We have investigated the signaling of OX1 receptors to cell death using Chinese hamster ovary cells as a model system. OX1 receptor stimulation with orexin-A caused a delayed cell death independently of cytosolic Ca\textsuperscript{2+} elevation. The classical mitogen-activated protein kinase (MAPK) pathways, ERK and p38, were strongly activated by orexin-A. p38 was essential for induction of cell death, whereas the ERK pathway appeared protective. A pathway implicated in the p38-mediated cell death, activation of p53, did not mediate the cell death, as there was no stabilization of p53 or increase in p53-dependent transcriptional activity, and dominant-negative p53 constructs did not inhibit cell demise. Under basal conditions, orexin-A-induced cell death was associated with compact chromatin condensation and it required \textit{de novo} gene transcription and protein synthesis, the classical hallmarks of programmed (apoptotic) cell death. However, though the pan-caspase inhibitor \textit{N}-benzoxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethyl ketone (Z-VAD-fmk) fully inhibited the caspase activity, it did not rescue the cells from orexin-A-induced death. In the presence of Z-VAD-fmk, orexin-A-induced cell death was still dependent on p38 and \textit{de novo} protein synthesis, but it no longer required gene transcription. Thus, caspase inhibition causes activation of alternative, gene transcription-independent death pathway. In summary, the present study points out mechanisms for orexin receptor-mediated cell death and adds to our general understanding of the role of G-protein-coupled receptor signaling in cell death by suggesting a pathway from G-protein-coupled receptors to cell death via p38 mitogen- and stress-activated protein kinase independent of p53 and caspase activation.

There is a growing interest in the ability of G-protein-coupled receptors (GPCRs)\textsuperscript{3} to affect synaptic plasticity and cell differentiation as well as cell growth, death, and survival (reviewed in Refs. 1–3). However, the mechanisms of GPCR signaling in these processes are poorly characterized. The pathways engaged seem to include classical GPCR signals like second messenger-dependent activation of protein kinase A and C but also (for GPCRs) more novel signal transducers like small G-proteins (of Ras and Rho families), PI3K (phosphatidylinositol 3-kinase) and non-receptor tyrosine kinases (e.g. Src).

In this study we have focused on the signaling of a novel GPCR, OX1 orexin receptor. Little is known about the intracellular signaling of OX1 receptor and its cognate receptor, OX2 receptor. Orexin receptor-expressing neuronal, endocrine, and muscle cells often seem to be excited by orexins, an effect that may be related to activation of Ca\textsuperscript{2+} or non-selective cation channels (reviewed in Refs. 4 and 5). Recently, orexin receptors have been suggested to regulate cell plasticity. ERK (extracellular signal-regulated kinase) is strongly activated in CHO cells heterologously expressing OX1 receptors (4, 6, 7). In the hippocampus, orexin receptors regulate synaptic plasticity (8). Long-lasting activation of OX\textsubscript{1} receptors leads to cell death in native transformed colon cancer and neuroblastoma cell lines and in recombinant immortal CHO cells (9). However, the signal pathways leading to this response are unclear except for an apparent involvement of caspases in the demise of colon cancer cell lines. We have therefore in this study investigated the mechanistic basis of the OX\textsubscript{1} receptor-mediated cell death in CHO cells. The results demonstrate that stimulation of OX\textsubscript{1} receptors with orexin-A induces activation of p38 mitogen- and stress-activated kinase(s) (MAPK and SAP-kinase(s)), which in its turn projects to delayed death of CHO cells via caspase-dependent and -independent pathways. The findings are important for our understanding of how GPCRs can take part in the control of cell fate determination in normal and transformed tissue.

**MATERIALS AND METHODS**

**Cell Culture**—CHO-OX\textsubscript{1} cells, expressing human OX\textsubscript{1} receptors, have been described before (10). They were cultured in Ham’s F-12 medium (Invitrogen, Paisley, UK) supplemented with 100 units/ml penicillin G (Sigma), 80 units/ml streptomycin (Sigma), 400 \( \mu \text{g}/\text{ml} \) Genetin (G418; Invitrogen) and 10% (v/v) fetal calf serum (Invitrogen) at 37 °C in 5% CO\textsubscript{2} in an air-ventilated humidified incubator in 260-ml plastic culture flasks (75 cm\textsuperscript{2} bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany). For different assays, the cells were cultured

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\( ^{2} \) The abbreviations used are: GPCR, G-protein-coupled receptor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester; BrdUrd, 5-bromouracil; CHO, Chinese hamster ovary; curcumin, diferuloylmethane; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; PKC, protein kinase C.

