evoked release of NPY-ir release was significantly attenuated by the D_2 receptor antagonist eticlopride in a concentration dependent manner (10^{-6} to 10^{-5} M), while similar concentrations of the D_1/D_5 receptor antagonist, SCH 23390 did not significantly alter the effect of APO on K^+-evoked release of NPY-ir. Clozapine is a D_4/D_2 antagonist, which can bind to D_2 and D_4 dopamine receptors, but has higher affinity and potency at the D_4 dopamine receptor [55]. Clozapine failed to alter the inhibitory effect of APO on K^+-evoked on NPY release. Compared to the high K^+ control, the antagonists SCH 23390, clozapine and eticlopride at 10^{-7} M all significantly decreased NPY-ir release. Similarly, SCH 23390 and clozapine at 10^{-6} and 10^{-5} M were significantly decreased from the high K^+ control. Eticlopride at 10^{-6} and 10^{-5} M were not significantly different from high K^+ but were significantly different than KCl plus apomorphine. These results further support the idea that it is the D_2 receptor subtype that mediates dopamine induced inhibition of NPY-ir release.

3.3. A pertussis toxin sensitive and voltage-dependent calcium channel pathway mediates apomorphine-induced inhibition of NPY-ir release

It is well known that PTX treatment produces ribosylation of the alpha subunit of GTP-binding proteins belonging to the $G\alpha_i$

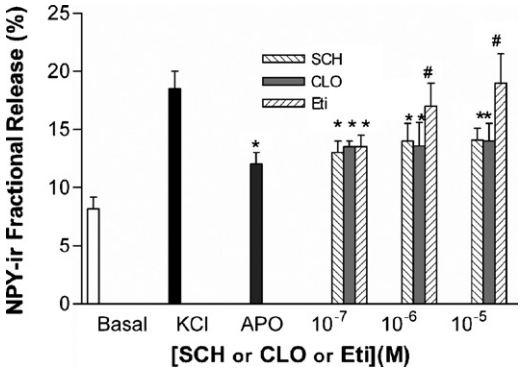

Fig. 2 – The effect of dopamine receptor antagonists on NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or in the presence of a high potassium buffer (50 mM) in the absence (KCl; solid black bar) or presence of APO (10^{-5} M; solid dark gray bar) or APO (10^{-5} M) plus increasing concentrations of the dopamine antagonists SCH23390 (SCH; 10^{-7} to 10^{-5} M; hatched gray bars), clozapine (CLO; 10^{-7} to 10^{-4} M; solid gray bar) or eticlopride (Eti; 10^{-7} to 10^{-4} M; hatched gray bars). The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar depicts the mean ± S.E.M. of 5–7 observations. $^{*}p < 0.05$ vs. K^+-evoked NPY release; (#) shows that Eti significantly reversed the inhibitory effect of APO ($p < 0.05$).

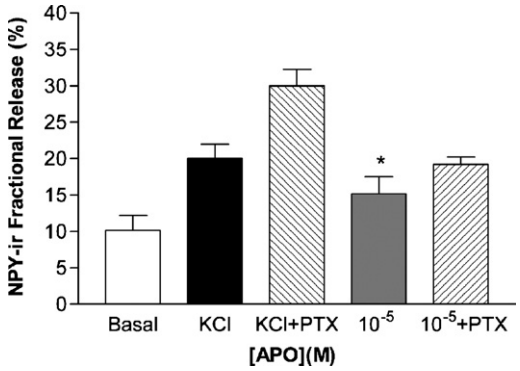

Fig. 3 – The effect of pertussis toxin on apomorphine-induced inhibition of NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or following a high potassium buffer (50 mM) in the absence (KCl; solid black bar) or presence of APO (10^{-5} M; solid gray bar), or in the presence of KCl (50 mM) in cells pretreated with pertussis toxin (PTX; 50 ng/ml; hatched black bars), or in the presence of APO (10^{-5} M) in cells pretreated with PTX (50 ng/ml; hatched gray bars) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar is the mean ± S.E.M. of 6–7 observations. $^{*}p < 0.01$ vs. K^+-evoked NPY release.

and $G\alpha_o$ family resulting in permanent inactivation of these G-proteins. As shown in Fig. 3, the inhibitory effect of APO on the K^+-evoked release of NPY-ir was blocked by pretreatment of PC12 cells with PTX at 50 ng/ml for 18 h. These results, therefore, suggest that G-proteins of $G\alpha_i$ and/or $G\alpha_o$ family may be involved in the dopamine-induced inhibition of depolarization-evoked release of NPY-ir.

