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ORIGINAL RESEARCH ARTICLE

Dopamine induces a biphasic modulation of hypothalamic ANF neurons: a ligand concentration-dependent effect involving D_5 and D_2 receptor interaction

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Increasing evidence now suggests that more than one subtype of dopamine receptors is co-expressed in some of the central neurons. The neurobiological effects on the host cells when these receptors are concurrently activated by their common physiological ligand, dopamine, however, remains elusive. Among the members of the family of dopamine receptors, coupling of D_1-like dopamine receptors to Gs and D_2-like receptors to Gi proteins are known to augment or suppress cellular functions respectively, through modulation of adenylyl cyclase activity and consequently cAMP generation. Simultaneous activation of D_1 and D_2 receptors in transfected cell lines expressing the two cloned receptors, however, produced antagonistic effects. This is in contrast to *in vivo* studies, in which concurrent activation of D_1-like and D_2-like receptors by their respective agonists may induce synergistic or antagonistic effects or both. We report here that in long-term rat hypothalamic cell cultures, activation of both D_1-like (D_1 and D_5) and D_2 receptors on atrial natriuretic factor-producing neurons by dopamine yields a biphasic response. The response is ligand concentration-dependent and involves type II adenylyl cyclases. This process is mediated primarily through antagonistic and synergistic interactions of D_5 and D_2 receptors as the event is mimicked by the concurrent activation of these two receptors co-transfected in CHO cells. Our present findings suggest a novel action of dopamine, and the biochemical processes involved may underlie some of the pharmacological actions of atypical anti-psychotic drugs. *Molecular Psychiatry* (2000) **5,** 39–48.

Keywords: dopamine receptor subtypes; atrial natriuretic factor producing neurons; G proteins; adenylyl cyclases; primary neuron cultures; triple staining; receptor gene co-expression; CHO cells

Introduction

Dopamine (DA) is an important neurotransmitter that is closely involved in the regulation of motor activities, higher cortical and neuroendocrine functions.[1,2] Whereas functional aberrations of the central DA system have been implicated in psychoses including schizophrenia, the neurobiological basis of the disease remains elusive.[3,4] This has been attributed, in part, to the complex actions of DA mediated through various receptor subtypes that may interact to produce intricate outcomes.[5–7] Unlike many neurotransmitters, DA is capable of exerting both stimulatory and inhibitory effects depending on receptor subtype involvement.[8–10] Thus, in the case of D_1-like receptors, which include the D_1- and D_5-receptor subtypes, the amine acts as a stimulator in many biological systems while exerting inhibitory effects in tissues expressing D_2-like receptors, which include the D_2-, D_3- and D_4-receptor subtypes. Increasing evidence now suggests that subtypes of the DA receptor are differentially expressed in various regions of the brain.[11–13] Whereas D_1 receptors are found abundantly in basal ganglia, D_5 receptors are primarily present in the hypothalamus and cortices.[14–17] More than one subtype of DA receptor can be expressed constitutively in the same cells. To date, up to three subtypes, namely D_1, D_5 and D_2 receptors have been localized in neurons of the basal ganglian.[12] The phenotype of the relevant cells remains unclear, however. The functional significance of this observation is still to be fully appreciated. More interestingly, studies of the Chinese Hamster Ovary (CHO) cell line concurrently expressing D_1 and D_2 receptors have demonstrated that an antagonistic effect on cAMP production is detected when the two receptors are activated at the same time by dopamine.[18] However, the neurobiological relevance of this to the central nervous system is not certain.

Previously we have localized individually D_1, D_5 or D_2 receptors in atrial natriuretic factor (ANF)-produc-

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ing neurons of the rat hypothalamus.[19–21] The neurons are stimulated or inhibited by D_1-like and D_2-like receptor activation respectively, through the modulation of the cAMP-dependent kinase A pathway. Using the same primary hypothalamic cell culture system,[22–24] we have now examined the effects of a concurrent activation of D_1-like and D_2-like receptors by DA, and by their respective agonists. The same studies have also been extended to include those performed in transfected CHO cells stably expressing D_5 and D_2 receptors.

Materials and methods

Cell cultures

Neonatal rat hypothalamic cultures were prepared[22,25,26] by enzymatic dispersion of hypothalami removed from 2 to 3-day-old Sprague–Dawley rat pups. The dispersed cells were maintained in Hepes buffered Dulbecco's Modified Eagle's Medium (HDME) supplemented with 8% fetal calf serum at 37°C in 10% CO_2. All release studies were conducted on the 7th day of culture and extended over a period of 4 days. The drugs were applied every 24 h to the cultures. Four hours after the last application of vehicle or drugs, conditioned media were removed and extracted for immunoreactive (ir) ANF release measurement by double antibody RIA.[27–29] To determine the intracellular cAMP, the cultures medium was replenished with fresh culture medium containing IBMX for 30 min at 37°C after 4-day treatment with various drugs. The cells were then incubated with the same drugs for another 15 min. The reaction was terminated by aspirating the medium, adding 1 ml of stop solution (0.01 M of HCl in ethanol) and placing at room temperature (25°C) for 30 min. The extracts were lyophilized and reconstituted for cAMP radioimmunoassay.[20] Intracellular cyclic AMP was then extracted and quantified using RIA modified from the method described by Marley.[30] Each of the experiments was performed at least three times; within an experiment, the results of an individual condition represented the average of three to four cultured wells. Results presented here are the mean of three or more separate experiments from different batches of cell preparations.