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\( ^{3} \) P13K, phosphatidylinositol 3-kinase; SAPK, stress-activated protein kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole; SB-334867, 1-(2-benzylbenzoxazol-5-y)-3-[3-(1,5)naphthyridin-4-yl]-urea hydrochloride; SP600125, 1,9-pyrazoloanthrone; U0126, 1,2-diamino-3,2-dicyano-1,4-phenylene-5-iminomercapto butadiene; Z-VAD-fmk, \textit{N}-benzoxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethyl ketone; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; PKC, protein kinase C.
on multiwell plates (24- or 96-well), on plastic culture dishes (inner diameter, 32, 52, or 82 mm), or on uncoated circular glass coverslips (diameter, 13 or 25 mm).

Chemicals—GF109203X (2-(1-[3-dimethylaminopropyl]-1H-indol-3-yl)-3-(1H-indol-3-yl)-maleimide), ionomycin, LY294002 (2-[4-morpholinyl]-8-phenyl-1-[4H]-benzopyran-4-one), and U0126 (1,4-diamino-2,3-dicyano-1,4-bis-[3-aminophenylmercaptojbutadiene) were from Calbiochem, and actinomycin D, aprotinin, BrdUrd (5-bromo-2'-deoxyuridine), curcumin (diferuloylmethane), leupeptin, and wortmannin were from Tocris Cookson (Bristol, UK), and thapsigargin was from RBI (Natick, MA). SB-334867 (1-[2-methylbenzoxazol-6-yl]-3-[1,5]naphthyridin-4-yl-urea) was a generous gift from Dr. Moshe Oren (Department of Molecular Cell Biology, The Weizmann Institute, Rehovot, Israel), pcDNA3-ΔN-p53 (human p53 lacking amino acids 1–39; Ref. 12) was from Dr. Pierre Hainaut (International Agency for Research on Cancer (WHO), Lyon, France), and pC53–175 and pC53–273 (p53 with point mutations S175H and S273H, respectively) and p53-EGFP (p53-driven expression of GFP (green fluorescent protein) by 13X p53-binding domain of p21-Waf in front of enhanced green fluorescent protein; Ref. 13) were from Drs. Klas Wi man and Vladimir Bykov (Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institute, Stockholm, Sweden). pEGFP-hM1 (human muscarinic receptor C-terminally fused to GFP) was produced within the laboratory.

Expression Vectors—pCMV-p53-wt (mouse wild-type p53) and pCMV-p53-DD (mouse p53 lacking amino acids 15–30; Ref. 11) were from Dr. Moshe Oren (Department of Molecular Cell Biology, The Weizmann Institute, Rehovot, Israel), pcDNA3-ΔN-p53 (human p53 lacking amino acids 1–39; Ref. 12) was from Dr. Pierre Hainaut (International Agency for Research on Cancer (WHO), Lyon, France), and pC53–175 and pC53–273 (p53 with point mutations S175H and S273H, respectively) and p53-EGFP (p53-driven expression of GFP (green fluorescent protein) by 13X p53-binding domain of p21-Waf in front of enhanced green fluorescent protein; Ref. 13) were from Drs. Klas Wi man and Vladimir Bykov (Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institute, Stockholm, Sweden). pEGFP-hM1 (human muscarinic receptor C-terminally fused to GFP) was produced within the laboratory.

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Transfection—Transfection of the cells was performed to introduce dominant-negative p53 or different GFP-expressing plasmids to the cells. The dishes/plates were washed with PBS, and the cells were transfected in Opti-MEM (Invitrogen) using Lipofectamine (Invitrogen). After 5 h, this medium was replaced with fresh Ham’s F-12 medium with all the usual supplements (see above). Transfection efficiency was 40–70% as determined using expression of green fluorescent protein and function of transfected proteins (e.g. receptors and cyclic nucleotide phosphodiesterases; data not shown). The total amount of DNA was kept the same in all the transfections using empty plasmids.

