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Apomorphine, dopamine and phenylethylamine reduce the proportion of phosphorylated insulin receptor substrate 1

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Abstract

We tested the ability of dopamine, apomorphine, phenylethylamine and pergolide to inhibit the proliferation of fetal calf serum-stimulated human breast cancer (MCF)-7 cells. While the first three compounds were able to block the proliferation of MCF-7 cells, pergolide failed to do so (up to 100 μM). The inhibitory effect of dopamine, apomorphine and phenylethylamine was also evident in serum-starved insulin-stimulated MCF-7 cells. Apomorphine also inhibited the proliferation of the human oestrogen receptor-negative breast cancer (MDA-MB231) and prostate carcinoma (LNCaP) cell lines. In a second set of experiments, we measured the ability of dopamine, apomorphine, phenylethylamine and pergolide to inhibit the phosphorylation (or increase the dephosphorylation) of the insulin receptor substrate (IRS)-1, a major intracellular substrate of the insulin-like growth factor (IGF)-1 receptor. Dopamine, apomorphine and phenylethylamine all reduced to zero the level of phosphorylated IRS-1 with potencies ranging between 0.01 and 1 μM. Finally, we found that fibroblasts from IRS-1 null (− / −) mice were less sensitive to the anti-proliferative effect of apomorphine compared to fibroblasts from wild type-mice, suggesting that the inhibition of IRS-1 phosphorylation by apomorphine is an important aspect of the activity of this compound.

Keywords: Dopamine; Apomorphine; Phenylethylamine; Pergolide; Insulin receptor substrate; Phosphorylation; Breast cancer cell; (Human)

1. Introduction

The oxidative metabolism of dopamine potentially yields reactive oxygen species (Halliwell and Gutteridge, 1985; Olanow and Tatton, 1999). The formation of reactive oxygen species by dopamine occurs either by auto-oxidation, with the formation of quinone and semiquinone, or by enzymatic reaction through monoamine oxidase, with the formation of H_2O_2, which via the Fenton reaction can react with iron forming the highly reactive $OH^{\cdot}$ radical (Jenner and Olanow, 1996; Olanow and Tatton, 1999). A variety of critical biomolecules can then be damaged by reactive oxygen species, leading to neurodegeneration. The formation of reactive oxygen species has also been implicated in the antiproliferative effect of dopamine against a number of cell lines (Snyder and Friedman, 1998; Graham et al., 1978). Dopamine and its oxidative metabolites may kill cells by causing membrane lipid peroxidation and disruption.

An additional dopamine agonist that has recently been considered for its antiproliferative activity is apomorphine. In 1990, Kondo et al. (1990) demonstrated that apomorphine inhibited the growth of several cell lines. Furthermore, apomorphine treatment resulted in a certain prolongation of the survival time of mice inoculated i.p. with leukaemia P388 cells, although its efficacy did not reach the standard criterion for anti-tumour activity. Apomorphine and dopamine have a strong oxidative potential and their oxidation results in the formation of reactive oxygen species (Liu and Mori, 1993). Therefore, the inhibitory activity has been ascribed to this effect.

Recently, in our laboratory, we have tested whether the production of reactive oxygen species could explain the anti-proliferative effect of dopamine and apomorphine against Chinese hamster ovary (CHO)-K1 cells (Scarselli et al., 1999; Maggio et al., 2000). Cell growth was inhibited by both dopamine and apomorphine; conversely, H_2O_2 (up to 100 μM) did not inhibit cell proliferation. Furthermore, we found that phenylethylamine, a compound closely correlated with dopamine but lacking the catechol ring, inhibited CHO-K1 cell proliferation as well. In an experimental

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setting in which dihydrorhodamine was used as a marker to follow the production of reactive oxygen species, dopamine and apomorphine (at a concentration that inhibited CHO-K1 cell growth) and phenylethylamine (up to 500 μM) did not oxidize the probe. These results suggest that other mechanisms beside the formation of reactive oxygen species could be responsible for the anti-proliferative effect of these compounds.

In this paper, we show that dopamine, apomorphine and phenylethylamine reduced the phosphorylated fraction of the insulin receptor substrate (IRS)-1 (a "docking" molecule which couples the insulin-like growth factor receptor IGF-1 to downstream SH2 proteins; Myers and White, 1995) in human breast cancer (MCF)-7 cells, suggesting a possible role of this effect in the anti-proliferative activity of these compounds.

2. Materials and methods

2.1. Materials

R(−)-Apomorphine hydrochloride and pergolide were purchased from Research Biochemicals International (Natick, MA). Dopamine and phenylethylamine were from Sigma (St. Louis, MO). Insulin was from Calbiochem (La Jolla, CA). All the solutions for the cell culture medium were from Sigma. Tissue culture supplies were from COSTAR.