Northern blot analysis

Following treatment, cells were lysed directly in the well by adding 0.5 ml of TRIZOL Reagent (Life Technologies, Grand Island, NY, USA). The cell lysate from three individual wells, each with 10^6 cells, was harvested by pipetting and pooled together as a single sample for the study. The cytoplasmic RNA was further extracted by chloroform and precipitated with isopropyl alcohol. Twelve micrograms of total RNA from each of the samples fractionated by electrophoresis through formaldehyde-agarose gel were transferred to nylon membrane and hybridized with digoxigenin-labeled ANF-antisense oligonucleotide probes[31–33] and visualized by colorimetric reaction. The signals of individual bands for pro-ANF mRNA were measured as integrated intensities by a computer based Image-Pro Plus version 3 (Media Cybernetics, MD, USA) taking the vehicle-treated controls as 100% for statistical comparison. After signal analysis, the same blots were reprobed with digoxigenin-labeled GAPDH mRNA oligonucleotide. The intensity readings of each band of pro-mRNA from various treatment groups were adjusted to that of corresponding bands of GAPDH mRNA; the values were then expressed as a percentage of that of the vehicle-treated controls.

Triple staining

Cells treated with vehicle were washed and fixed with 3% phosphate-buffered paraformaldehyde.[23,33] After prehybridization, the cells were incubated overnight at 37°C with antisense oligonucleotide probes (200 pmol ml^{-1}) labeled with digoxigenin and biotin for D_5 mRNA (5′-TGGCAGCACACACTAGCACGTTCC, 857–880) and D_2 mRNA,[19] respectively. The cultures were then washed for 3 h with decreasing concentrations of SSC. For simultaneously detecting D_5, D_2 mRNA and ir-ANF, Fab segments of sheep antiserum raised against digoxigenin and conjugated with rhodamine (TRITC; 1:40; Boehringer-Mannheim) and fluorescein conjugated strepABC complexes (FITC; 1:40; DAKO) were added together with ANF antiserum (R178, 1:50) of rabbit in Tris buffer containing 1% BSA and 0.3% Triton X-100 overnight at 4°C. The cells were then washed and incubated subsequently with gold conjugated swine anti-rabbit IgG antiserum (1:200) for 60 min. At the end of the incubation, wells were washed and applied with silver enhancement. After a short incubation, the intensity of gold staining was monitored through a microscope and the reaction was terminated by washing with tap water. The cells were then mounted in DAKO fluorescent Mounting Medium and microphotographed with a Nikon DIAPHOT model TMD microscope. FITC and TRITC were viewed with Epi-fluorescence filter combination B-2E and G-2A respectively.

The specificity of the ir-ANF staining was demonstrated by the absence of specific staining when the primary antiserum was replaced with Tris buffer, normal rabbit serum, or anti-ANF antiserum preabsorbed with an excess amount of synthetic rat ANF (99–126). To eliminate the possibility of false positive staining in the *in situ* hybridization, the replacement of antisense probe with sense probe, pretreatment of fixed cultures with RNAase, coincubation with an excess of unlabeled oligo probes, application of anti-digoxigenin antiserum or FITC-conjugated strepABC complex without probe was carried out. For triple staining, negative controls which employed either primary antiserum or oligonucleotide probe alone in the presence of one of the non-corresponding second antisera were included. In all cases, no significant staining was observed above background.

Construction of mammalian expression D_5 receptor vector, CHO cell transfection and measurement for cAMP

A $PAXD_2$ plasmid containing full-length cDNA encoding the human D_{2A} DA receptor was given by Dr Gillian Hayes of Garvan Institute of Medical Research in Sydney, Australia.[34] To construct a $PAXD_5$ plasmid, total RNA was extracted from cultured rat hypothalamus by TRIZOL and amplified by RT-PCR. Primers corresponding to 5′-(TTCTCGAGCTACTCAGCGCGAC ATGC, 672–697, *Xho*I site underlined) and 3′-(TTCTATGCAGCAGACTACAGAAAGC, 2240–2264) untranslated regions of rat D_5 receptor cDNA were used in the PCR to amplify the full-length cDNA encoding the rat D_5 receptor. Hot start PCR were initiated as described before,[21] followed by 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were purified using the QIAquick Spin purification kit from Bresatec and the nucleotide sequence was confirmed by dideoxy sequencing of double strand DNA using the DNA sequencing system from Promega following manufacturer's instructions. Briefly, the samples were analyzed by electrophoresis on a 6% polyacrylamide, 7 M urea gel, dried onto Whatman 3 M paper, and exposed to X-ray film (Kodak BioMax, MR-1, Kodak) for 16 h at −70°C. The sequence of the amplified cDNA was identical to the published rat D_5 receptor sequence. The cDNA was then modified by adding a *Hind*III linker at 3′ end and cloned into the *Sal*I and *Hind*III sites of PAX.[34]

CHO cells were grown in α-medium (Gibco RBI) supplemented with 8% FCS in six well dishes (35 mm; Costar, Cambridge, MA, USA; 2×10^6 cells $dish^{-1}$) and transfected at 90–100% confluence using a modified calcium phosphate method with $pAXD_5$ and $pAXD_2$ or wild-type pAX vector (MOCK). Following transfection (4 h), CHO cells were shocked with 15% glycerol in PBS for 3 min, washed with PBS, and fed with fresh culture medium. After a recovery period (24 h), cells were trypsinized and replated in α-medium containing geneticin (G418) to isolate stable transformants. Selection of stably transformed cells was over a period of 4 weeks in the presence of 800 $\mu g\ ml^{-1}$ G418, after which time clonal cell lines were isolated by dilution cloning. Successful co-transfection of D_5 and D_2 receptors in CHO cells was confirmed by *in situ* hybridization with both digoxigenin-labeled oligonucleotide complementary to D_5 cDNA and biotinylated oligonucleotide of D_2 receptor cDNA (as described in Triple Staining). Furthermore, by employing reverse transcripted polymerase chain reaction (RT-PCR), we have also demonstrated the presence of D_5 and D_2 receptor mRNA in the total RNA extracts of the transfected CHO cells, but not in that of the mock transfected equivalence. To determine the intracellular cAMP, the transfected cells were seeded in 24-well cell culture clusters (16 mm; Costar; 0.4×10^6 $well^{-1}$). Two days later, the culture medium was replaced by fresh α-medium containing IBMX for 30 min at 37°C and the cells were then incubated with various drugs for 15 min. The reaction was terminated by adding 0.5 ml of stop solution and the intracellular cyclic AMP was then extracted and quantified using RIA (as described in Cell Cultures).

