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Direct dopamine D_2-receptor-mediated modulation of arachidonic acid release in transfected CHO cells without the concomitant administration of a Ca^{2+}-mobilizing agent

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1 In CHO cells transfected with the rat dopamine D_2 receptor (long isoform), administration of dopamine *per se* elicited a concentration-dependent increase in arachidonic acid (AA) release. The maximal effect was 197% of controls ($EC_{50}=25$ nM). The partial D_2 receptor agonist, (−)-(3-hydroxyphenyl)-N-*n*-propylpiperidine [(−)-3-PPP], also induced AA release, but with somewhat lower efficacy (maximal effect: 165%; $EC_{50}=91$ nM).

2 The AA-releasing effect of dopamine was counteracted by pertussis toxin, by the inhibitor of intracellular Ca^{2+} release, 8-(*N N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), by excluding calcium from the medium, by the phospholipase A_2 (PLA_2) inhibitor, quinacrine, and by long-term pretreatment with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In addition, it was antagonized by the D_2 antagonists, raclopride and (−)-sulpiride – but not by (+)-sulpiride – and absent in sham-transfected CHO cells devoid of D_2 receptors.

3 The results obtained contrast to the previous notion that dopamine and other D_2 receptor agonists require the concomitant administration of calcium-mobilizing agents such as ATP, ionophore A-23187 (calcimycin), thrombin, and TRH, to influence AA release from various cell lines.

Keywords: Dopamine; (−)-3-PPP; haloperidol; dopamine D_2 receptors; arachidonic acid; CHO cells; inverse agonism

Introduction

Arachidonic acid (AA) is both an essential component of cell membrane phospholipids and a factor of importance for intracellular transduction as well as extracellular signalling (Axelrod *et al*., 1988; Di Marzo, 1995; Katsuki & Okuda, 1995). The hydrolysis of membrane arachidonoyl-*sn*-2-phospholipids, leading to the liberation of AA from cell membranes, is catalyzed by the enzyme, phospholipase A_2 (PLA_2); activation of PLA_2, which can be obtained by an elevation of intracellular Ca^{2+} in conjunction with an activation of G_i-proteins, thus leads to a marked increase in AA release. In line with this concept, substances that increase intracellular Ca^{2+} concentrations by stimulation of Ca^{2+}-channel activating phosphorylating kinases (12-*O*-tetradecanoylphorbol-13-acetate, TPA), by Ca^{2+} ionophoresis (ionophore 6S-[6α(2S*,3S*),8β(R*),-9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]-undec-2-yl]methyl]-4-benzoxazolecarboxylic acid, A-23187; calcimycin) or *via* G-protein activation (ATP, TRH, thrombin), have all been shown to elevate cellular AA release *in vitro* (Murray-Whelan *et al*., 1995; Di Marzo, 1995; Vial & Piomelli, 1995).

In striatal neurons (Schinelli *et al*., 1994), as well as in CHO cells transfected with cDNA for the dopamine D_2 receptor (Felder *et al*., 1991; Kanterman *et al*., 1991; Piomelli *et al*., 1991; Lahti *et al*., 1992; Di Marzo *et al*., 1993, Di Marzo 1995; Katsuki & Okuda, 1995), activation of D_2 receptors has been shown to induce a G_i-mediated potentiation of the AA response to Ca^{2+}-mobilizing compounds (such as ATP, A-23187, TPA, thrombin, and TRH). On the other hand, D_2 receptor activation has consistently been reported *not* to influence AA release *per se*; thus, simultaneous Ca^{2+} mobilization has been regarded as an indispensable prerequisite for D_2-induced AA release to occur. How D_2 receptors influence PLA_2 activity and subsequent AA release is an issue of considerable importance since this response may prove useful for investigations of D_2 receptor function *in vitro*. In addition, given the possible importance of AA for signal transduction in brain (Axelrod *et al*., 1988; Di Marzo, 1995; Katsuki & Okuda, 1995) and for the pathophysiology of DA-related psychiatric disorders (Gattaz & Brunner, 1996; Horrobin, 1996; Ross *et al*., 1996), D_2/AA interactions may be of physiological importance.

Antagonists at G-protein-coupled receptors such as the dopamine D_2 receptor, have traditionally been assumed to counteract the effect of an agonist, but not to influence intracellular transduction *per se*. During the last years, however, in transfected systems, many compounds previously regarded as neutral antagonists at G-protein-coupled receptors have been shown to exert effects opposite to those induced by an agonist also in the complete absence of agonists, so-called *inverse agonism* (Kenakin, 1996). For example, this phenomenon has been demonstrated in prolactin-secreting GH_4C_1 cells transfected with cDNA for the D_2 receptor. Thus, whereas D_2 activation reduces cyclic AMP formation and prolactin release in this cell line, the D_2 antagonist, haloperidol, has been shown to exert the opposite effects also in the complete absence of D_2 agonists (Nilsson & Eriksson, 1993, Nilsson *et al*., 1996).