2.2. Cell cultures

Human oestrogen receptor positive and negative breast cancer MCF-7 and MDA-MB231 cells (from Dr. Antonino Belfiore, Institute of Internal Medicine and Endocrine and Metabolic Diseases, University of Catania School of Medicine, Catania, Italy) were cultured in Dulbecco's Modified Eagle's Medium (DMEM with sodium pyruvate) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The human prostate carcinoma LNCaP cell line was cultured in RPMI-1640 medium, supplemented with 5% fetal calf serum, penicillin and streptomycin. Fibroblasts from IRS-1 null (−/−) and wild-type (+/+) mice (a kind gift from Dr. Jens C. Brüning, Klinik II und Poliklinik für Innere Medizin der Universität zu Köln, Germany) were grown in DMEM containing glucose 4500 mg/l, supplemented with 10% fetal calf serum, penicillin and streptomycin.

All cells were grown in 25- or 75-cm^2 flasks in a water-jacketed incubator at 37 °C, in a 5% CO_2 atmosphere. In proliferation experiments, cells were plated at a density of 10^4 in 12-well plates (22.6-mm diameter) and in a volume of 1 ml/well. After 24 h, cells were exposed to the treatment. After 72 h, viable cells were counted with a hemocytometer.

Cell viability was evaluated by the Trypan blue exclusion test. All inhibitors were prepared and added to the medium in the absence of light to avoid oxidation of the compounds.

2.3. Cell cycle analysis

MCF-7 cells were plated in a 175-cm^2 flask (3×10^5 in 20 ml medium) and stimulated with 100 ng/ml insulin. On the following day, apomorphine (or saline for control) was added at a final concentration of 5 μM and the cells incubated for an additional 72 h. On the day of the assay, cells were washed twice with phosphate-buffered saline (PBS) and lifted from the flask with trypsin. Then they were spun down at $2000 \times g$ for 5 min and resuspended in 5 ml PBS (this step was repeated three times). After the third centrifugation, cells were resuspended in PBS containing 100 mg/ml RNAse A (Boehringer) and 50 mg/ml propidium iodide (Sigma) and stained for 30 min at room temperature. Cells were analysed with a FACSort flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed with CELLOQuest software programs (Becton-Dickinson) to determine percentages of cells in the different phases of the cell cycle.

2.4. Western blot analysis of IRS-1 and its phosphorylated form

For signal transduction analysis experiments, MCF-7 cells were grown for 3 days in 60 mm Petri dishes in DMEM/fetal calf serum 1%. The day of the assay, medium was aspirated, and replaced with serum-free DMEM containing 100 ng/ml of insulin. After a preincubation of 2 h in the presence of insulin, graded concentrations of the test substance were added to the medium. At the end of the experiment, cell proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 5 mM EDTA, 1 mM Na_3VO_4, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol and 0.2% Triton X-100). Cell lysates were stored at −80 °C until processed for the immunoprecipitation assay. Cell extracts were centrifuged for 15 min at $15{,}000 \times g$ at 4 °C to remove insoluble material. The supernatants were incubated overnight at 4 °C with a mouse anti-IRS-1 antibody (Upstate Biotechnology, Lake Placid, NY). The complexes were precipitated with anti-mouse immunoglobulin G-agarose beads for 1 h at 4 °C. After three washes in 500 μl of lysis buffer, precipitates were eluted from the beads by boiling in 20 μl of elution buffer (20% glycerol, 10% β-mercaptoethanol, 4.6% sodium dodecyl sulfate, 0.125 M Tris, pH 6.8), subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, and then transferred onto a Hybond enhanced chemoluminescence (ECL) nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) for 45 min at a fixed current of 150 mA. Membranes were blocked in 200 mM NaCl, 50 mM Tris pH 7.5, 1% bovine serum albumin overnight at 4 °C.

After the blocking procedure, membranes were incubated for 2 h at room temperature with an anti-IRS-1 antibody (to evaluate IRS-1 expression) in antibody buffer (50 mM Tris

Table 1
Antiproliferative effect of dopamine agonists and phenylethylamine against MCF-7 cells

	FCS (IC_{50} μM)	Insulin (IC_{50} μM)
Dopamine	59.3 ± 6.5	64.4 ± 5.2
Apomorphine	1.3 ± 0.3	2.26 ± 0.21
Phenylethylamine	61.2 ± 6.3	54.1 ± 4.1
Pergolide	>100	>100

The growth of MCF-7 cells was stimulated by 10% fetal calf serum (FCS) or 100 ng/ml insulin. Inhibitors were added 24 h after plating, and 3 days later viable cells were counted. Apomorphine was used up to 25 μM, while the other compounds were up to 100 μM.

pH 7.6, 200 mM NaCl, 0.05% Tween-20, 1% bovine serum albumin). Blots were then washed three times for 10 min in washing buffer (50 mM Tris pH 7.6, 200 mM NaCl, 0.05% Tween-20), followed by incubation for 45 min with a specific secondary antibody conjugated to horseradish peroxidase (Amersham Life Science) diluted 1:4000 in the blocking buffer at room temperature. After three washes of 10 min, blots were incubated with ECL reagents, in accordance with the manufacturer's instructions (Amersham Life Science), for 1 min before exposure to Kodak X-Omat AR film (3–30 min).