Results

Using a well characterized system of long-term monolayer cell cultures prepared from neonatal rat hypothalamic tissues as previously described,[22,23] we have examined the effect of the endogenous ligand for both D_1-like and D_2-like receptors, dopamine, on the secretion of immunoreactive (ir) ANF from the culture. After four days of incubation with DA over a wide range of concentrations (10^{-10} to 10^{-5} M), ir-ANF levels in the conditioned medium showed biphasic changes (Figure 1a). At low concentrations, in the range of 10^{-10} to 10^{-7} M, DA inhibited the basal release of ir-ANF in a dose-related manner to approximately half that of the control value ($P < 0.01$). In contrast, higher concentrations (10^{-6} and 10^{-5} M) of DA reversed this inhibitory effect and showed a dose-related enhancement of ir-ANF release. At the concentration of 10^{-5} M, DA increased ir-ANF release approximately 35% above that of the vehicle-treated control cultures ($P < 0.01$) (Figure 1a).

The biphasic effect of DA treatment suggests that more than one subtype of DA receptor may be involved in the regulation of ir-ANF secretion from the cultures. To further characterize this effect, hypothalamic cultures were pre-incubated with SCH-23390, a D_1-like receptor antagonist, 20 min before the application of DA. Figure 1b shows that the blockade of D_1-like receptors effectively abolished the ascending-phase of ir-ANF release and results in an overall dose-related suppression of DA with an ED_{50} of approximately 10^{-7} M. This observation suggests the involvement of D_1-like receptors in this event. The accentuated effect of DA suppression in the presence of SCH-23390 was similar to that seen in cultures treated with quinpirole alone,[19] a D_2-like receptor agonist. The findings suggest that under these circumstances, the overall suppression effect of DA is likely to be mediated through D_2-like receptors. Consistent with this interpretation is the observation that the suppressive effect induced by low doses of DA (10^{-9} to 10^{-7} M) is abolished by concurrent incubation with sulpiride, a D_2-like receptor antagonist (Figure 1b). These results together suggest that the inhibitory effect of DA (the descending-phase) is mediated through the activation of D_2-like receptors. In contrast to the marked suppression mediated by D_2-like receptors, the lack of significant augmentation of ir-ANF release above the baseline level in the presence of sulpiride, suggests that DA-mediated D_1-like receptor stimulating effect is, at best, modest. It is, however, important to point out that whereas the stimulating effect of a high concentration (10^{-5} M) of DA, was blocked completely by SCH-23390, a specific D_2-like receptor antagonist, it was also suppressed significantly ($P < 0.05$) but to a much lesser extent by sulpiride, the D_2-like receptor antagonist (Figure 1b). These

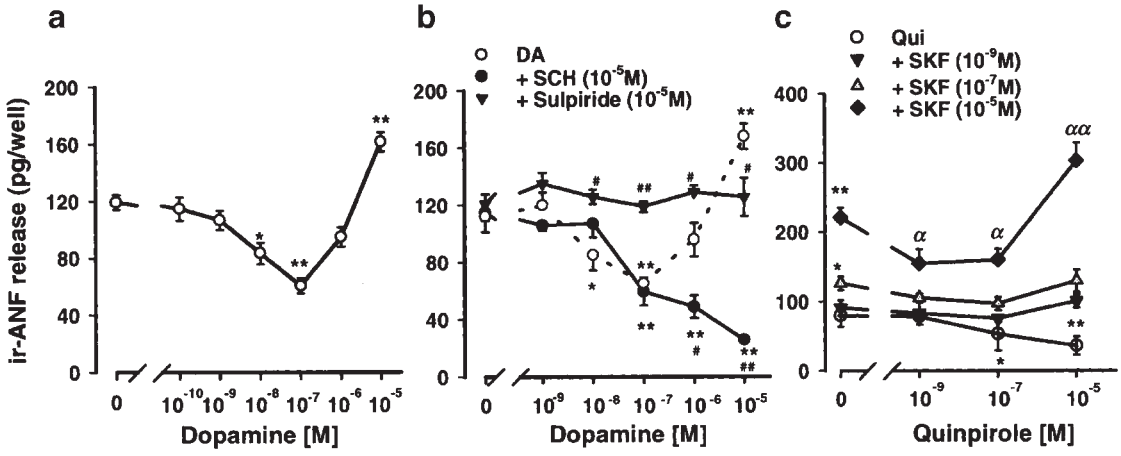

Figure 1 Immunoreactive atrial natriuretic factor (ir-ANF) secretion from hypothalamic cultures incubated with (a) dopamine (DA) alone or (b) in the presence or absence of SCH-23390 (SCH, D_1-like antagonist) and sulpiride (D_2-like antagonist). (c) Quinpirole (Qui, D_2-like agonist)-treated cultures with or without SKF-38393 (SKF, D_1-like agonist) supplement. Asterisks and hatches denote values significantly different from cultures treated with vehicle and with corresponding doses of DA respectively; α indicates values significantly different from SKF-38393 10^{-5} M treatment alone. Values shown represent mean ± SEM, $n = 4$. *, #, α, $P < 0.05$; **, ##, $\alpha\alpha$, $P < 0.01$.

findings suggest that the stimulation mediated by the high dose (10^{-5} M) of DA may involve a synergistic interaction of D_1-like and D_2-like receptors through their common physiological ligand.