Different Staining Protocols for Dead and Living Cells—Cell viability was assayed using MTT, which stains viable mitochondria blue, and morphological observation. Spectrophotometric MTT was used to assay the total number of cells alive. This method, although displaying linear a relationship between the absorbance and cell viability, cannot thus distinguish between treatments that reduce the number of cells alive by killing them or by simply inhabitating the cell growth. Therefore, spectrophotometric MTT was supplemented with manual counting of stained and non-stained cells. The data from these two methods were consistent in this study (see e.g. Fig. 18), and the data have therefore been pooled for figures other than Fig. 1. The cells were stained with 0.5 mg/ml MTT for 1 h in the cell culture medium. For spectrophotometric MTT, the crystals formed were dissolved in 65 mM HCl in isopropl alcohol, and the specific absorbance was measured at 570 nm (the nonspecific absorbance, to be subtracted, was measured at 690 nm; Lab-systems Multiskan MS). For manual counting, the stained cells were photographed with color reversal film and the numbers of stained and non-stained cells were counted from the photographs.

For nuclear staining (Figs. 1, 5, and 8), the cells were stained with 5 μg/ml Hoechst 33342 for 30 min, and the fluorescent condensed and non-condensed nuclei counted from digital images (Zeiss Axioplan 2 fluorescence microscope with a CCD camera). In some experiments the dead/dying cells were stained with 2.5 μg/ml propidium iodide (for 5–30 min), and the fluorescent cells were counted from the images (Zeiss Axioplan 2 or Olympus FV1000 confocal microscope) and compared with the total number of cells from Hoechst staining. The results with propidium iodide were in accordance with the results from MTT staining and are thus not presented.

All the compounds were added as a single initial dose. All the inhibitors were added 30 min before the addition of orexin-A or thapsigargin. When no inhibition was seen with an inhibitor, another protocol, designed to circumvent possible breakdown, was tested. In this the inhibitors, in addition to the initial dose, were added once a day in half of the original concentration. This did not improve the efficacy of any of the inhibitors.

BrdUrd Incorporation for Cell Growth—The cells growing on 24-well plates were labeled with 10 μM BrdUrd for 12–16 h. The medium was discarded, and the cells were fixed with 4% paraformdehyde, washed with PBS, and 2 mM HCl was added for 20 min at room temperature. The cells were rinsed again, blocked with PBS containing 2% (w/v) bovine serum albumin and 0.1% Triton X-100, and stained with mouse primary anti-BrdUrd IgG (B2531 or B8434; 1:1000; Sigma). After a wash, the cells were labeled with the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG F(ab′)2 fragments (F2772; 1:500; Sigma). All the cells (dead and alive) were stained using Hoechst 33342 as described above. The cells were digitally photographed under fluorescence microscope, and the dividing cell (BrdUrd fluorescein isothiocyanate) and total cell numbers (Hoechst 33342) were manually counted from the images.

Flow Cytometric Analysis of Cell Cycle—The attached cells on 82-mm plates were harvested using PBS + 0.02% (w/v) EDTA, pooled with the spontaneously detached cells (= medium from the dishes), spun down, and washed with PBS, after which they were fixed with ice-cold 70% ethanol for 30 min. Ethanol was carefully washed away with PBS, and 1 mg/ml RNase was added for 5 min in room temperature before staining with 50 μg/ml propidium iodide. The intensity distribution was analyzed using standard protocols of BD Biosciences FACScalibur instrument. A minimum of 10,000 cells was run from each sample.