The present study had two purposes. Firstly, the possibility of evoking AA release from D_2-transfected CHO cells by means of D_2 receptor stimulation without the concomitant administration of Ca^{2+}-mobilizing compounds was investigated. Secondly, the possibility of studying inverse agonism at dopamine D_2 receptors using the AA response as an

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experimental paradigm was analysed. To summarize, it was demonstrated that DA can induce pertussis toxin-reversible and Ca^{2+}-dependent stimulation of AA release from D_2-transfected CHO cells without the concomitant administration of a Ca^{2+}-mobilizing agent and that D_2 antagonists, such as haloperidol, exert the opposite effect.

Methods

Cell culture

Chinese hamster ovary (CHO) cells (CHO $^{pro-}5$ strain, subclone CHO 10001 fibroblasts) expressing rat dopamine D_2 receptors (long isoform) due to transfection (approximately 500 fmol mg^{-1} protein, [^{3}H]-spiperone binding; 'CHO-L6 cells'; in this paper referred to as CHO-D2 cells), and CHO 10001 cells transfected with the p3C vector only (but with no DA receptor insert) ('CHO-3C cells', serving as 'sham-transfected' controls), were obtained from Dr R. M. Huff (Pharmacia & Upjohn, Kalamazoo, MI, U.S.A; Chio *et al*., 1990; Lahti *et al*., 1992, Chio *et al*., 1993; Lajiness *et al*., 1993). Untransfected CHO cells (CHO-UV 135 fibroblast line) were obtained from the Coriell Cell Repository, National Institute of General Medical Sciences, Camden, NJ, U.S.A. Specific binding of [^{3}H]-spiperone was undetectable in wild type cells. All cell lines were kept frozen in liquid N_2 before being thawed and propagated, without ever reaching confluence, in 80 cm^2 Nunclon flasks (Nunc A/S, Roskilde, Denmark) containing α-MEM (modified Eagle's medium with ribonucleosides and deoxyribonucleosides) (Biochrom KG, Berlin, Germany) + 10% fetal calf serum (FCS) (Sigma Chemical Company, St Louis, MO, U.S.A.) supplemented with penicillin G/streptomycin (PeSt) and l-glutamine (Biochrom) (1 vol% each) (= complete medium) at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Experimental incubations

After trypsinization, stock grown cells were seeded at a density of 1×10^5 cells per ml into 24-well plates (0.5 ml per well). Twenty-two hours later, the growth medium in the wells was replaced by 0.5 ml serum-free complete medium containing 0.5% fatty-acid-free BSA (bovine serum albumin) (Calbiochem, La Jolla, CA, U.S.A.) and 0.5 μCi ml^{-1} [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (6.66–8.88 TBq $mmol^{-1}$) (DuPont NEN®, Wilmington, DE, U.S.A.). After 3 h of preincubation with labelled arachidonic acid, the cells were washed (15 min twice) with EBSS (Earle's balanced salt solution) (Biochrom) containing 0.5% BSA; thereafter the cells were incubated for 30 min with experimental drugs dissolved in EBSS/BSA (incubation volume: 0.5 ml). Dopamine HCl, ATP, indomethacin, TPA and pertussis toxin (PTX) were obtained from Sigma, (−)-(3-hydroxyphenyl)-N-*n*-propylpiperidine HCl [(−)-3-PPP], haloperidol, (−)-raclopride L-tartrate, (+)-, and (−)-sulpiride, nimodipine, quinacrine dihydrochloride and 8-(*N N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) HCl were obtained from RBI. Eticlopride HCl was purchased from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). All drugs were solubilized directly into EBSS/BSA except for haloperidol, sulpiride and nimodipine, which were solubilized in ethanol (maximal final concentrations 0.3%, 0.2% and 0.05%, respectively), and indomethacin, which was solubilized in methanol (maximal final concentration 0.05%). Controls were given the corresponding vehicle. Treatment groups were distributed randomly on the plates. In the concentration-response experiments, four wells on each plate were always given 1 μM DA to ascertain that the AA response was of the same magnitude on different plates. When groups were administered more than one compound, the regimen is indicated in the corresponding figure legend. After experimental incubation, the reactions were terminated by placing the plates on ice. Media were gently aspirated and centrifuged ($300 \times g$; 5 min) and the radioactivity in the supernatant layers (325 μl samples), reflecting [^{3}H]-arachidonic acid release, were counted utilizing standard liquid scintillation spectrometer techniques (LKB Wallac 1215 with quench correction by automatic external standardization).

Calculations and statistics

The method of detecting release of incorporated [^{3}H]-arachidonic acid as an index of living whole cell membrane AA metabolism is well established. Thus, HPLC and TLC analyses have revealed that high amounts (≈ 90–99%) of released radioactivity in the presence of BSA is attributable to free AA (Kanterman *et al*., 1991; Piomelli *et al*., 1991; Muruyama *et al*., 1995). Baseline control levels of d.p.m. (disintegrations per min) values (from wells receiving vehicle) on different plates belonging to experiments performed on the same day generally displayed only small differences. However, as a rule, all d.p.m. values were normalized to per cent of controls (wells given vehicle only) on the same plates in order to eliminate possible errors due to inter-plate variabilities. Data are presented as means ± s.e.mean of normalized d.p.m. values where *n* denotes number of independent determinations from each separate group. Differences between groups were analysed statistically using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) test (> two groups) or Student's *t*-test (two groups). In the monophasic concentration-response experiments (DA, (−)-3-PPP), percentage-normalized data were analysed and fitted by non-linear least-squares regression (the Levenberg-Marquardt algorithm) to a logistic sigmoid curve according to the equation $\text{Effect} = M/\{1 + (C_{50}/[C])\}$, where E = Effect (Y), M = maximum AA value, [C] = drug concentration (X), and C_{50} = the concentration causing half-maximal response, assuming no effect (basal = 100%) at negative infinity. Graphs were generated and parameters computed using the KaleidaGraph™ program (Abelbeck Software, Reading, PA, U.S.A.).