To examine whether the biphasic effect of DA can be mimicked by a concurrent activation of D_1-like and D_2-like receptors with their respective agonists, the D_1-like receptor agonist SKF-38393, and quinpirole, a D_2-like receptor agonist were applied in hypothalamic cultures under the same experimental conditions as described above. Over the range of concentrations from 10^{-9} to 10^{-5} M, application of SKF-38393 alone markedly increased, whereas quinpirole suppressed ir-ANF secretion in a dose-dependent manner as shown in Figure 1c. When applied together, low doses of quinpirole (10^{-9} and 10^{-7} M) consistently suppressed the stimulatory effect of 10^{-5} M SKF-38393. By contrast, at a high dose of 10^{-5} M, the D_2-like receptor agonist now acted as an enhancer to significantly augment the SKF-38393-induced ir-ANF release by approximately 40% ($P < 0.01$). Simultaneous activation of the D_1-like and D_2-like receptors by their respective agonists therefore also results in a biphasic modulation of ir-ANF release in a ligand concentration-dependent manner.

To evaluate whether the biphasic effect induced by the interaction of D_1-like and D_2-like receptors may also affect the production of the peptide, Northern blot analysis[19,20] was performed to examine the abundance of pro-ANF mRNA signals in the cultures that were treated with a protocol similar to that described in the secretion studies. The intensity of pro-ANF mRNA signal for each lane was standardized to that of the constituent housekeeping gene, GAPDH, after re-probing the membrane with GAPDH antisense oligonucleotides (Figure 2). Treatment with 10^{-7} M of DA markedly reduced the abundance of pro-ANF mRNA to approximately 40% of the vehicle-treated controls whereas at a higher concentration of 10^{-5} M, the amine doubled the level of pro-ANF mRNA expression (Figure 2a). When applied alone, 10^{-5} M of SKF-38393 significantly increased, whereas quinpirole treatment decreased the abundance of pro-ANF mRNA. In contrast, the enhanced pro-ANF mRNA expression by high dose of SKF-38393 (10^{-5} M) was markedly potentiated by a concurrent application of a high dose of 10^{-5} M of quinpirole in cultures. In all the above conditions, only a single band of signal for pro-ANF mRNA, similar in size to that of rat cardiac origin (not shown) was detected. Replacement of the antisense probes with sense oligonucleotides failed to show any hybridization signals for pro-ANF mRNA (data not shown).

Whereas we have localized D_1 and D_2 receptors in ANF-producing neurons in the past,[19,20] our recent evidence suggests that D_5 receptors are also present in hypothalamic ANF neurons.[21] To address whether D_5 receptors may play a more important role than their D_1 receptor counterparts in functional modulation of hypothalamic ANF neurons, a specific antisense oligonucleotide complementary to D_5 receptors was synthesized and applied to our hypothalamic cultures to differentially suppress the expression of D_5 receptors. Figure 3 shows that the stimulatory but not the inhibitory effects of DA on ir-ANF release are abolished in cultures treated with antisense but not sense oligonucleotides for D_5 receptors.

To confirm that ANF neurons indeed possess both D_2 and D_5 receptors, we used a triple staining technique of *in situ* hybridization for both D_2 and D_5 receptor mRNA and immunostaining for ir-ANF.[20] Figure 4c shows a representative cell stained for ir-ANF that is also positive for both D_2 (Figure 4a) and D_5 receptor (Figure 4b) mRNA. As more than 80% of the ir-ANF positive cells simultaneously stain for D_2 and D_5 receptor mRNA signals, the result confirms that most of the ir-ANF positive cells in our cultures express the two receptors concurrently.

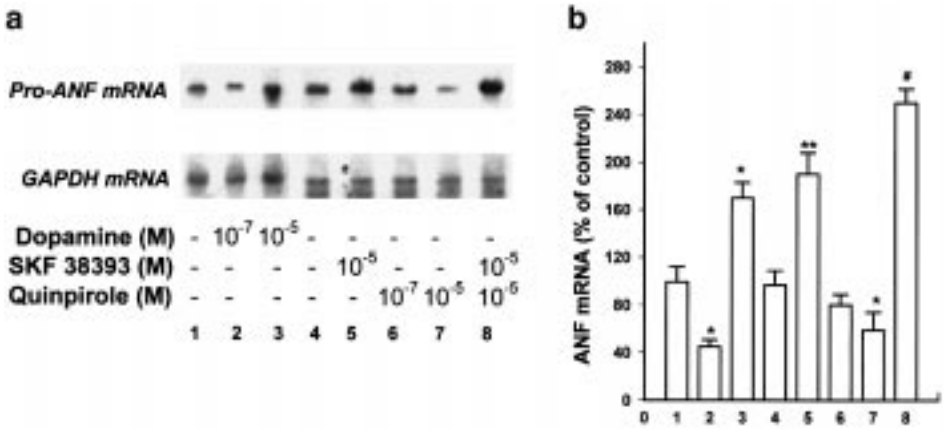

Figure 2 Levels of pro-ANF mRNA and corresponding GAPDH mRNA in hypothalamic cultures treated with (a) dopamine (10^{-7} M and 10^{-5} M), SKF-38393 (D_1-like agonist, 10^{-5} M), quinpirole (D_2-like agonist, 10^{-7} M and 10^{-5} M) alone or co-treated with SKF-38393 (10^{-5} M) and quinpirole (10^{-5} M). (b) Bar graph showing statistical significance with asterisks and hatches to denote values significantly different from vehicle-treated control group and from the group treated with SKF-38393 respectively. Values shown represent mean ± SEM, $n = 3$. *, #, $P < 0.05$; **, $P < 0.01$.