To analyse IRS-1 phosphorylation, the anti-IRS-1 antibody was removed by stripping the blots twice for 30 min in 10 ml of glycine-stripping buffer (0.2 M glycine, 0.1% sodium dodecyl sulfate, 1% Tween 20; pH 2.2). The blots were then washed three times for 5 min in Tris-buffered saline Tween washing buffer (10 mM Tris pH 8, 0.15 N NaCl and 0.2% Tween 20) and blocked at 4 ° C overnight in 2% bovine serum albumin/Tris-buffered saline blocking buffer (2% bovine serum albumin, 10 mM Tris pH 8, 0.15N NaCl). Finally, the membranes were incubated with a mouse monoclonal anti-phosphotyrosine antibody (a gift of Dr. Oreste Segatto, Institute Regina Elena, Roma, Italy), and processed as previously described.

3. Results

We tested the ability of dopamine, apomorphine, pergolide and phenylethylamine to inhibit the proliferation of MCF-7 cells grown in the presence of 10% fetal calf serum. As shown in Table 1, dopamine, apomorphine and phenylethylamine inhibited MCF-7 cell growth; among them apomorphine was the most active, with an IC_{50} of 1.3 ± 0.3 μM. After 3 days of incubation in the presence of these compounds, the morphology of the cells changed substantially, from a star-like to a round shape. Pergolide, up to 100 μM, did not inhibit MCF-7 cell proliferation.

The anti-proliferative effect of dopamine, apomorphine and phenylethylamine was tested also against MCF-7 cells grown in the presence of insulin alone. Initially, we titrated the amount of insulin needed to sustain MCF-7 cell growth similar to that of cells stimulated with 10% fetal calf serum. As shown in Fig. 1A, at a concentration of 50 ng/ml insulin, the rate of growth was not significantly different from that of cells grown in the presence of 10% fetal calf serum, and the growth rate reached a plateau at 100 ng/ml insulin. Dopamine, apomorphine and phenylethylamine all inhibited the growth of serum-starved insulin (100 ng/ml)-stimulated MCF-7 cells (Table 1, Fig. 1B). Again, pergolide up to 100 μM did not inhibit MCF-7 cell proliferation. (Table 1).

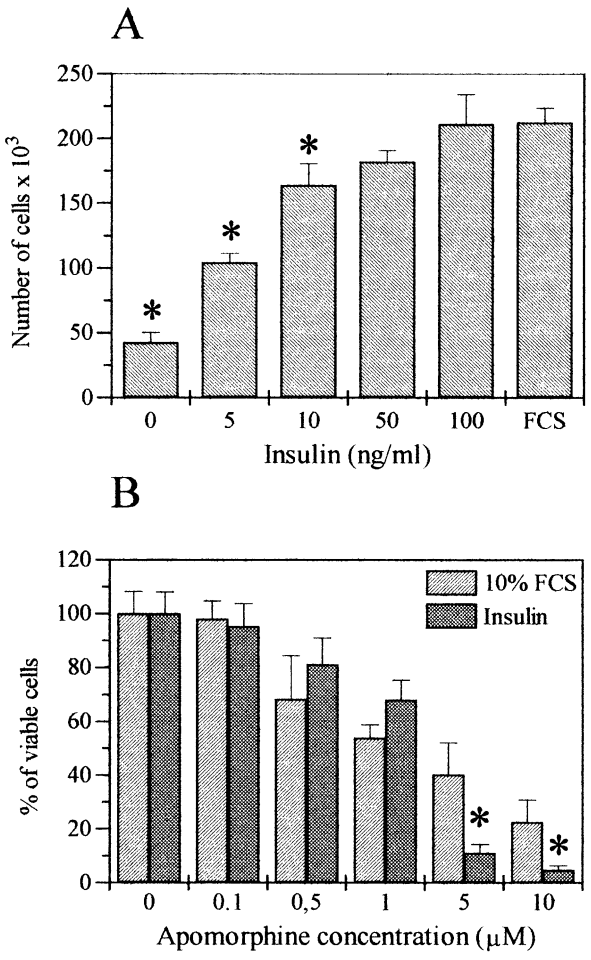

Fig. 1. Panel A: dose–response curve of insulin-stimulated growth of serum-starved MCF-7 cells. Cells were seeded in 12-well plates at a density of 10^4 cells/well and allowed to grow in the presence of 10% fetal calf serum or the indicated concentration of insulin. After 4 days, the cells were counted with a hemocytometer. The numbers on the ordinate refer to the actual number of cells in each well after 3 days of incubation. Panel B: dose–response curve for the anti-proliferative effect of apomorphine against MCF-7 cells grown in the presence of insulin or fetal calf serum. Cells were plated as described above and grown in the presence of 10% fetal calf serum or 100 ng/ml insulin. The day after plating, the cells were challenged with the indicated concentration of apomorphine. After an additional 3 days, the cells were counted with a hemocytometer, and cell viability was assessed with Trypan blue. The 100% level represents the number of viable cells counted on the fourth day after plating in wells with no addition of apomorphine. Vertical bars are means ± S.E. * Significantly different from fetal calf serum (FCS) ($P < 0.05$, ANOVA + Fisher test).