Results

Effects of ATP, DA and ATP + DA on basal AA release from CHO-D2 cells

In CHO-D2 cells, ATP (12 μM) induced a marked increase in AA release (860% of vehicle). The response to ATP was augmented by coadministration of DA (1 μM) (140% of ATP only). DA (1 μM) given *per se* also induced a significant increase in AA release (224% of vehicle) (Figure 1). In sham-transfected CHO-3C cells, DA (1 μM) exerted no significant effect on AA release: 97.2 ± 5.4% *vs* vehicle: 100.0 ± 2.6%, $n = 12$, mean absolute [^{3}H]-AA activity = 1866 d.p.m.). In wild type CHO-UV 135 fibroblasts, DA (1 μM) induced a slight inhibition of AA release: 90.8 ± 1.5% *vs* vehicle: 100.0 ± 1.5%, mean absolute [^{3}H]-AA activity = 3546 d.p.m., $P < 0.01$, $n = 12$.

Effects of growth time on the influence of DA and ATP on AA release from CHO-D2 cells

In CHO-D2 cells seeded as described, the AA response to DA (1 μM) was markedly reduced when the experiment was performed on day two (77.4% effect compared to day one after seeding) and almost abolished on day three (5.5% compared to day one after seeding; 104.8% compared to vehicles = 100%; non-significant) (Figure 2a). Also, the AA response to ATP (12 μM) declined gradually as a function of growth time so that the response to ATP was lower 2 or 3 days after seeding, as compared to 1 day after seeding (83% and 55%, respectively) (Figure 2b). Morphologically, the cells displayed a pattern of complete confluence with tightly packed cells on day three.

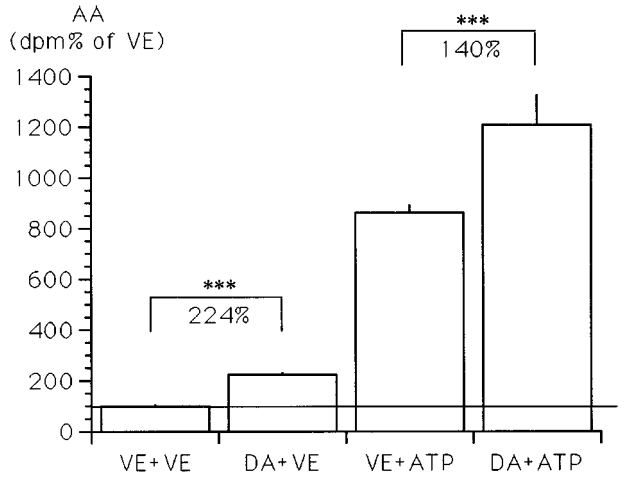

Figure 1 Effects of ATP (12 μM), dopamine (DA) (1 μM), and ATP + DA on basal arachidonic acid (AA) release from CHO-D2 cells. Wells were given vehicle (VE) or DA in a volume of 400 μl; thereafter vehicle or ATP were added in a volume of 100 μl. Concentrations refer to final volumes. Bars represent mean values ± s.e.mean. $n = 12$ in each group. Mean absolute [^{3}H]-AA activity for the control group given vehicle + vehicle only was 1656 d.p.m. ***$P < 0.001$. Vehicle + ATP differed significantly from vehicle + vehicle ($P < 0.001$).

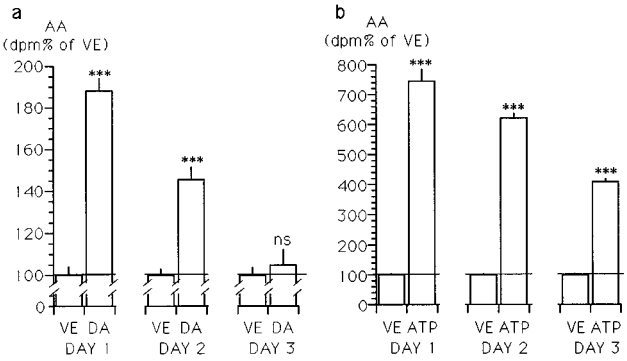

Figure 2 Effects of growth time on the influence of (a) DA (1 μM), and (b). ATP (12 μM) on basal arachidonic acid (AA) release from CHO-D2 cells. Cells were seeded as described in Methods. Experiments were performed after 1, 2 or 3 days of growth. Bars represent mean values ± s.e.mean. $n = 12$ in each group. Mean absolute [^{3}H]-AA activity for the control groups given vehicle only, were 2247, 3882, and 2525 d.p.m. on day one, two, or three, respectively. ***$P < 0.001$, ns = not significant *vs* vehicle. The AA responses to DA on the three different days all differed significantly from each other ($P < 0.001$). The AA response to ATP on day two differed significantly from that on day one ($P < 0.05$); the response on day three differed from that on day one ($P < 0.001$) as well as from that on day two ($P < 0.001$). Note differences in scale.