Western Blotting—The experiments were performed essentially as described in Refs. 7 and 14. Briefly, the cells, cultured on 24-mm plates, were rapidly washed with ice cold PBS supplemented with phosphatase and protease inhibitors (1 mM Na3 orthovanadate, 10 mM NaF, 250 μM p-nitrophenol phosphate, leupeptin) and lysed. The protein concentration was determined (DC protein assay; Bio-Rad) and adjusted accordingly. The samples were diluted in Laemmli buffer (50 mM Tris-HCl, pH 6.8, + 100 mM dithiothreitol, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.1% (w/v) bromphenol blue), boiled, separated by SDS-PAGE, and transferred to methanol-soaked polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were
Mechanism of OX₁ Receptor-mediated Cell Death

Primary antibody incubation was done in TPBS in +4 °C overnight and secondary antibody incubation in TPBS-milk at room temperature for 1 h after two 20-min washes with TPBS. Specific protein detection was performed using antibodies specific for phosphorylated (active) states of MAP/SAP-kinases (MAPK/SAPK) and total MAPK/SAPK and p53. The following primary antibodies were used: for active ERK1/2, rabbit polyclonal anti-pThr183-pTyr185-ERK1/2 antibody (V8031; 1:5000; Promega, Madison, WI); for total ERK, rabbit polyclonal anti-total MAPK (ERK1/2) antibody (NA 9340; 1:2000; Oncogene Research Products, San Diego, CA); for active p38 MAPK/SAPK, rabbit polyclonal anti-dually phosphorylated-p38 antibody (V1211; 1:1000; Promega); for total p38 MAPK/SAPK, rabbit polyclonal anti-total p38 antibody (#9212; 1:1000; Cell Signaling Technology, Beverly, MA); for active c-Jun N-terminal kinases (JNKs), mouse monoclonal anti-pThr183-pTyr185-JNK antibody (sc-6254; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA); for total JNK, mouse monoclonal anti-total JNK antibody (654285; 1:1000; Pharmingen); and for p53, mouse monoclonal anti-p53 antibodies (sc-99 or sc-126; 1:1000; Santa Cruz Biotechnology).
was counted for each sample. Student’s non-paired two-tailed t test was used for pairwise comparisons and analysis of variance, followed by Tukey’s post-hoc test, for multiple comparisons. Significances: ns, not significant; p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001. Significances are indicated only for the data where the results are not self-evident. In the figures, mean ± S.E. is given. SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA) was used for non-linear curve-fitting.

RESULTS

**OX1 Receptor Activation Induces Death of CHO Cells**—To avoid complex effects from growth factors and other mediators present in serum, CHO-K1 cells, stably transfected with the OX1 orexin receptor, were prior to the exposure to other treatments serum-starved for 24 h. CHO cells were surprisingly resistant to the removal of serum as such, after 72–96 h, 94% of the serum-starved cells were cycling (see below). Orexin-A markedly reduced the fraction of the 12-h period. Cell cycle analysis also showed that serum-starved cells incorporated BrdUrd in a manner (Fig. 1A). Cell growth was markedly, but far from completely, reduced as evaluated from manual counting of cell numbers and MTT staining (data not shown). 30–40% of the serum-starved cells incorporated BrdUrd in a 12-h period. Cell cycle analysis also showed that serum-starved cells were cycling (see below). Orexin-A markedly reduced the fraction of viable cells in a concentration- (EC_{50} ≈ 20 nM) and time-dependent manner (Fig. 1, A and B). The response to orexin-A was the same independently of whether the cell death was evaluated using spectrophotometric measurement of MTT staining (Fig. 1B, empty symbols) or manual counting of live and total numbers of cells (Fig. 1B, filled symbols). Thus, orexin-A indeed induced cell death and not inhibition of cell growth.

This response to orexin-A was fully blocked with 10 μM OX1 receptor antagonist, SB-334867 (data not shown), confirming the involvement of OX1 receptors. A similar slow decrease in cell viability as with orexin-A was observed with thapsigargin (Fig. 1A), a compound that persistently elevates cytosolic Ca^{2+} and induces cell death in most cell types, probably via mitochondrial and endoplasmic reticulum stress. Presence of serum reversed the negative effects of both orexin-A and thapsigargin on cell viability (Fig. 1C). Nuclear staining with Hoechst 33352 revealed sharp and compact chromatin condensation, suggesting a caspase-dependent classical apoptotic mechanism of cell death (reviewed in Refs. 15 and 16) for both treatments (Fig. 1D). The pan-caspase inhibitor Z-VAD-fmk (25 μM), fully inhibited chromatin condensation (Fig. 1D), confirming the engagement of caspases in this response.