Multiple subtypes of Ca^{2+} channels have been distinguished on the basis of pharmacology, electrophysiological properties and molecular cloning [26]. NGF-differentiated PC12 cells have been demonstrated to possess L-type (dihydropyridine sensitive) and N-type (ω-conotoxin-GIVa sensitive) voltage-dependent Ca^{2+} channels [21,22,27,28]. D_2 receptor activation has been shown to block various calcium channels [29–32]. We therefore explored the possibility that blocking calcium channels may be a potential mechanism by which dopamine agonist inhibits the K^+-evoked release of NPY-ir from PC12 cells. It was observed that both nifedipine (Fig. 4) and Ctx-GVIa (Fig. 5) alone could attenuate the K^+-evoked increase in the release of NPY-ir. However, the combination of APO and the L-type channel blocker, nifedipine (Fig. 4) or APO and the N-type channel blocker, Ctx-GVIa produced (Fig. 5) no further inhibition. These data suggest that the activation of D_2 receptors by APO may block both L and N calcium channels.

3.4. *Apomorphine-induced inhibition of NPY-ir release is mediated by PKC- and CaM kinases and independent of adenylate cyclase/PKA activation*

To further our understanding of the mechanism by which APO attenuates the K^+-evoked release of NPY-ir from PC12 cells, we examined the effect of various concentrations of the PKC agonist, PMA. Under basal conditions PMA resulted in an increase in NPY-ir release. PMA, at concentrations of 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} and 5×10^{-5} M, increased the fractional

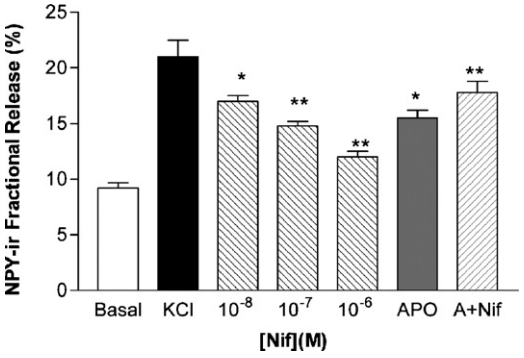

Fig. 4 – The effect of L-type voltage-gated calcium channel inhibitors on apomorphine-induced inhibition of NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or in the presence of a high potassium buffer (KCl; 50 mM; solid black bar) alone or in the presence of varying concentrations of nifedipine (Nif; 10^{-8} to 10^{-6} M; hatched black bars), APO (10^{-6} M; solid gray bar) or APO (10^{-6} M) plus nifedipine (10^{-6} M; A + Nif; hatched gray bar) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar is the mean ± S.E.M. of 5–8 observations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. K^+-evoked NPY release.

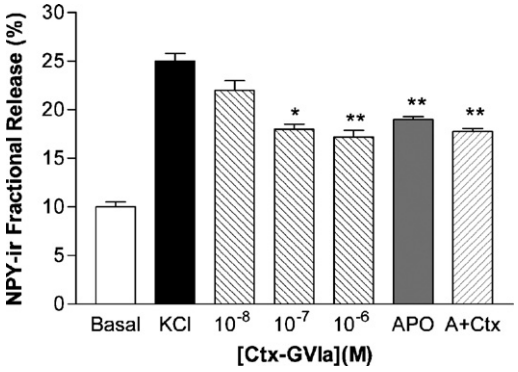

Fig. 5 – The effect of N-type voltage-gated calcium channel inhibitors on apomorphine-induced inhibition of NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or in the presence of a high potassium buffer alone (KCl; 50 mM; solid black bar) or in the presence of varying concentrations of Ctx-GVIa (10^{-8} to 10^{-6} M; hatched black bars), APO (10^{-6} M; solid gray bar) or APO (10^{-6} M) plus Ctx-GVIa (10^{-6} M; A + Ctx; hatched gray bars) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar is the mean ± S.E.M. of 5–7 observations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. K^+-evoked NPY release.