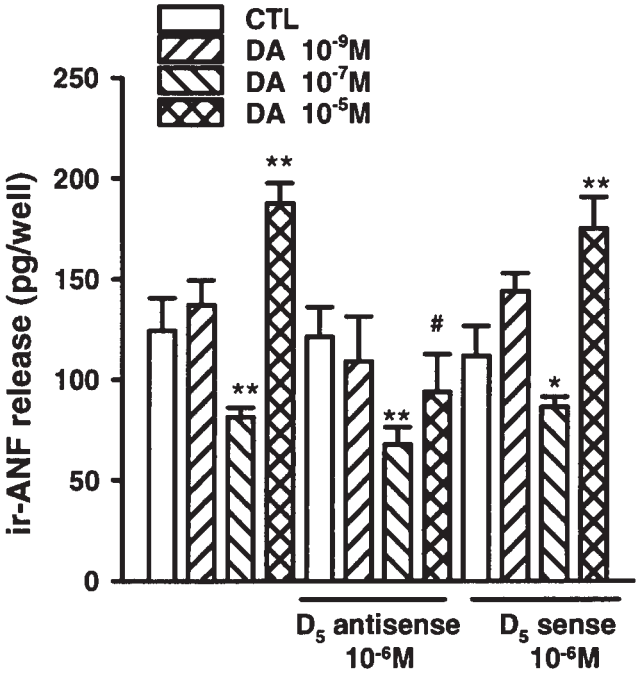

Figure 3 Effects of dopamine (DA) alone or in the presence of phosphorothioate-modified oligonucleotide antisense or sense for D_5 receptor mRNA (5′-TGTCGCGCTGAGTAGCTCG, 676–696) on the secretion of immunoradioactive atrial natriuretic factor (ir-ANF) from rat hypothalamic cultures. The cultures were treated with antisense or sense oligonucleotides at the concentration of 10^{-6} M approximately 2 h before the supplement of dopamine. This protocol was repeated daily for 4 consecutive days over the period of the study and the media were collected for radioimmunoassay as described in Materials and Methods. Similar results were obtained when non-phosphorothioate-modified oligonucleotide antisense or sense was used in the study. Asterisks and hatches denote significant changes from the control group and from the cultures treated with similar levels of DA respectively. Values shown represent mean ± SEM, $n = 3$. *, #, $P < 0.05$; **, $P < 0.01$.

To establish that the biphasic effect is indeed mediated through the interaction of D_5 and D_2 receptors, rat DA D_5 and human D_2 receptor cDNA have been cloned and co-transfected into CHO cells.[21,34] Figure 5a shows that in these cells, SKF-38393 significantly elevates cAMP levels in a dose-related manner, whereas quinpirole produces an opposite effect by suppressing the level of cAMP. In addition, simultaneous activation of the two receptors with DA (Figure 5b), or SKF-38393 in conjunction with quinpirole (Figure 5c), induces biphasic changes in cAMP levels as seen in hypothalamic cell cultures treated in the same manner (Figure 6)

It is now evident that coupling of D_1-like receptors to Gs, or D_2-like receptors to Gi proteins, respectively, stimulates or inhibits the activity of adenylyl cyclases.[35–37] To confirm that this remains true in our culture system, Gs and Gi proteins were activated directly with various chemicals. Figure 7a shows that the biphasic change of ir-ANF release is reproduced when the D_1-like receptor agonist is replaced with cholera toxin, a chemical that acts upon Gs proteins directly.[38] Similar results occur in studies where the D_2 receptor agonist is replaced with mastoparan (Figure 7b), a peptide of wasp venom origin that produces a direct activation of Gi proteins.[39] These observations suggest that the biphasic interaction of D_5 and D_2 receptors is likely to be mediated through Gs and Gi proteins.

As the synergistic effect is also reproduced by replacing the D_1-receptor agonist with forskolin that induces a direct pan-activation of all subtypes of adenylyl cyclases (shown in Figure 7a), the possibility that the cyclases may play a central role in this biphasic effect needs to be considered. This notion is consistent with the finding that the D_2 agonist failed to potentiate the stimulating effect of Bu_2cAMP, a synthetic analogue of cAMP or with IBMX, which raises intracellular cAMP level distal to the activation of adenylyl cyclases (Figure 7c).

To examine the involvement of the type II isoform

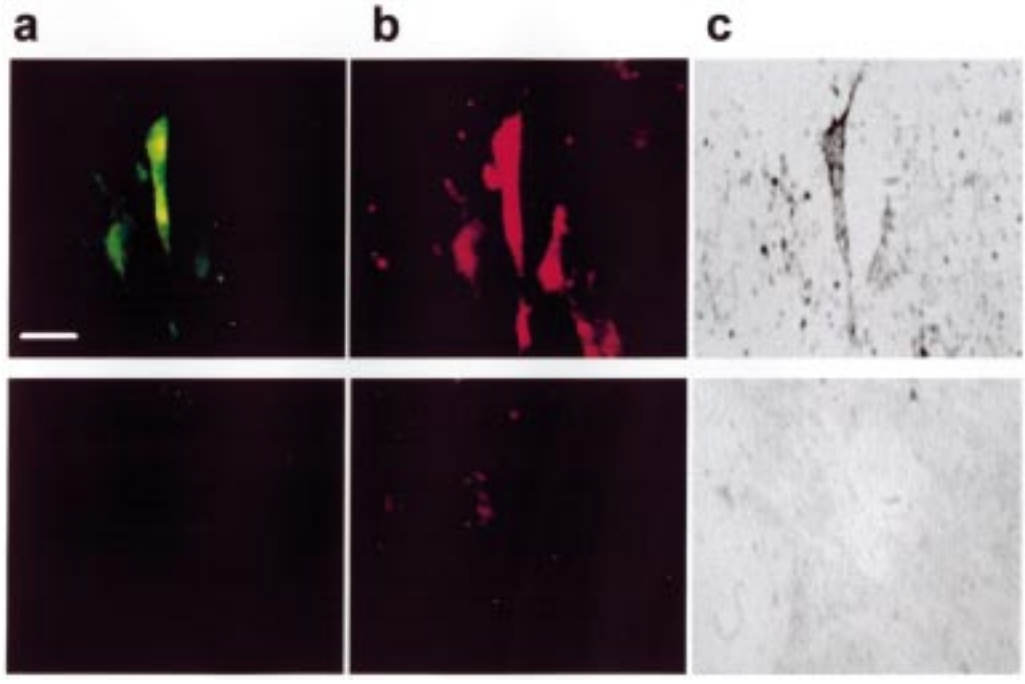

Figure 4 Cultured hypothalamic neurons showing triple staining for (a) *in situ* hybridization of D_2 receptor mRNA in fluorescence and (b) of D_5 receptor mRNA in rhodamine, and (c) immunoreactive atrial natriuretic factor in immunogold silver staining. Lower panel shows absence of signal in cultures (a) pre-treated with RNAase; (b) hybridized with sense oligonucleotide of D_5 receptor; and (c) incubated with anti-ANF antiserum preabsorbed with an excess amount (1 μg) of synthetic rat ANF (1–28). Scale bar = 20 μm.