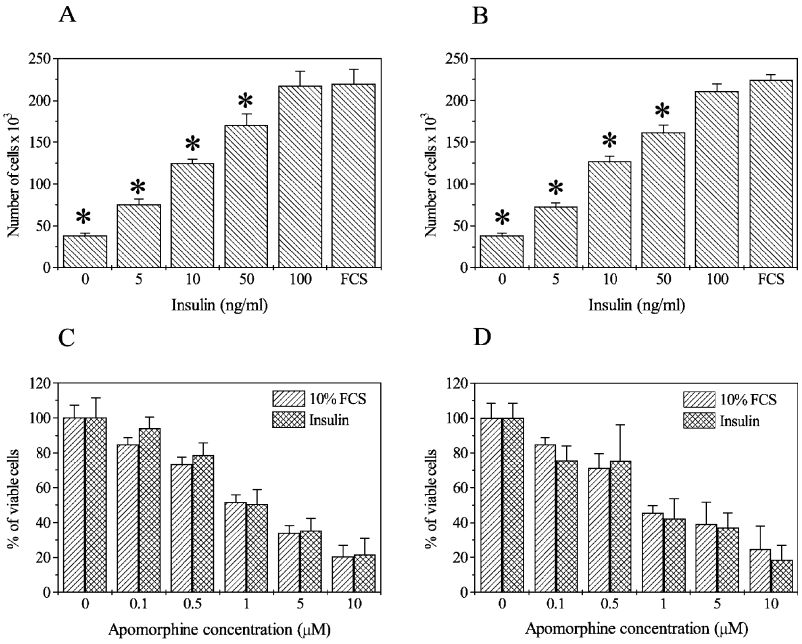

Fig. 2. Panels A and B: concentration–response curve of insulin-stimulated proliferation of serum-starved MDA-MB231 (A) and LNCaP (B) cells. Cells were plated in 12-well plates at a density of 10^4 cell/well and grown in the presence of 10% fetal calf serum or the indicated concentration of insulin. After 4 days, the cells were counted with a hemocytometer. Panels C and D: concentration–response curve for the anti-proliferative effect of apomorphine in MDA-MB231 (C) and LNCaP cells (D) grown in the presence of 10% fetal calf serum or 100 ng/ml insulin. At 24 h after plating, cells were challenged with the indicated concentrations of apomorphine. After an additional 72 h, cells were counted with a hemocytometer and cell viability was assessed with Trypan blue. The 100% level represents the number of viable cells counted on the fourth day of incubation in the absence of apomorphine. Vertical bars are means ± S.E. * Significantly different from fetal calf serum (FCS) ($P < 0.05$, ANOVA + Fisher test).

Apomorphine was also tested in other two cell lines, the MDA-MB231 (a human oestrogen receptor negative breast cancer) and LNCaP (a prostate carcinoma). Preliminary experiments indicated that these two cell lines grew in the presence of insulin (in serum-starved condition) as well as MCF-7; at a concentration of 100 ng/ml, the rate of growth was not significantly different from that of cells grown in the presence of 10% fetal calf serum (Fig. 2, panels A and B). Apomorphine had an anti-proliferative effect on both the MDA-MB231 and LNCaP cell line (Fig. 2, panels C and D), the potency of inhibition was similar when cells were grown either in 10% fetal calf serum or 100 ng/ml of insulin.

To investigate the effect of apomorphine on the cell cycle of serum-starved insulin (100 ng/ml)-stimulated MCF-7 cells, we studied the change in the intensity of DNA fluorescence using flow cytometric analysis of propidium-iodide-stained purified cell nuclei after 72 h of exposure to apomorphine 5 μM. The column graph of Fig. 3 shows that apomorphine induced a modest but significant increase in the percentage of cells in the G2/M phase (from 21.8 ± 4.5 to 35.3 ± 3.6) while it did not modify the percentage of cells in the S and G0/G1 phases. The percentage of cells with hypodiploid DNA was very small in control MCF-7 cells (3.4 ± 1.2) and was not modified by 5 μM apomorphine (3.1 ± 1.3). The effect of apomorphine on the cell cycle was reversible after replacement of the agent with fresh medium (data not shown).

In a second set of experiments, we tested the ability of dopamine, apomorphine, phenylethylamine and pergolide to modify the amount of the phosphorylated fraction of IRS-1. MCF-7 cells were grown in 1% fetal calf serum and on the day of the assay they were stimulated for two h with DMEM/insulin (100 ng/ml) solution. Afterwards, an increasing concentration of dopamine, apomorphine, phenylethylamine and