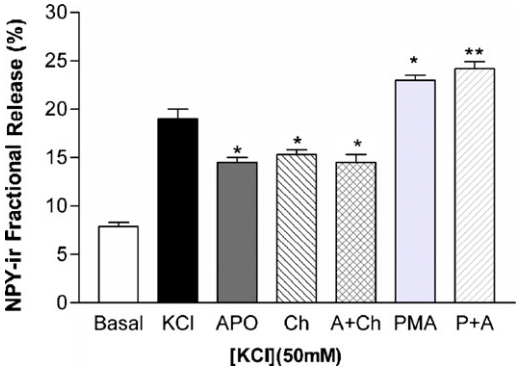

Fig. 6 – The effect of PKC inhibitors and activators on apomorphine-induced inhibition of NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or in the presence of high potassium buffer alone (KCl; 50 mM; solid black bar) or in the presence of APO (10^{-5} M; solid dark gray bar), chelerythrine (Ch; 5×10^{-5} M; hatched dark gray bars), APO (10^{-5} M) plus Ch (5×10^{-5} M; A + Ch; dark gray cross-hatched bars), PMA ($5 \times 5 \times 10^{-5}$ M; light gray bar), PMA ($5 \times 5 \times 10^{-5}$ M) plus APO (10^{-5} M; PMA + A; light gray hatched bar) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar is the mean ± S.E.M. of 6–8 observations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. K^+-evoked NPY release.

release of NPY 112 ± 2%, 120 ± 5%, 135 ± 4%, 140 ± 7% and 144 ± 5% of basal, respectively. In addition, PMA enhanced the K^+-evoked release of NPY-ir (Fig. 6). However, in the presence of PMA, APO no longer had an inhibitory effect on the K^+-evoked NPY-ir release. Although both APO and the PKC inhibitor, chelerythrine chloride, by themselves had inhibitory effects on the K^+-evoked release of NPY-ir, the combination of these two agents did not produce an additive effect (Fig. 6). These results suggest that PKC may play a role in the APO induced inhibition of NPY-ir release.

To investigate whether the dopamine agonist, APO, also acts through the CaM kinase II pathway, we used the selective inhibitor of CaM kinase II, KN-62. It was observed that KN-62 itself produced a concentration dependent (10^{-8} to 10^{-5} M) inhibition of the K^+-evoked release of NPY-ir from PC12 cells (Fig. 7). However, the combination of KN-62 and APO did not produce any further inhibition (Fig. 7).

If cAMP-activated protein kinase A (PKA) is a major pathway for dopamine-mediated inhibition of NPY-ir release, it was reasoned that direct stimulation of this pathway would increase NPY-ir release and direct inhibition of this pathway would produce the opposite effect. Several agents were utilized to test this hypothesis. We observed that neither the direct adenylate cyclase/PKA activator forskolin nor the active cAMP analog [adenosine 3′5′-cyclic monophosphorothioate Sp-isomer (Sp-cAMP)] produced any significant effect on the K^+-evoked NPY-ir release over a wide concentration range. For instance, forkolin at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M altered the fractional release of K^+-induced NPY-ir release 21.2 ± 1.5%, 20 ± 0.8%, and 18.5 ± 0.7%, respectively, compared to KCl control alone (20.8 ± 0.9%). Similarly, the concentration effect of Sp-cAMP (10^{-6}, 10^{-5}, and 10^{-4} M) on NPY-ir release was 17.2 ± 0.5%, 18.8 ± 0.9%, and 16 ± 0.7%, respectively, compared to the KCl control.

Additionally, the adenylate cyclase/PKA inhibitors, Rp-cAMPS or SQ 22536, did not produce a statistically significant

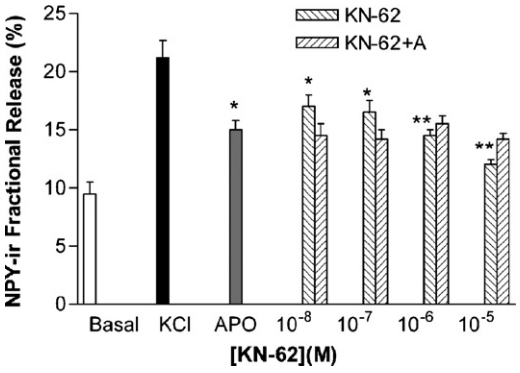

Fig. 7 – The effect of a CaM kinase inhibitor on apomorphine-induced inhibition of NPY release. The percent fractional release of NPY-ir under basal conditions (basal) or in the presence of a high potassium buffer alone (KCl; 50 mM; solid black bar) or in the presence of APO (10^{-5} M; hatched dark gray bar) or KN-62 alone (10^{-8} to 10^{-5} M; hatched gray bars) or KN-62 (10^{-8} to 10^{-5} M) plus APO (10^{-5} M; KN-62 + A; hatched dark gray bars) was measured. The percent fractional release was calculated on the basis of: (fractional release/[fractional release + total release])×100. Each bar is the mean ± S.E.M. of 7–8 observations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. K^+-evoked NPY release.