**OX1 Receptor-mediated Cell Death Requires de Novo Gene Transcription and Protein Synthesis**—The protein synthesis inhibitors anisomycin and cycloheximide concentration-dependently inhibited orexin-A-induced loss of cell viability (data not shown). A full reversal was obtained at 100 nM anisomycin or 1 μM cycloheximide, concentrations which themselves did not induce cell death (Fig. 2A). To investigate whether the orexin-A-induced cell death also required de novo synthesis of miRNA, we treated CHO cells with an inhibitor of gene transcription, actinomycin D. At the concentration of 10 nM, actinomycin D only weakly affected cell viability, but it fully reversed orexin-A-induced cell death (Fig. 2B). We thus conclude that de novo gene transcription and protein synthesis are required for the cell death induced by OX1 receptor activation under these conditions.

**OX1 Receptor-mediated Cell Death Occurs Independently of Ca^{2+} Elevation, PKC, and PI3K**—Our previous studies with CHO cells have indicated that OX1 receptor stimulation induces strong Ca^{2+} elevation via Ca^{2+} influx and release (10, 17) and activation of ERK, PKC, and PI3K (4, 7, 14). Chelation of intracellular Ca^{2+} with 1 μM BAPTA-AM significantly improved cell viability in response to thapsigargin, as expected, but did not affect orexin-A-mediated cell death (Fig. 3A). Thus, orexin-A-induced cell death does not seem to be mediated by persistent Ca^{2+} elevation. For inhibition of PI3K and PKC we used wortmannin (100 nM), LY294002 (10 μM), and GF109203X (1 μM), which at these concentrations should be effective inhibitors of the respective enzymes in CHO cells (7, 18–22). The PI3K inhibitors wortmannin and LY294002 and the PKC inhibitor GF109203X were without an effect (Fig. 3B), suggesting that PI3K and PKC pathways are not...
**FIGURE 4.** Activation MAPK/SAPK pathways upon OX1 receptor stimulation with orexin-A. A and B, representative Western blots of the active species. C and D, time curves of the phospho-MAPK/SAPK immunoreactivity. The phospho-data are expressed in correlation to the corresponding non-phosphorylated MAPK/SAPK species. ns, not significant; *, *p < 0.05; **, *p < 0.01; ***, *p < 0.001.

**FIGURE 5.** Involvement of different MAPK/SAPK pathways in orexin-A- and thapsigargin-mediated cell death (72 h). A, the effect of inhibition of ERK activation (1–10 μM U0126). The data from 1 and 10 μM U0126 were pooled together, since the effect was the same. B, the effects of inhibition of JNK (curcin, SP600125) and p38 MAPK/SAPK (SB203580). Only significant results are marked. C, the effect of SB203580 on chromatin condensation. D, the concentration-response curve for cell rescue by SB203580. IC50 = 246 ± 85 nM, Hill coefficient = 0.71 ± 0.18. ns, not significant; ***, *p < 0.001; ctrl, control.
(centrally) involved in the cell death induced by orexin-A or thapsigargin.

OX1 Receptor Induces Cell Death via the p38 MAPK/SAPK Pathway—Activation of MAPK pathways was assessed with Western blotting using antibodies specific for active (phosphorylated) MAPK species. p44/42 (ERK1/2) and p38 MAPKs were rapidly and strongly activated by orexin-A (Fig. 4, A, C, and D). In contrast to phosphorylation of p38, which was a long lasting response, the ERK response had much decayed already at 3 h to fully cease at 6–24 h (Fig. 4, A and C). CHO cells also expressed 46- and 55-kDa isoforms of JNKs. Cell stress induced by UV light or hyperosmolarity (500 mM sucrose) led to a rapid and strong phosphorylation of both distinguishable species (data not shown). However, OX1 receptor stimulation only induced a very weak phosphorylation of the 46-kDa species alone (Fig. 4, B and E). Correlation of the phosphorylated MAPK/SAPK species to actin gave qualitatively and quantitatively similar results as correlation to the respective non-phosphotylated MAPK/SAPK species (data not shown).