Concentration-dependent increase of basal AA release from CHO-D2 cells induced by DA and (−)-3-PPP, respectively

Confirming the effect of DA *per se* observed in the previous experiments, DA added without the simultaneous administration of any Ca^{2+}-mobilizing agent induced a concentration-dependent increase in AA release from CHO-D2 cells with a maximal response of 197% (compared to vehicle) from approximately 1 μM (EC_{50}: 25.2 ± 2.6 nM) (Figure 3). The partial D_2 agonist, (−)-3-PPP, also induced a concentration-dependent AA response reaching a maximum of 165% (compared to vehicle) at 1–3 μM (EC_{50}: 90.7 ± 18.3 nM). When (−)-3-PPP was added at concentrations exceeding 3 μM, the AA response was markedly reduced. In sham-transfected CHO-3C cells, (−)-3-PPP (1 μM) exerted no significant effect on AA release: 94.3 ± 2.4% *vs* vehicle: 100.0 ± 2.7%, mean absolute [^{3}H]-AA activity = 1890 d.p.m., $n = 12$. In wild type CHO-UV 135 fibroblasts, (−)-3-PPP (10 μM) induced a slight inhibition of AA release: 90.3 ± 2.2% *vs* vehicle: 100.0 ± 1.3%, mean absolute [^{3}H]-AA activity = 4353 d.p.m.), $P < 0.001$, $n = 12$.

Effects of pertussis toxin, TMB-8, calcium-free medium, quinacrine, long-term TPA pretreatment, nimodipine, and indomethacin on AA responses to D_2 activation in CHO-D2 cells

Various concentrations of DA or (−)-3-PPP given alone induced a concentration-dependent release of AA. The AA responses to DA and (−)-3-PPP were effectively inhibited by pretreatment with PTX (200 ng ml^{-1}). Moreover, the AA

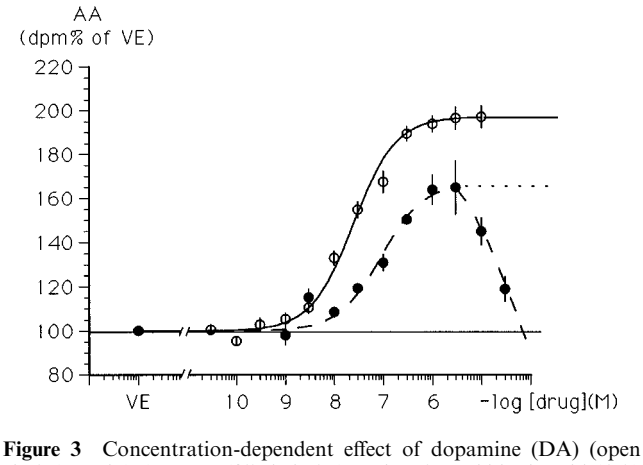

Figure 3 Concentration-dependent effect of dopamine (DA) (open circles), and (−)-3-PPP (filled circles) on basal arachidonic acid (AA) release (means ± s.e.mean) from CHO-D2 cells. Solid curve represents best fit as described in Methods. Maximal effect for DA = 197%; EC_{50} = 25.2 ± 2.6 nM. Left part of broken curve represent similar fit for (−)-3-PPP, yielding a maximal effect of 165% (indicated by hatched line); EC_{50} = 90.7 ± 18.3 nM. $n = 12$ for each concentration of DA or (−)-3-PPP, $n = 144$ for vehicles (VE) in DA experiments, and 108 for vehicles in (−)-3-PPP experiments, respectively. Mean absolute [^{3}H]-AA activity for the control groups given vehicle only, were 1938 d.p.m. (DA curve), and 2208 d.p.m. ((−)-3-PPP curve). $P < 0.01$ for [DA] = 3 nM and for [(−)-3-PPP] = 10 nM; $P < 0.001$ for [(−)-3-PPP] = 3 nM; $P < 0.001$ for all other DA or (−)-3-PPP concentrations > 3 nM compared to vehicles.

response to DA was effectively counteracted by excluding Ca^{2+} from the incubation medium, or by co-incubation with the inhibitor of intracellular Ca^{2+} mobilization, TMB-8 (100 μM) or with the PLA_2 inhibitor, quinacrine (50 μM). Also, it was counteracted by 22 h pretreatment with the PKC-activating phorbol ester, TPA (1 μM). In contrast, pretreatment with the cyclooxygenase inhibitor, indomethacin (4 μM) or coincubation with the L-type Ca^{2+} channel antagonist, nimodipine (500 nM), did not counteract the AA response to DA (Figure 4).