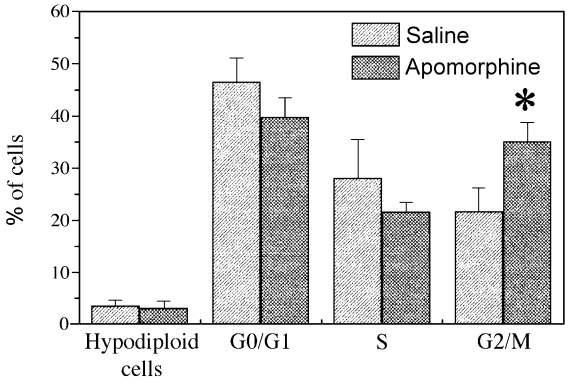

Fig. 3. Effect of apomorphine (5 μM) on DNA content of serum-starved insulin-stimulated MCF-7 cells, as determined by flow cytometry following staining with propidium iodide. Each pair of columns represent the percentage of cells in each phase of the cell cycle. Values are means ± S.E. of four experiments. * Significantly different from saline ($P < 0.05$, Student's *t*-test for unpaired data).

pergolide was added to the medium for two additional h. The reaction was stopped by the addition of lysis buffer. The total amount of IRS-1 and the phosphorylated fraction were detected by immunobloting with an anti-IRS-1 and an anti-phosphotyrosine antibody, respectively. As shown in the panels A, B, C and D of Fig. 4, dopamine, apomorphine, phenylethylamine and pergolide did not substantially alter the total amount of IRS-1 during the 2-h incubation. Conversely, all the drugs except pergolide reduced to zero the fraction of phosphorylated IRS-1. Pergolide up to 100 μM did not modify the amount of phosphorylated IRS-1 (Fig. 4, panel D). Apomorphine, in line with its higher potency to inhibit MCF -7 cell proliferation, reduced the phosphorylation of IRS-1 with a potency ranging between 0.01 and 0.1 μM. The poten-