Each MAPK/SAPK pathway was then targeted using specific inhibitors. Inhibitor of MKK1 (mitogen-activated kinase kinase 1) activation (and thus the ERK pathway), U0126 (1–10 \( \mu M \)), sensitized CHO cells to orexin-A-induced cell death (Fig. 5A), suggesting that activation (or the basal activity) of the ERK pathways is protective against OX1 receptor-mediated cell death. The JNK inhibitor SP600125 (1 \( \mu M \)) and the putative JNK inhibitor curcumin (10 \( \mu M \)) did not affect orexin- or thapsigargin-mediated cell death (Fig. 5B). In contrast, the p38 inhibitor SB203580 (10 \( \mu M \)) fully inhibited orexin-A-mediated, but not thapsigargin-mediated, cell death (Fig. 5, B–D). This effect was concentration-dependent; half-maximal inhibition of the cell death was obtained with ~250 nm SB203580 (Fig. 5D).

We further assessed the number of cells in each phase of cell cycle using flow cytometry. As indicated by the MTT assays and BrdUrd incorporation, removal of serum did not induce cell cycle arrest (see above under “OX1 Receptor Activation Induces Death of CHO Cells”). This was confirmed in the flow cytometric assay. Also, no significant difference was seen in the distribution of viable control cells and cells treated with orexin-A, SB203580, or both in the different phases of cell cycle (Fig. 6). These data also confirm that SB203580 does not save CHO cells from orexin-A-mediated cell death by inducing cell cycle arrest. This is supported by the fact that SB203580, alone or together with orexin-A, did not inhibit basal BrdUrd incorporation (data not shown).

Thus, orexin-A-mediated activation of p38 MAPK/SAPK pathway causes programmed cell death. This is in agreement with other studies showing that p38 MAPK/SAPKs affect, via different targets, for instance, cellular response to stress (23). One of the central targets of p38 MAPK/SAPK-mediated cell death/cell cycle arrest is p53. p53 protein can be phosphorylated and therewith stabilized and/or functionally activated by p38 MAPK/SAPKs. We observed that p53 protein level was slightly, although non-significantly, elevated in orexin-A-stimulated CHO cells (Fig. 7, A and B). To investigate the transcriptional activity of p53, we transfected the cells transiently with a p53-dependent reporter plasmid (p53-GFP). We could not see any specific increase in GFP fluorescence upon 48- or 72-h incubation with orexin-A (data not shown).
suggesting that there was no activation of the transcriptional activity of p53. We also transiently transfected the cells with truncated p53 constructs, known to be the most efficient dominant-negative constructs (11, 12, 24). No effect on orexin-A-induced cell death was seen in the spectrophotometric MTT assay (Fig. 7C). We also tested two commonly used dominant-negative p53 point mutants, S175H and S273H, neither of which inhibit cell demise (data not shown). Since only 40–70% of the cells are transfected, we wanted to make sure that the activity of these constructs was not masked by low transfection efficiency. We thus cotransfected the cells with GFP-plasmids together with mutant p53 constructs to specifically identify the transfected cells. Cells transfected with GFP- (cytosolic GFP) and CAAX-GFP- (lipid-linked membrane fluorescence) expressing plasmids lost their GFP fluorescence upon cell death; however, cells transfected with pEGFP-hM1 plasmid (transmembrane protein-linked GFP) retained the GFP fluorescence even during cell death. Thus, the latter plasmid was used in transfections together with mutant p53 and the cell death evaluated by microscopy of GFP fluorescence and MTT. However, the results were not improved; also GFP-positive cells showed cell death (data not shown). We thus conclude that p53 is not involved as a downstream effector of p38 MAPK/SAPK in OX1 receptor signaling to cell death. This may be related to the previously published reports on defective p53 signaling in CHO cells (see “Discussion”).

Caspase-dependent and -independent Signaling to OX1 Receptor-mediated Cell Death—As indicated above (Fig. 1D), caspase activity upon orexin-A stimulation leads to sharp and compact chromatin condensation, a hallmark of classical apoptosis. The pan-caspase inhibitor Z-VAD-fmk (gray bars) are compared. A, quantitation of Hoechst-stained cells with condensed (apoptotic) and non-condensed nuclei as compared with the counting of non-stained (dead) and stained (viable) cells in MTT assay. The significances for Hoechst are calculated against the corresponding basal values (white for white bars etc.). B–D, the dependence of cell death on p38 MAPK/SAPK (10 μM SB203580, B), protein synthesis (1 μM cycloheximide; C) and gene transcription (10 nM actinomycin D; D). ns, not significant; **, p < 0.01.