PTX, TMB-8, incubation with calcium-free balanced salt solution or quinacrine inhibited basal AA release *per se* (PTX: 80.1 ± 1.2%, $P<0.001$; TMB-8: 79.0 ± 2.0%, $P<0.001$; Ca^{2+}-free medium: 63.1 ± 6.9%, $P<0.001$; quinacrine: 85.0 ± 3.2%, $P<0.001$, *vs* vehicles; $n=12$ for each group). In contrast, indomethacin, nimodipine or chronic TPA did not influence AA release when administered alone.

Blockade of DA- and (−)-3-PPP-induced AA release in CHO-D2 cells by D_2 antagonists

The AA responses to DA (30 nM) or (−)-3-PPP (30 nM) were effectively inhibited by co-incubation with the D_2 antagonist, raclopride (300 nM). The D_2 antagonist, (−)-sulpiride (100 nM), also effectively antagonized the AA-releasing effects of DA (100 nM) while (+)-sulpiride (100 nM) had no effect (Figure 5). Raclopride (300 nM) exerted a small inhibitory effect on basal AA release *per se* (93.8% of vehicles). In contrast, at this concentration, neither stereoisomer of sulpiride influenced basal AA release.

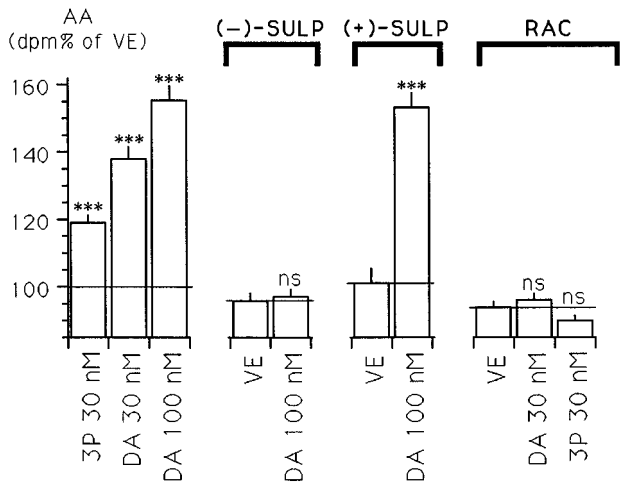

Figure 5 Effects of raclopride (RAC) and the two stereoisomers of sulpiride (SULP) on arachidonic acid (AA) release induced by dopamine (DA) or (−)-3-PPP (3P) in CHO-D2 cells. Raclopride (300 nM) was co-administered with DA (30 nM) or (−)-3-PPP (30 nM) or given with vehicle (VE) only. (−)-sulpiride (100 nM) or (+)-sulpiride (100 nM) were administered together with DA or with vehicle only. Bars represent mean values ± s.e.mean. $n=12$ in each group. Mean absolute [^{3}H]-AA activity for the control group given vehicle only (indicated by 100% baseline) was 2322 d.p.m. ***$P<0.001$, ns = not significant *vs* controls groups given vehicle or vehicle + treatment as indicated. Wells given raclopride + vehicle differed significantly from wells given vehicle only ($P<0.05$). Groups treated with the respective stereoisomer of sulpiride did not differ significantly from the group given vehicle only.