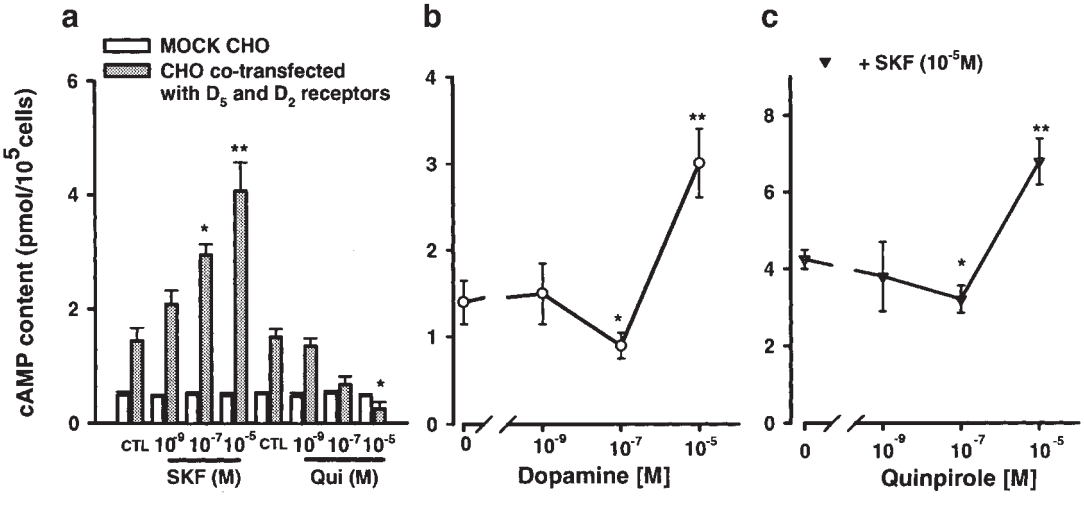

Figure 5 Cell contents of cAMP in CHO cells transfected with or without D_5 and D_2 receptors. Effects of (a) SKF-38393 (SKF), a D_1-like receptor agonist or quinpirole (Qui), a D_2-like receptor agonist. Transfected CHO cells treated with (b) dopamine, and (c) quinpirole in conjunction with SKF-38393. Asterisks denote significant changes from the group treated with vehicle (CTL) or SKF-38393 alone. Values shown represent mean ± SEM, $n = 4$. *, $P < 0.05$; **, $P < 0.01$.

of adenylyl cyclase (ACII) in our studies, we constructed a 30-mer antisense oligonucleotide complementary to the second membrane-spanning domain of ACII and added it to our cultures to suppress the expression of ACII in ANF neurons. Figure 8 illustrates that this approach significantly inhibits the suppressing and augmenting effect of DA alone. Besides, it also abolishes the inhibiting and potentiating effect of quinpirole on SKF-38393-induced ir-ANF secretion. In contrast, application of sense oligonucleotides under similar conditions failed to modulate the effects of these modalities (data not shown).

Discussion

In the present studies, we have exploited the paradigm of long-term primary cultures of rat hypothalamic cells

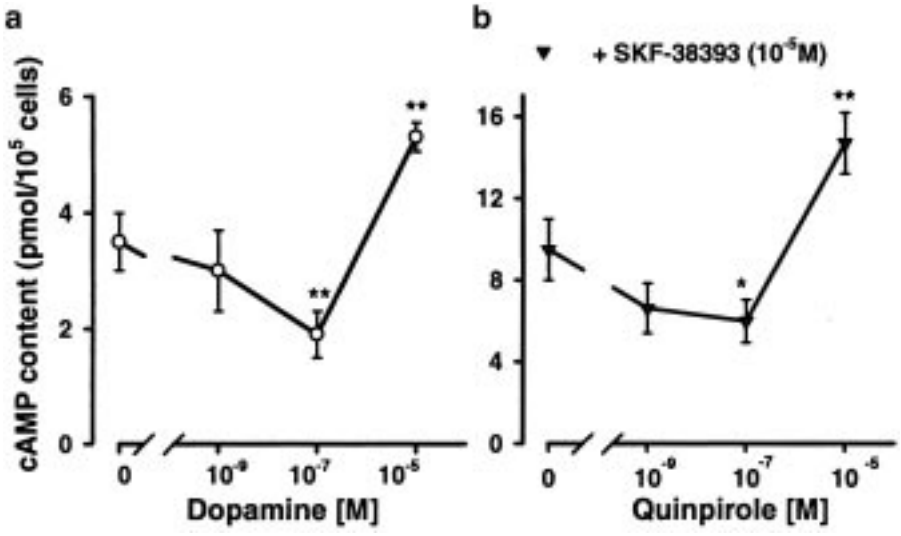

Figure 6 Cell contents of cAMP in primary hypothalamic cell cultures (a) treated with DA; (b) cotreated with SKF-38393 and quinpirole. The asterisks denote significant changes in comparison with vehicle or SKF-38393-treated hypothalamic cultures. Values shown represent mean ± SEM, $n = 4$. *, $P < 0.05$; **, $P < 0.01$.