increase in the K^+-evoked NPY-ir release. SQ 22536 at concentrations of 10^{-6}, 10^{-5}, and $10^{-4.3}$ M modified the fractional release of K^+-induced NPY-ir release $23 \pm 0.3\%$, $21 \pm 1.5\%$, and $20.8 \pm 0.9\%$, respectively, compared to KCl control alone ($20.5 \pm 0.8\%$). The concentration effect of Rp-cAMPS (10^{-6}, 10^{-5}, and $10^{-4.3}$ M) altered the fractional release of K^+-induced NPY-ir release $17 \pm 1\%$, $17.4 \pm 0.8\%$, and $17 \pm 0.7\%$, respectively, compared to KCl control alone. This is consistent with results in which we observed that activation of dopamine D_1/D_5 receptors, which is known to have a stimulatory effect on cAMP/PKA pathway, also failed to produce any effect on NPY-ir release. Furthermore, this is consistent with our observation in which D_1/D_5 dopamine receptor agonists known to stimulate the cAMP/PKA pathway did not have an effect on the K^+-evoked release of NPY-ir (Fig. 1).

4. Discussion

The purpose of the present study was to investigate the effect of dopamine agonists on NPY-ir release from PC12 cells and the mechanism of the regulation. We have demonstrated that the dopamine agonists, APO and NPA, inhibited NPY-ir release from PC12 cells most likely by activation D_2 receptors through a pharmacological approach. The effect of APO was blocked by the D_2 receptor antagonist eticlopride in a concentration-dependent manner but not by the D_1/D_5 antagonist, SCH 23390 or the D_4/D_2 antagonist, clozapine. Clozapine, which has some D_2 receptor activity, did not partially antagonize the APO-induced inhibitors of the K^+-evoked release of NPY-ir. A possible explanation for this effect may be that the effects of apomorphine on D_4 receptors are opposite the effects at D_2 receptors. Although the D_4 and D_2 receptors are linked to the same G-protein family, it has been observed that among the variants of the D_2-like dopamine receptor family – the D2L and D2S – there is differential inhibition of adenylate cyclase inhibition [62]. Moreover, it appears that the short form of the D_2 receptor is more efficient at this inhibition [62], and that this effect is carried out by differential coupling to a specific G-protein of in the Gi/o family [63].

The D_2 receptor has been shown to inhibit calcium channels [40–42]. Dopamine appears to regulate the opening and closing of ion channels on lactotroph cells by inhibition of Ca^{2+} currents [33,39] via inhibition of voltage-gated Ca^{2+} channels [30]. On lactotroph cell membrane patch preparations [31] dopamine initiates dual inhibition of L-type and N-type Ca^{2+} channels which is consistent with a direct action of receptor-mediated, G protein-coupled channel modulation [33]. Additionally, in cAMP-differentiated NG108-15 cells, D_2 receptors partially inhibit two types of high threshold Ca^{2+} channels, Ctx-GVIa-sensitive and dihydropyridine sensitive channels [32].

The present study suggests that the D_2 receptor is most likely the principal dopamine receptor that is involved in the inhibition of NPY-ir release. Therefore, we investigated whether the negative modulation of NPY-ir release is mediated by voltage-dependent Ca^{2+} channels. We observed that both N- and L-type Ca^{2+} channel blockers reduced the K^+-evoked release of NPY-ir from PC12 cells. However, the combination of APO and either of the Ca^{2+} channel antagonists, Ctx-GVIa (N-type) or nifedipine (L-type), failed to produce an additive inhibitory effect on the evoked release. This observation supports the hypothesis that the effect of APO on depolarization-induced release of NPY-ir is at least partially due to blockade of L- and N-type calcium channels. Although we favor the idea that the effect of APO on depolarization induced release of NPY-ir is at least partially due to blockade of L- and N-type Ca^{2+} channels, interpretations are obviously complex. It is also possible that APO may also inhibit the release of intracellular Ca^{2+} from endoplasmic reticulum (ER) following increases of IPs or activation of voltage gated Ca^{2+} channels triggering release of Ca^{2+} from the ER.

It would appear that apomorphine may be reducing or blocking part of the nifedipine effect. It has been demonstrated in the literature that dopaminergic receptors can both block and facilitate calcium influx through L-type channels and that this action is dopamine receptor specific. For example, in lactotrophs activation of the D2S receptor inhibits dihydropyridine-sensitive L-type channels and thus diminishes extracellular calcium influx [56]. Conversely, when the D1 receptor is activated calcium entry through the L-type calcium channel is increased [61]. In our experiments it appeared that apomorphine decreased or blocked part of the dihydropyridine-sensitive effect. Based upon the demonstration that activation of the D1 receptor facilitates calcium influx through L-type calcium channels it maybe that apomorphine treatment may indirectly activate dopamine D1 receptors leading to this effect in PC12 cells.