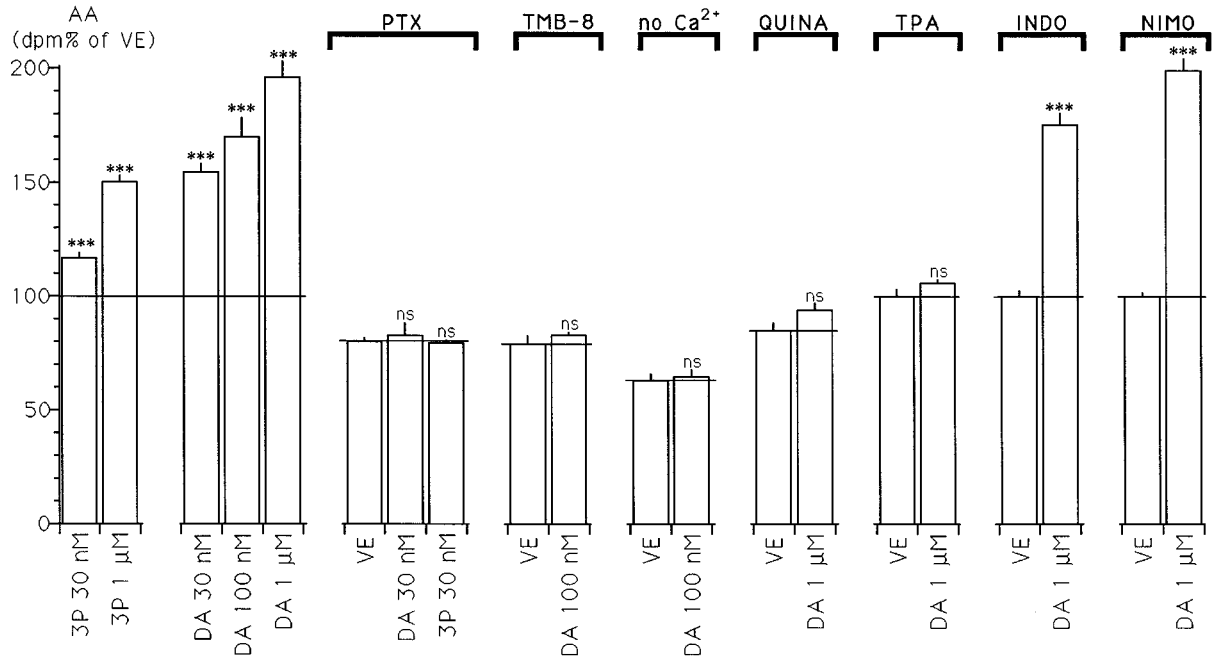

Figure 4 Effects of pertussis toxin (PTX), TMB-8, Ca^{2+}-free experimental incubation ('no Ca^{2+}'), quinacrine (QUINA), long-term TPA, indomethacin (INDO), and nimodipine (NIMO) on the AA response to dopamine (DA) or (−)-3-PPP (3P). PTX (200 ng ml^{-1}) dissolved in complete medium was given as 22 h pretreatment before experimental washes and present also during the AA preincubation period but not during the 30-min experimental incubations. TMB-8 (100 μM) was co-administered with DA. Ca^{2+}-free balanced salt solution was applied containing dissolved DA for the experimental incubation, and used also for the preceding washes (without DA). Quinacrine (50 μM) was co-administered with DA. TPA (1 μM) was given in complete medium as 22 h long-term pretreatment and added also during the 3-h [^{3}H]-AA labelling period. Indomethacin (4 μM) was present during both the 3-h [^{3}H]-AA labelling period and the 30-min experimental incubation. Nimodipine (500 nM) was co-administered with DA. Bars represent mean values ± s.e.mean. $n=12$ for each group. Mean absolute [^{3}H]-AA activity for the control group given vehicle (VE) only (indicated by 100% baseline), was 2139 d.p.m. ***$P<0.001$, ns = not significant *vs* controls groups given vehicle or vehicle + treatment as indicated. PTX + VE differed significantly from wells given VE only ($P<0.001$), TMB-8 + VE differed significantly from VE ($P<0.001$), Ca^{2+}-free VE differed significantly from VE ($P<0.001$), QUINA + VE differed significantly from VE ($P<0.001$). Groups treated with long-term TPA, INDO, or NIMO did not differ significantly from the control group given vehicle only.

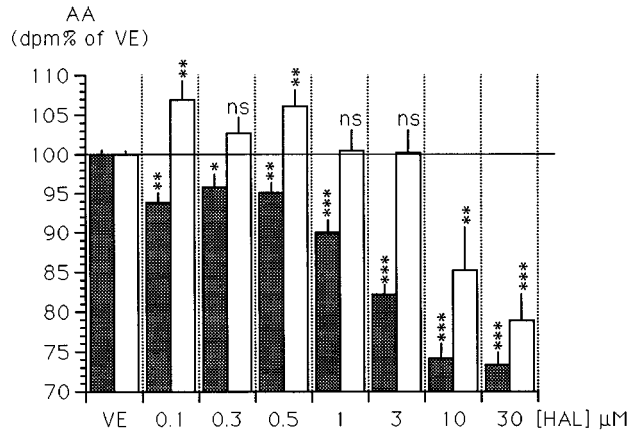

Figure 6 Effects of various concentrations of haloperidol (HAL) on basal arachidonic acid (AA) release from CHO-D2 cells (shaded bars), and from sham-transfected CHO-3C control cells (white bars). Bars represent mean ± s.e.mean. $n=10$ for each concentration of haloperidol, $n=70$ for vehicles (VE). Mean absolute [^{3}H]-AA activity for the control group given vehicle only was 2105 d.p.m. in CHO-D2 cells, and 2851 d.p.m. in CHO-3C cells. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, ns = not significant *vs* vehicles. The effects of haloperidol in CHO-D2 cells differed significantly from the effects obtained in CHO-3C cells at 100 nM ($P<0.001$), 300 nM ($P<0.05$), 500 nM ($P<0.001$), 1 μM ($P<0.001$), 3 μM ($P<0.001$), and 10 μM ($P<0.05$).

Reduction in basal AA release from CHO-D2 cells induced by various concentrations of haloperidol

Haloperidol (from 0.1 μM) *per se* induced a concentration-dependent reduction in AA release from CHO-D2 cells (Figure 6). In CHO-3C cells, haloperidol significantly reduced AA release at high concentrations only (10 μM and 30 μM). In another CHO fibroblast line tested (wild type CHO-UV 135), a response similar to that observed in CHO-3C cells was found. Thus, AA release was reduced at high concentrations (10 and 30 μM) of haloperidol but not influenced by concentrations $\leqslant 3$ μM (not shown).