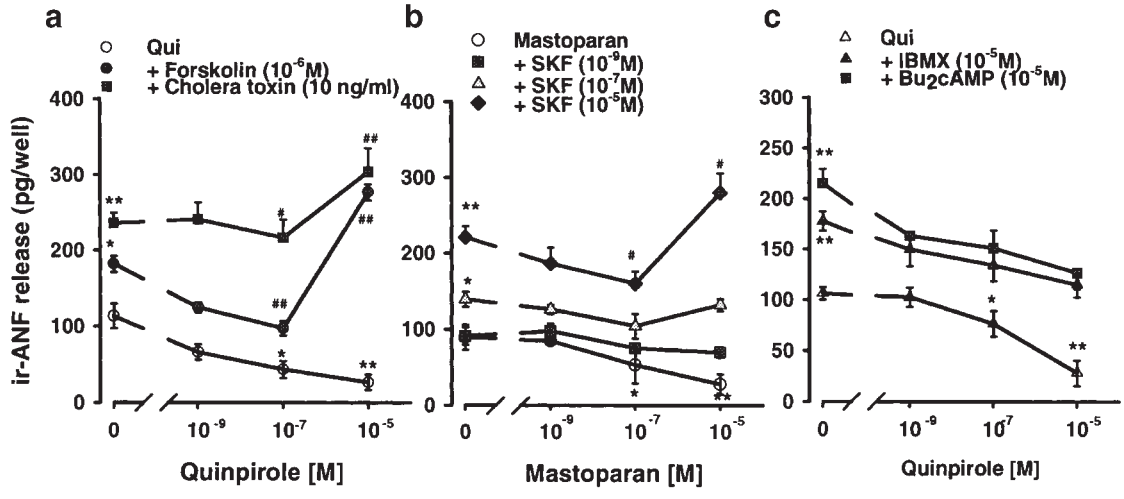

Figure 7 Immunoreactive atrial natriuretic factor (ir-ANF) secretion from cultures treated with (a) quinpirole (Qui, D_2-like agonist) alone or in the presence of forskolin or cholera toxin; (b) mastoparan, SKF-38393 (SKF, D_1-like agonist) alone or in combination; and (c) Qui together with or without 3-isobutyl-l-methyl xanthine (IBMX) or dibutyryl cAMP (Bu_2cAMP). The asterisks and hatches denote values significantly different from cultures treated with vehicle, with corresponding controls and with corresponding doses of SKF-38393 respectively. Values shown represent mean ± SEM, $n = 4$. *, #, $P < 0.05$; **, ##, $P < 0.01$.

to show that ANF neurons possess D_5 and D_2 receptors. This has been achieved through a combined approach of double staining by employing *in situ* hybridization and immunocytochemistry. Taken together with our previous localization of D_1 receptors on hypothalamic ANF neurons,[20] our present findings suggest that ANF neurons share common features with the neurons of the basal ganglia,[12] in that both of them possess D_5, D_1 and D_2 receptors concurrently. Of greater interest is our findings that activation of both D_1-like (D_1 and D_5) and D_2 receptors by their natural ligand, dopamine, yields a biphasic response on ir-ANF release and pro-ANF mRNA expression in a ligand concentration-dependent manner.

However, the concurrent action of dopamine on three subtypes of DA receptors in ANF neurons raises several interesting issues worthy of further discussion. First, in the absence of specific ligands, some of the D_1-like receptor agonist mediated effects should have been attributed, at least in part, to D_5 receptors. Second, the possibility that under certain conditions, the biological action of one of the receptor subtypes may overshadow others also needs to be considered. The above argument is consistent with the notion that D_1 and D_5 receptor subtypes exhibit different biochemical characteristics[35,40,41] and their distributions in the brain differ substantially.[14–17] In this context, it is noteworthy that by comparison, D_5 but not D_1 receptors are predomi-

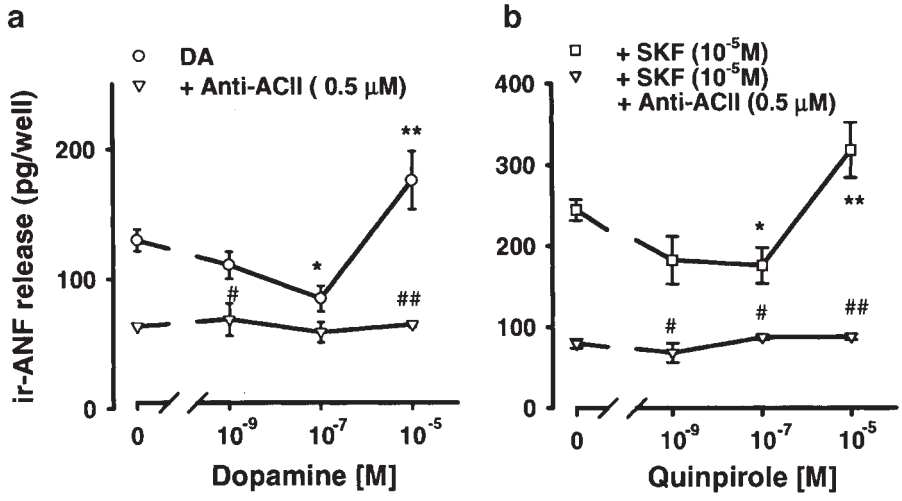

Figure 8 Effects of antisense oligonucleotides against adenylyl cyclase type 2 (Anti-ACII) on immunoreactive atrial natriuretic factor (ir-ANF) release induced by (a) dopamine (DA); and (b) combined treatment of SKF-38393 (SKF, D_1-like agonist) and quinpirole (D_2-like agonist). The asterisks and hatches denote values significantly different from cultures treated with vehicle and with corresponding doses of DA or of quinpirole in conjunction with SKF-38393 respectively. Values shown represent mean ± SEM, $n = 4$. *, #, $P < 0.05$; **, ##, $P < 0.01$.

nant in the rat hypothalamus.[14,15] This notion is also true in our hypothalamic cultures, as confirmed by the double staining approach with semi-quantitative *in situ* hybridization and immunocytochemical studies of D_5 and D_1 receptor mRNA in ANF neurons (data not shown). Taken together, the likelihood that D_5 receptors may play a more important role than their D_1 receptor counterparts in functional modulation of hypothalamic ANF neurons needs to be considered. This argument is consistent with the findings that the suppression of D_5 receptor expression by antisense oligonucleotide supplemented to our cultures effectively abolishes the synergistic effect of dopamine.