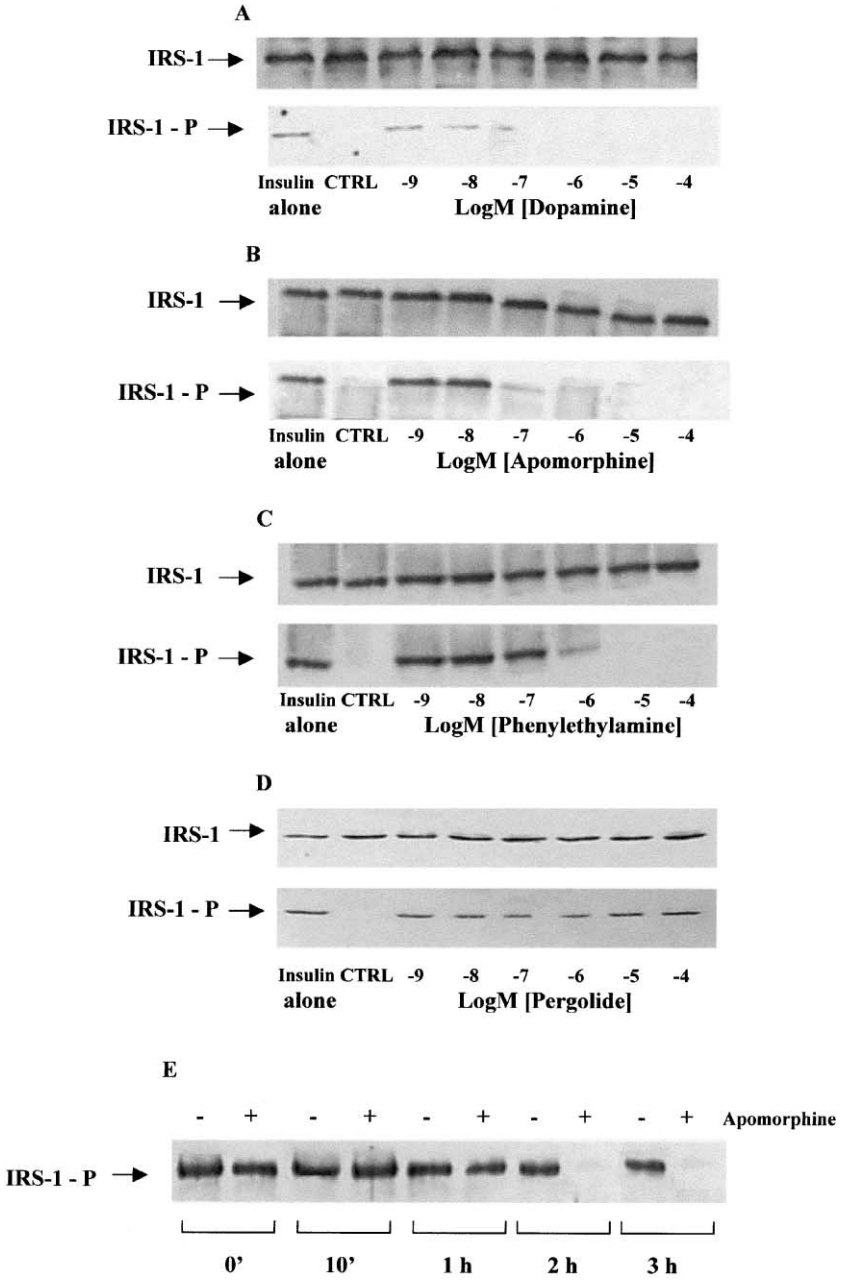

Fig. 4. Concentration-dependent effects of dopamine (A), apomorphine (B), phenylethylamine (C), and pergolide (D) on IRS-1 protein levels and IRS-1 protein phosphorylation in MCF-7 cells. MCF-7 cells were plated in 60-mm Petri dishes and grown in DMEM/fetal calf serum 1%. At the day of the assay, the DMEM/fetal calf serum solution was replaced with DMEM/insulin (100 ng/ml) to stimulate IRS-1 phosphorylation, and after 2 h the inhibitor was added. After an additional 2 h of incubation, the reaction was stopped by the addition of lysis buffer. In the absence of insulin (CTRL) IRS-1 phosphorylation was undetectable. Panel E: Time course of the reduction of insulin-stimulated IRS-1 phosphorylation by apomorphine. MCF-7 cells were treated as above and after the preincubation with DMEM/insulin (100 ng/ml) solution, they were challenged at different times with 10 μM apomorphine. The − and + signs represent untreated and apomorphine-treated cells, respectively. Each blot is representative of three or four replicates.

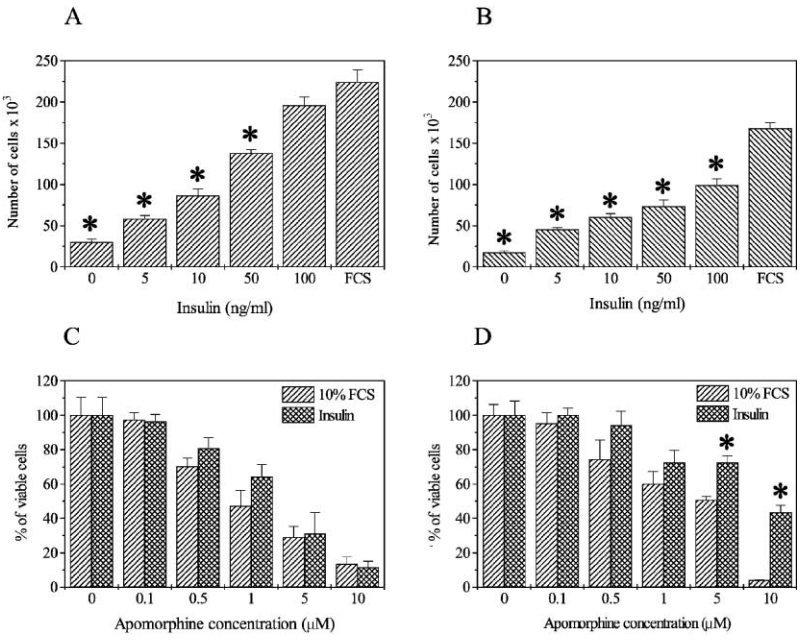

Fig. 5. Panels A and B: concentration–response curve for insulin-stimulated proliferation of serum-starved fibroblasts from wild-type (A) and IRS-1 null (B) mice. Cells (10^4 /well) were grown either in the presence of 10% fetal calf serum or the indicated concentration of insulin. After 4 days, cells were counted with a hemocytometer. Panels C and D: concentration–response curve for the antiproliferative effect of apomorphine in fibroblast cultures from wild-type (C) and IRS-1 null (D) mice grown either in the presence of 10% fetal calf serum or 100 ng/ml insulin. Cells were challenged with the indicated concentrations of apomorphine. After 72 h, cells were counted in a hemocytometer and cell viability was assessed by means of the Trypan blue method. The 100% level represents the number of viable cells counted on the fourth day of culture in the absence of apomorphine. Vertical bars are means ± S.E. * Significantly different from fetal calf serum (FCS) ($P < 0.05$, ANOVA + Fisher test).

cy of dopamine and phenylethylamine ranged between 0.1 and 1 μM. For all the compounds, the effect increased sharply in the range of two concentrations.

We also measured the time course of the reduction of IRS-1 phosphorylation in the presence of apomorphine at a concentration of 1 μM. The effect of apomorphine was maximal after 2 h of incubation, at which time no phosphorylated IRS-1 was detectable in the blot (Fig. 4, panel E).

We performed an additional experiment with fibroblasts from IRS-1 null (− / −) mice in comparison with fibroblasts from wild-type (+/+) mice. In serum-starved fibroblasts from wild-type mice, insulin induced a concentration-dependent increase in cell growth (Fig. 5A). At 100 ng/ml, the growth rate was not different from that of cells stimulated with 10% fetal calf serum. Also in fibroblasts from IRS-1 null mice insulin induced a concentration-dependent increase in cell growth, but at the concentration of 100 ng/ml the growth rate was significantly lower than in cells treated with 10% fetal calf serum (Fig. 5B). Furthermore, in the presence of fetal calf serum, fibroblasts from wild-type mice grew faster than fibroblasts from IRS-1 null mice. Apomorphine inhibited the growth of fibroblasts from wild-type mice in the presence of fetal calf serum or insulin with comparable potency (Fig. 5C). In contrast, apomorphine (up to the concentration of 10 μM) did not inhibit the growth of insulin-stimulated fibroblasts from IRS-1 null mice to the same extent as it did in fibroblasts grown in the presence of fetal calf serum (Fig. 5D).