FIGURE 8. OX1 receptor activation can induce both caspase-dependent and -independent cell death (72 h). Control cells (white bars) and cells treated concomitantly with the pan-caspase inhibitor Z-VAD-fmk (gray bars) are compared. A, quantitation of Hoechst-stained cells with condensed (apoptotic) and non-condensed nuclei as compared with the counting of non-stained (dead) and stained (viable) cells in MTT assay. The significances for Hoechst are calculated against the corresponding basal values (white for white bars etc.). B–D, the dependence of cell death on p38 MAPK/SAPK (10 μM SB203580, B), protein synthesis (1 μM cycloheximide; C) and gene transcription (10 nM actinomycin D; D). ns, not significant; **, p < 0.01.

8A). Thus, it appears that although OX1 receptors can induce caspase activity, they can also trigger cell death via caspase-independent mechanisms. In the presence of Z-VAD-fmk, OX1 receptor-mediated cell death was still reversed by SB203580 (p38 MAPK/SAPK inhibition; Fig. 8B) and cycloheximide (protein synthesis inhibition; Fig. 8C). However, inhibition of gene transcription with actinomycin D no longer rescued the cells (Fig. 8D), suggesting that inhibition of caspasers alters the cell death execution.

DISCUSSION

In the present study, we show induction of cell death upon activation of OX1 orexin receptors and partially characterize the signal pathways. The cell demise shows the classical signs of apoptotic cell death: (i) compact, caspase-dependent chromatin condensation and (ii) dependence on protein synthesis (and gene transcription). On the other hand, when the caspase-dependent processes are blocked, cell death is still induced, suggesting that OX1 receptors can also direct the signal to non-caspase-dependent cell death pathways, this being the first time this has been shown for GPCRs.

Studies on the mechanisms by which orexin-A induces cell death revealed that intracellular Ca2+ elevation was not involved. This is interesting, since orexin receptors have been associated with strong Ca2+ signaling in all native cells and heterologous expression systems where this has been investigated (including CHO cells; see e.g. Refs. 4 and 25), and Ca2+, e.g. via mitochondrial Ca2+ overload, can induce both programmed and necrotic cell death in essentially all cell types (reviewed in Refs. 26 and 27). In contrast, the cell death response to thapsigargin was at least in part mediated by Ca2+, confirming that CHO cells are susceptible to Ca2+-induced cell death. OX1 receptor stimulation also acti-
vated the classical MAP-/SAP-kinases, ERK, JNK, and p38, and of these, p38α/β were centrally involved in the induction of OX1 receptor-mediated cell death. The mechanisms by which GPCRs regulate MAPK pathways are not fully clear at the moment, and for p38 only speculations exist. Grα11-11, Grα2/211-11, and Gβγ-dependent upstream signaling has been suggested to be involved, with possible downstream effectors such as ASK1 and non-receptor tyrosine kinases (reviewed in Refs. 2 and 23). Orexin receptors are able to interact with members of at least three different families of G-proteins, i.e. Gi/o, Ga, and Gαq (28, 14, 29), suggesting considerable versatility in orexin receptor signaling.