The above notion is further supported by findings from our transfection studies. In our CHO cells stably expressing D_5 and D_2 receptors, concurrent stimulation with their respective agonists or with dopamine produces a biphasic change of cAMP levels mimicking that seen in hypothalamic neuron cultures. This is in contrast to the earlier report that under similar conditions, an antagonistic but not synergistic action is seen in transfected CHO cells expressing D_1 and D_2 receptors concurrently when challenged with dopamine.[18] Taken together, the results strongly suggest that the biphasic effect found in our hypothalamic ANF neurons is primarily mediated through D_5 and D_2 receptors, and not D_1 and D_2 receptor interactions.

In our present studies, the hypothalamic cultures were stimulated daily for 4 consecutive days so that the abundance of ir-ANF release could be determined by our RIA in a reproducible and confident manner. Whereas DA-induced biphasic changes of ir-ANF release may be due to alterations in DA receptor abundance following repeated DA exposures, it appears unlikely for the following reasons. First, in vehicle-treated control or drug-naïve cultures subjected to the same 4-day experimental protocol, our in-house findings showed that an acute 15-min stimulation with DA induced a biphasic pattern of cAMP changes similar to that found in cultures treated repeatedly with DA. Secondly, our unpublished observations show that in these drug-naïve cultures, 4-h exposure to a single DA treatment also induced biphasic changes of pro-ANF mRNA abundance mimicking that seen in repeated DA-stimulated cultures. Since changes of cAMP contents and pro-ANF mRNA abundance correlate well with the release of ir-ANF from our hypothalamic cultures,[22,23] it is argued that in our present studies, alterations in the number and density of DA receptors do not contribute significantly to the biphasic responses of our hypothalamic cultures, including that of ir-ANF release. It is interesting to note that the antagonistic interaction of D_5 and D_2 receptors appears to take place when D_2 receptors are modestly activated by low concentrations of D_2 receptor agonist, an event known to induce a weak activation of Gi proteins. However, the synergistic interaction of the two receptors requires high concentrations of D_2 agonist. Under the latter condition, Gi proteins are expected to be activated strongly, and presumably this process would produce excess copies of $\beta\gamma$ subunits of Gi protein origin. The argument that the biphasic effect of D_5 and D_2 receptor interaction is mediated through Gs and Gi proteins is further substantiated by our studies conducted at post-receptor levels. For example, we have shown that a direct activation of the Gs and Gi proteins by cholera toxin and mastoparan in conjunction with D_2 and D_1 receptor agonists, respectively, induces the biphasic release of ir-ANF from our cultures. Furthermore, a similar biphasic effect is also detected when D_2 receptor agonist is used together with forskolin, a pan-adenylyl cyclase activator, but not with the cAMP analogue. All of the above discussion supports the argument that the biochemical events involved in the synergistic

interaction are confined mainly to the level of G proteins and adenylyl cyclases but not beyond the step of cAMP generation.

The underlying mechanism of the subunits of G-protein interaction in our system remains unclear and further exploration is beyond the scope of the present studies. However, the synergism shows similarities to that reported in transfected tumor cell systems. In the latter, the type II isoform of adenylyl cyclase (ACII) is synergistically activated by high levels of $\beta\gamma$ subunits in the presence of Gsα,[42,43] both of them have been co-transfected into the tumor cells of interest. Relevant to this point is the fact that ACII is expressed abundantly in the rat hypothalamus.[44–46] The proposition that the synergistic effect of co-activation of D_5 receptor (coupling to Gs-protein) and D_2 receptor (coupling to Gi) on hypothalamic ANF neurons may involve a similar mechanism therefore needs to be considered. The present findings of the blocking effect of ACII antisense on the biphasic response induced by dopamine and its agonists strongly suggest that both the antagonistic and synergistic effect of D_5 and D_2 receptor interaction are mediated, at least in part, through the modulation of the activity of ACII.

Our present results add a new dimension to the knowledge of D_5 and D_2 receptor interaction in DA-responsive neurons of the central nervous system. They have further substantiated the general principle of synergistic interaction of Gsα and $\beta\gamma$ subunits of Gi proteins of an adenylyl cyclase isoform mediated through dopamine receptor subtypes and its neurophysiological relevance. However, it is also important to point out that our findings have extended the interaction to include that of an antagonism which takes place in the presence of a low or modest availability of the subunits of Gi proteins. Whereas the physiological significance of D_5 and D_2 interaction requires further elucidation, in general, our present findings suggest that at low levels, DA may behave as an inhibitor to these neurons, whilst high availability of the neurotransmitter augments the function of the neurons.

Functional aberrations in DA-producing or DA-sensitive neurons of the central nervous system have been strongly implicated in mental illnesses such as schizophrenia, as many of these patients respond favorably to typical or atypical anti-psychotic drugs.[3,4] Of interest is the fact that the therapeutic efficacy of all of these drugs appears to act by interfering with binding of DA to D_2 or D_5/D_1 receptors in critical regions of the central nervous system.[5–7] Thus, although the full complexity of the regulation of ANF neurons by DA involving D_5 and D_2 receptor interaction requires more investigation, it has considerable implications for explaining the neurobiochemical aberrations which affect neurons in other critical regions of the central nervous system and which underlie various mental illnesses.

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