4. Discussion

Several reports have shown that dopamine agonists have an antiproliferative effect on different cell lines. While in some cases the antiproliferative effect is mediated by the stimulation of dopamine receptors (Schrell et al., 1990; Drewett et al., 1993), an oxidative mechanism has been hypothesized in other cases (Snyder and Friedman, 1998; Graham et al., 1978). A large body of evidence shows that dopamine can produce reactive oxygen species (Halliwell and Gutteridge, 1985; Olanow and Tatton, 1999).

We have recently addressed the question of whether or not the production of reactive oxygen species by dopamine and dopamine-related compounds could be responsible for their anti-proliferative effect (Maggio et al., 2000). To study this effect, we chose the CHO-K1 cell model because of the particular resistance of this cell line to reactive oxygen species, as shown by the fact that H_2O_2 (up to 5 mM) does not alter their proliferation (Sanchez-Gongora et al., 1996). Dopamine and its receptor agonists, apomorphine and bromocriptine, inhibited the growth of CHO-K1 cells. Interestingly, phenylethylamine, an analogue of dopamine lacking the catechol ring, inhibited cell proliferation as well. We also showed that, under the same conditions, dopamine, apomorphine and phenylethylamine did not produce reactive oxygen species.

In this study, we extended our analysis to the MCF-7 cell line. Initially, we tested the ability of dopamine, apomor-

phine, pergolide and phenylethylamine to inhibit the proliferation of MCF-7 cells grown in the presence of 10% fetal calf serum. Dopamine, apomorphine and phenylethylamine inhibited MCF-7 cell growth; among them, apomorphine was the most potent. Pergolide, up to 100 μM, did not inhibit MCF-7 cell proliferation, indicating that the effect of the other two dopamine agonists (dopamine and apomorphine) was not receptor mediated. This was also demonstrated by the observation that R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) and sulpiride, dopamine D1 and D2 receptor antagonists, respectively, did not modify the anti-proliferative effect of apomorphine, a dopamine D1 and D2 receptor agonist (data not shown).

Dopamine, apomorphine and phenylethylamine inhibited also the growth of serum-starved insulin stimulated MCF-7 cells. Again, pergolide up to 100 μM did not inhibit MCF-7 cell proliferation. While the IC_{50}'s were similar to those observed when cells were grown in the presence of 10% fetal calf serum, there was a substantial difference in inhibition. In cells grown in the presence of fetal calf serum, the inhibition was linearly correlated with the concentration of the inhibitor; in cells grown in the presence of insulin, the inhibition increased sharply at a certain concentration. This effect can be clearly appreciated in the graph of Fig. 1B: when the concentration of apomorphine increased from 1 to 5 μM, the inhibition of serum-starved insulin-stimulated MCF-7 cells decreased from 68 ± 7% to 11 ± 3%. This difference could be explained by the fact that fetal calf serum contains several growth factors with redundant effects on MCF-7 cell proliferation; if the intracellular path of one of them is inhibited, the others could still support growth.

Analysis of the cell cycle of serum-starved insulin-stimulated MCF-7 showed that apomorphine induced a significant accumulation of these cells in the G2/M phase. This effect, which is not sufficient to consider apomorphine as a phase-specific inhibitor of the cell cycle, was previously seen with CHO-K1 cells (Scarselli et al., 1999) and it is similar to that seen with the commonly used tyrosine kinase inhibitor genistein (Matsukawa et al., 1993).

The anti-proliferative effect of apomorphine was also tested on other two cell lines: the MDA-MB231 (a human oestrogen receptor-negative breast cancer) and LNCaP (a prostate carcinoma). The inhibitory potency of apomorphine was similar in serum-starved insulin-stimulated cells and in cells grown in the presence of fetal calf serum. These results, together with data obtained with CHO-K1, African green monkey kidney cells (COS-7), rat glial tumor (C6) and rat pheochromocytoma (PC12) cells (Maggio et al., 2000), indicate that apomorphine is an anti-proliferative compound with a wide spectrum of activity.

As previously described (Maggio et al., 2000), phenylethylamine does not possess the oxidative potential of dopamine and apomorphine, though it completely blocks the proliferation of MCF-7 cells. If we hypothesize that a common mechanism underlines the anti-proliferative effects of dopamine, apomorphine and phenylethylamine, we have to exclude the production of reactive oxygen species as the causative mechanism of the inhibition of MCF-7 cell growth.

The inhibitory effects of dopamine, apomorphine and phenylethylamine against serum-starved insulin-stimulated MCF-7 cells prompted us to explore the possibility that these compounds could interfere with the phosphorylation of IRS-1, a major substrate of insulin and IGF-1 receptors (Myers and White, 1995) which is responsible for the proliferative effect of insulin in these cells. Therefore, in a second set of experiments, we tested the ability of dopamine, apomorphine, phenylethylamine and pergolide to reduce the fraction of phosphorylated IRS-1. Dopamine, apomorphine, phenylethylamine and pergolide did not substantially alter the total amount of IRS-1. Conversely, all the drugs except for pergolide reduced to zero the phosphorylated fraction of IRS-1. Pergolide up to 100 μM did not modify the amount of phosphorylated IRS-1. Apomorphine, in line with its higher potency to inhibit MCF-7 cell proliferation, reduced the fraction of phosphorylated IRS-1 with a higher potency than dopamine and phenylethylamine. For all the compounds, the inhibition increased sharply in the range of two concentrations. This is in agreement with results for the anti-proliferative effect.