At 3 μM, both D_2 antagonists, haloperidol and eticlopride, separately induced a significant inhibition of AA release in CHO-D2 cells. However, the effect of eticlopride ($94.5\pm2.3\%$ of vehicles, $P<0.05$) was less pronounced than that of haloperidol ($83.5\pm1.7\%$ of vehicles, $P<0.001$). Moreover, co-incubation of eticlopride at the same concentrations significantly ($P<0.02$) reduced the effect of haloperidol (to $89.3\pm1.4\%$ of vehicles: $100.0\pm1.4\%$, $P<0.001$) ($n=12$ for all groups). Mean absolute [^{3}H]-AA activity for the control group was 2096 d.p.m.

Discussion

The main finding of the present study is that exposure of D_2-transfected CHO cells to DA or to the partial D_2 agonist, (−)-3-PPP, robustly stimulates AA release without requiring the concomitant administration of a Ca^{2+}-mobilizing agent. Supporting that the effect is mediated by D_2 receptors, it was counteracted by co-administration of the D_2 antagonist, raclopride, steroselectively antagonized by sulpiride and absent in sham-transfected CHO cells devoid of D_2 receptors. The EC_{50} for DA-induced AA release was similar to the values previously reported for the effect of DA on A-23187-evoked AA release (25 *vs* 24 and 29 nM) (Di Marzo *et al*., 1993; Kanterman *et al*., 1991).

The effect of both DA and (−)-3-PPP on AA release was abolished by PTX pretreatment, suggesting that is mediated by G-proteins belonging to the G_i/G_o subclasses. The effect was also antagonized by the inhibitor of intracellular calcium mobilization, TMB-8 (Griffiths *et al*., 1994) and by removal of calcium from the incubation medium, hence indicating that it is calcium-dependent (Di Marzo, 1995; Vial & Piomelli 1995). The observation that the L-type calcium channel antagonist, nimodipine, did not influence the effect of DA on AA release in CHO-D2 cells confirms the previous observation that CHO cells do not express this type of Ca^{2+} channel (Perez *et al*., 1993).

It has generally been assumed that G_i-protein activation, as induced for example by D_2 receptors (Lajiness *et al*., 1995), influences AA mobilization only when intracellular levels of calcium are simultaneously elevated (Gupta *et al*., 1990; Glaser *et al*., 1993; Murray-Whelan *et al*., 1995; Vial & Piomelli, 1995). Yet, the present observation that D_2 activation does increase AA levels *per se* is not surprising given the fact that D_2 receptors are reported not only to activate G_i-proteins but also to *increase* intracellular levels of Ca^{2+} in transfected CHO cells (Hayes *et al*., 1992). Nevertheless, the finding that D_2 agonism may elicit AA release in the absence of a Ca^{2+}-mobilizing agent does contrast with many previous reports showing that activation of D_2 receptors (in contrast to e.g. α_1 (Perez *et al*., 1993), α_2 (Jones *et al*., 1991), or 5-HT_{2C} (Berg *et al*., 1996) receptors), does not influence AA release in transfected CHO cells, unless intracellular Ca^{2+} levels are simultaneously elevated by other agents such as A-23187 or ATP (Felder *et al*., 1991; Kanterman *et al*., 1991; Piomelli *et al*., 1991; Lahti *et al*., 1992; Di Marzo, 1995; Katsuki & Okuda, 1995).

It is not unlikely that the extent to which the cells investigated are in the exponential growth phase, with a high proportion of the cells being in the metabolically active cell cycle G1 phase (Acton *et al*., 1979; Jackowski, 1996), may influence the D_2-mediated regulation of AA release. Thus, one factor that may be of importance for the discrepancy between our results and those from other workers with respect to the effect of DA given alone on AA release is the handling of the cells. In the present experiments, frequent passages and low seeding density was rigorously applied in order to keep the cells from reaching even subconfluence. The importance of this factor for the AA response to DA gains support from the present experiment showing that the AA response to DA, but not to ATP, was indeed completely absent in cells that had reached confluence. In line with this concept, we have noted a dramatic reduction in D_2 receptor density (Ekman *et al*., unpublished observation) in transfected CHO cells allowed to reach confluent or subconfluent growth.

Relatively large (nanomolar) amounts of radioactively labelled AA were added to the cells. AA may be converted to prostaglandin E_2 (PGE_2) which in turn may sensitize CHO cells to the AA-releasing effect of D_2 activation (Di Marzo & Piomelli, 1992; Piomelli & Di Marzo, 1993). Thus, the possibility that the formation of PGE_2 from the externally added AA contributes to the D_2-induced AA release should be taken into consideration. However, our finding that administration of the cyclooxygenase inhibitor, indomethacin, at a concentration reported to counteract the conversion of AA to prostaglandins (Di Marzo & Piomelli, 1992; Piomelli & Di Marzo, 1993) did not counteract the AA response to DA indicates that the effect of D_2 activation on AA release is not dependent on an enhanced formation of PGE_2.