p38 MAPK/SAPKs are involved in the cellular response to different types of stress caused, for example, by cytotoxic agents, UV irradiation, osmotic shock, oncogenic stress, and even death receptor activation (Ref. 30 and reviewed in Refs. 23, 31, and 32). In the situations of stress, p38 activation can lead to either cell cycle arrest or cell death (reviewed in Refs. 23, 31, and 32). A major part of p38 activity in these situations is targeted toward transcription factors either via direct phosphorylation or via other kinases. p53 is often implicated both in p38-dependent cell cycle arrest and cell death, but whether other transcription factors targeted by p38 could serve these functions is very much unknown, although not without precedent (33, 34). As the results of the present study suggested gene transcription-dependent cell death in the presence of caspase activity, we focused our attention on p53. The link between p38 and p53 may occur at several levels. p38 phosphorylates p53, which may, first, stabilize it, i.e. by inhibiting its interaction with mdm2/hdm2, which otherwise targets it to degradation, or, second, functionally activate the protein (35–38). Third, p38 may elevate p53 by increased de novo synthesis (39). Our results strongly indicate that p53 is not involved in p38 MAPK/SAPK signaling to cell death from OX1 receptors, as (i) we do not see any significant stabilization of p53 proteins, (ii) there is no significant p53-dependent transcriptional activity in our reporter assay, and (iii) dominant-negative p53 constructs do not inhibit cell demise. A question arises why p53 does not seem to be targeted by p38, as p53 is so often implicated in p38 signaling. One likely explanation to this is offered by previous studies showing T211K mutation in the p53 of CHO cells, making these cells resistant to UV-induced G1 arrest (40, 41). In the absence of caspase activity (Z-VAD-fmk), death of CHO cells was still dependent on p38. Signaling of p38 to apoptotic/necrotic cell death independent of caspase activation has previously been observed for, for instance, ionotropic glutamate receptor stimulus (42), 2-deoxyglucose/NaCN (43), and Cd2+ (44).

OX1 receptors also strongly activated ERK, although this was of shorter duration than activation of p38 MAPK/SAPK(s). In contrast to the p38, which was central in induction of cell death in OX1 signaling, ERK activity appeared to be antagonistic to the cell death. The balance between ERK and p38 pathways has previously been shown to be crucial in the regulation of cell survival versus demise in a number of studies (see e.g. Refs. 45–47).

It is striking that the cell death is much delayed (24 h and onward), whereas p38 activation and p53 expression are rapid responses (30 min and onward). One factor likely leading to the delay in cell death is the requirement of gene transcription and protein synthesis. Also, different cells are clearly dying at different times, as seen from the gradual time dependence of the cell death. Although not targeted in this study, it is tempting to suggest that the long lasting activation of p38 may supply a constant driving force for the apoptosis. One likely explanation to the gradual cell death is that each cell needs to be in a particular phase of the cell cycle to be primed for the apoptosis. We did not observe any major differences between the cell cycle phases of the control cells and cells treated with orexin-A. However, the gradual cell death is likely to mask subtle changes in cell cycles of individual cells, and thus these data are inconclusive. Thapsigargin-mediated cell death was independent of the p38 pathway and protein synthesis, yet it was also delayed. Therefore, similarly to many other cells, delayed death may be as an “intrinsric” property of CHO cells, at least in some degree independent of the pathway leading to it.

Separation of cell death in apoptotic and necrotic cell death has been challenged by the discovery of intermediate and unclassifiable forms of cell death (reviewed in Refs. 15 and 16). For instance, there are several studies showing cell death clearly associated with caspase activation but where blocking of caspases does not inhibit the cell death (reviewed in Refs. 15 and 16). This is similar to the results of the current study. Thus, either the cell death is mediated by concurrent activation of caspase-dependent and -independent processes or the cell death program is altered upon caspase inhibition. We observed that the determinants of the cell death were similar in the presence and absence of caspase activity, i.e. p38 MAPK/SAPK activity and protein synthesis appeared to be required, except for the fact that gene transcription was no longer required when caspase activity was blocked. Thus, we conclude that OX1 receptor activation-triggered cell death program is altered in CHO cells upon caspase inhibition. A previous study has shown that OX1 receptor stimulation induces caspase activation and apoptotic cell death in a number of transformed and otherwise immortal cells (9). Although we can here confirm the cell death response, our data suggest that the caspase activation is not necessarily required for induction of cell death. Also, in sharp contrast to the findings of Rouet-Benzineb et al. (9), the death of our CHO cells is fully reversed by fetal calf serum.

In conclusion, we have in this study shown that OX1 orexin receptors, expressed in CHO cells, induce cell death with classical and non-classical apoptotic features via p38 MAPK/SAPK(s). The data further show that p38-mediated cell death can proceed without requirement for activation of p53. p53 is often mutated in cancer, and it can also be incapacitated by posttranslational modifications or indirect effects (reviewed in Refs. 48 and 49), wherewith cancer therapy relying on functional p53 will fail. The ability of p38 MAPKs to “bypass” p53 makes p38 a more feasible target in cancer therapy.

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