The potency of dopamine, apomorphine and phenylethylamine for the anti-proliferative effect and the reduction of the phosphorylated fraction of IRS-1 were slightly different. If we suppose that the anti-proliferative effect of these compounds on MCF-7 cells grown in the presence of insulin is due to the reduction of the fraction of phosphorylated IRS-1, it remains to be explained why the potency to exert an anti-proliferative effect and to decrease the fraction of phosphorylated IRS-1 was different. One possibility could be that these compounds undergo auto-oxidation and enzymatic destruction. The anti-proliferative effect was measured during 3 days of exposure to the inhibitors, and therefore it is very likely that processes of drug metabolism and auto-oxidation could slowly have reduced their inhibitory activity. Dopamine and phenylethylamine, for example, are substrates of monoamine oxidase and may have been slowly inactivated by this enzyme. Consistent with this interpretation, deprenyl, an irreversible monoamine oxidase type B inhibitor, potentiates the anti-proliferative effect of dopamine in SH-SY5Y cells (Lai and Yu, 1997). Furthermore, dopamine and apomorphine are transformed into 3-*O*-methyldopamine and apocodeine, respectively, by catechol-*O*-methyltransferase. This enzyme is a ubiquitous enzyme that is crucial to the metabolism of carcinogenic catechols and catecholamines and is present in MCF-7 cells (Xie et al., 1999).

Dopamine and apomorphine are auto-oxidized into melanin-like compounds, and therefore after 3 days of incubation, most of the original drug would have disappeared (Graham et al., 1978; Gassen et al., 1996). This assertion is not in contrast with the fact that previously, using dihydrorhodamine, we did not detect the oxidation of dopamine and apomorphine (Maggio et al., 2000). At the concentration of

these two compounds that inhibited cell growth, it is likely that the antioxidant activity of the cells is enough to prevent a detectable oxidation of the probe.

We also measured the time course of the reduction of phosphorylated IRS-1 in the presence of apomorphine at a concentration of 10 μM. The effect of apomorphine was maximal after 2 h of incubation, at which time no phosphorylated IRS-1 was detectable in the blot (Fig. 2, panel E). This suggests that IRS-1 is actively dephosphorylated and that when the balance between the phosphorylation and dephosphorylation processes is in favour of the latter (due to the presence of the inhibitor), the fraction of phosphorylated IRS-1 quickly decreases to zero. From our results, we cannot predict whether the final effect that we observed in the blot is due to an inhibition of the phosphorylation of IRS-1 or to an increase in its dephosphorylation.

In order to see how essential IRS-1 was for the induction of proliferation and IRS-1 phosphorylation was for the inhibition elicited by apomorphine, we tested fibroblasts from IRS-1 null (−/−) mice in comparison with fibroblasts form wild-type (+/+) mice. Despite the absence of IRS-1, insulin induced the growth of IRS-1 null fibroblasts, though the rate of growth was significantly lower than that for wild-type fibroblasts. Similar results were obtained with fetal calf serum. The data obtained with insulin are in line with the data of Brüning et al. (1997), who first developed this experimental model and described the reduction of the growth response to IGF-1 stimulation in these cells. Furthermore, in fibroblasts from null mice insulin up to 100 ng/ml stimulated cell growth by about 60% compared to fetal calf serum. This implies that IRS-1 has an important, though not essential, role in the growth of these cells, and that the lack of it reduces their proliferation.

In fibroblasts from wild type-mice, the anti-proliferative effect of apomorphine was not significantly different among cells stimulated with insulin or fetal calf serum. In contrast, in fibroblasts from IRS-1 null mice the anti-proliferative effect of apomorphine was reduced when cells were grown in the presence of insulin compared to cells stimulated with fetal calf serum. If we assume that the inhibition of IRS-1 phosphorylation by apomorphine is crucial for the anti-proliferative effect of this compound, we have to suppose that the contribution of phophorylated IRS-1 to the growth of the wild-type fibroblasts in the presence of insulin is relevant. In contrast, in IRS-1 null fibroblasts, insulin could stimulate proliferation by alternative pathways that are less sensitive to the inhibitory effect of apomorphine.

Furthermore, since the inhibitory potency of apomorphine was similar among wild-type and IRS-1 null fibroblasts grown in the presence of fetal calf serum, it is likely that the overall contribution of phosphorylated IRS-1 to growth is small due the limited amount of insulin present in fetal calf serum. Nevertheless, the sensitivity to the inhibitory effect of apomorphine of the alternative pathways, which are stimulated by the multiple growth factors present in the serum, should be comparable to that of insulin-stimulated IRS-1 phophorylation.

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