A potential mechanism by which dopamine receptors negatively modulate voltage-dependent calcium channels with subsequent inhibition of NPY-ir release may be through a PTX-sensitive mediated pathway utilizing a specific $G\alpha_{i/o}$ subtype. In lactotroph cells it has been observed that the D_2-mediated inhibition of voltage-dependent L-type calcium channels is mediated by the $G\alpha_o$ subtype [56]. Our observation that the inhibitory effect of APO on the K^+-evoked release of NPY-ir was blocked by pretreatment of PC12 cells with PTX supports the concept that a $G\alpha_{i/o}$ subtype is involved. However, the specific $G\alpha_{i/o}$ subtype mediating this effect remains unknown, and further investigation into the $G\alpha_{i/o}$ subtype/s involved in the dopamine receptor mediated-inhibition of NPY-ir release is required.

In a number of systems it is well established that activation of D_2 receptor results in multiple signaling mechanisms including inhibition of adenylate cyclase [34,35], inhibition of phosphoinositide turnover [36–38], and activation of potassium channels [39]. We further investigated the potential signaling mechanisms involved in the dopamine mediated inhibition of NPY-ir release. The CaM kinase II inhibitor KN-62 produced a concentration dependent inhibition of the K^+-evoked release of NPY-ir but the combination of KN-62 and APO produced no further inhibition. It is well known that CaM kinase plays a regulatory role in neurotransmitter release as well as other processes [43]. It has been suggested that calcium influx through voltage-gated calcium channels leads to elevation of intracellular calcium levels with a resultant activation of CaM kinase. Activated CaM kinase can phosphorylate a variety of substrates such as synapsin I as well as other neural specific phosphoproteins which are involved in

the regulation of neurotransmitter release [44]. In the present studies it is hypothesized that APO lowers CaM kinase activity through the initial inhibition of voltage-gated Ca^{2+} channels.

Increases in PKC levels have also been suggested to lead to activation of transmitter release in various systems [45–47]. In the present studies, our observations suggest a role for PKC in the dopamine-mediated inhibition of NPY-ir release. Down stream effectors of G-protein-coupled receptors have been shown to modulate voltage-dependent calcium channel activity. For example, in rat central and peripheral neurons PKC activation prevents βγ-mediated inhibition of N-type channels [57]. Inhibition of PKC may be a potential mechanism by which dopamine-mediated inhibition of NPY-ir occurs as the non-additive effect of APO and chelerythrine suggest a common pathway. Alternatively, inhibition of depolarization-induced increases in PKC may also play a role in the dopamine-induced attenuation of NPY-ir release. However, it is unlikely that K^+-induced depolarization and PMA are increasing NPY-ir release by a similar mechanism. This is because depolarization has been reported to increase the activity of CaM kinase [48] and increase diacylglycerol [49] and cyclic AMP [50] while PMA inhibits depolarization-induced Ca^{2+} influx [51–53] and activation of CaM kinase [48].

In contrast to a role for both CaM kinase and PKC, activation or inhibition of the cAMP pathway or cyclic AMP dependent protein kinase does not appear to be involved in the inhibitory effect of APO on the evoked release of NPY-ir. Athough D_2 receptor activation has been shown to inhibit adenylate cyclase and cAMP production in LD2S fibroblasts [58] and lactotrophs [59], studies have suggested that inhibition of adenylate cyclase does not contribute to inhibition of transmitter release [54]. Furthermore, in cerebellar granule cells Ca^{2+} channel-mediated mobilization of intracellular Ca^{2+} stores has been shown to be cAMP independent [60].

A unifying mechanism that may explain the cAMP-independent inhibitory effect of APO on the evoked release of NPY-ir is differential coupling of G-protein isoforms to the D_2 receptor for divergent signaling events. The D_2 receptor has been shown to couple to distinct G-proteins to mediate specific signaling events. For example, in GH4ZR7 cells the G_o protein, via the Gβγ subunits, has a primary role in the D_2-mediated inhibition of L-type calcium channels [56]. In contrast, in the same cells adenylate cyclase inhibition is dependent on the Gi_2 and Gi_3 G-proteins but is independent of the Gβγ subunits in this signaling pathway [56]. Further dissection of the signaling events in the APO-mediated inhibition of NPYir release will form the basis for the continuation of this work

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