An alternative, non-PLA_2-mediated, pathway (Katsuki & Okuda, 1995) leading to the generation of free AA involves the action of diacylglycerol (DAG) lipase and monoacylglycerol

lipase on DAG synthesized by activated phospholipase C. This reaction, however, is not likely to occur in CHO cells (Gupta *et al*., 1990; Jones *et al*., 1991; Lin *et al*., 1992; Vial & Piomelli, 1995), and especially not when no Ca^{2+}-mobilizing agent (such as ATP) is present (Murray-Whelan *et al*., 1995). The assumption that the AA-releasing effects of DA in CHO-D2 cells is mediated by PLA_2 is lent support from the finding that it was effectively antagonized by the specific PLA_2 inhibitor, quinacrine (Jones *et al*., 1991). Also, further support for the notion that the effects of DA on AA release are mediated by the same pathways as when DA is administered in conjunction with Ca^{2+}-mobilizing agents, was obtained by the finding that the effect was abrogated by PKC desensitization induced by long-term phorbol ester pretreatment (Kanterman *et al*., 1991; Felder *et al*., 1991).

We have previously shown that the D_2 antagonist, haloperidol, in the absence of D_2 agonists may exert an influence on prolactin release and cyclic AMP formation in D_2-transfected cells opposite to that induced by agonists (Nilsson and Eriksson, 1993; Nilsson *et al*., 1996). Tentatively, this effect may be the result of so-called inverse agonism (Kenakin, 1996). Recently, the assumption that D_2 receptors can indeed be the subject of inverse agonism has gained support also from studies by others (Giambalvo & Wagner, 1994; Hall & Strange, 1996a, b; Malmberg *et al*., 1996; Hall & Strange, 1997; Kozell & Neve, 1997). Notably, apart from the study by Giambalvo & Wagner (1994) using synaptoneurosomes prepared from rat neural tissue, all these examples of D_2 receptor-mediated inverse agonism have been demonstrated in transfected systems characterized by an over-expression of receptors. Thus, to what extent inverse agonism is a phenomenon of relevance also for constitutively-expressed D_2 receptors remains to be clarified (for discussion, see Nilsson *et al*., 1996; Hall & Strange, 1997).

In the present study, haloperidol alone was shown to induce a concentration-dependent reduction of basal AA release from D_2-transfected cells in concentrations from 0.1 μM. Supporting the assumption that this response is indeed D_2-mediated, at concentrations between 0.1 and 3 μM, haloperidol failed to reduce AA release from sham-transfected CHO-10001 cells or from wild type CHO-UV 135 cells. Also, the effect of haloperidol in CHO-D2 cells was reduced by co-administration of a D_2 antagonist exerting less effect on AA release *per se* (eticlopride), and mimicked by the D_2 antagonists raclopride and (−)-sulpiride (at 1 μM, data not shown). Moreover, the assumption that receptor/G_i-protein complexes in CHO-D2 cells are indeed the subject of considerable spontaneous activity is underpinned by the present observation that administration of PTX alone induced a significant reduction in AA release (Bates *et al*., 1991; Mullaney *et al*., 1996). Of interest here is a recent report by Hall & Strange (1996a) in which a similar PTX regimen as applied in our experiments (200 ng ml^{-1} overnight) revealed a marked precoupling of D_2 receptors in transfected CHO cells.

When interpreting the reduction in AA release observed after administration of haloperidol alone, it should, however, be underlined that the lowest concentration shown to be effective (100 nM), is considerably higher than the K_i value ($\approx$1 nM) for haloperidol *vis-à-vis* the D_2 receptor. This discrepancy between lowest effective concentration and K_i value, respectively, may cast some doubt on the involvement of D_2 receptors in the haloperidol-induced suppression of AA release. However, a lack of correlation between potency with respect to inverse agonism and K_i values has previously been reported for several compounds acting on different G-protein-coupled receptors (Vogel *et al*., 1995; Chiu *et al*., 1996; Griffon *et al*., 1996; Nilsson *et al*., 1996; Malmberg *et al*., 1997; Pregenzer *et al*., 1997). Moreover, it has been suggested that such a correlation should in fact not be expected, given the fact that the fraction of the receptors mediating the response to an inverse agonist are probably in a different state than those studied in radioligand binding experiments (see Kenakin, 1996; Bockaert *et al*., 1997; Chidiac *et al*., 1997; Gürdal *et al*., 1997; Leff *et al*., 1997; Pardo *et al*., 1997; Shea & Linderman, 1997).

Although the effect of haloperidol on AA release should be interpreted with caution, the possibility that it may be due to inverse agonism should thus not be excluded. In contrast, the effect of higher concentrations of haloperidol ($\geqslant$10 μM) on AA release in probably not mediated by D_2 receptors, since these concentrations induced AA release in sham-transfected cells. Tentatively, the previous observation that high concentrations of haloperidol may interact with various Ca^{2+} mobilization mechanisms (Fletcher *et al*., 1994; Koizumi *et al*., 1995; Sczecan & Strumwasser, 1996) may be of importance in this context. Notably, DA and (−)-3-PPP also induced a reduction in AA release from wild type CHO-UV 135 cells when administered at micromolar concentrations.

To conclude, the present findings show that AA release from CHO cells kept in an exponential growth phase is influenced by transfected D_2 receptors in the absence of Ca^{2+}-mobilizing agents. The D_2 receptor antagonist, haloperidol (at concentrations $\geqslant$100 nM), was shown to increase AA release in the absence of agonist, an effect that may be due to inverse agonism.

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