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Signaling pathways leading to the induction of ornithine decarboxylase: Opposite effects of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors

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Abstract

Treatment of serum-starved, human ECV304 cells with histamine or ATP elicited a transient induction of ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis, to an extent similar to that provoked by phorbol myristate acetate or serum re-addition. All these agents also provoked an increase in active phosphorylated p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK. The involvement of p44/42 MAPK and p38 MAPK in the induction of ODC was investigated by using selective inhibitors. U0126 and PD98059, two specific p44/42 MAPK kinase inhibitors, prevented the induction of ODC elicited by any stimulus employed, whereas SB203580 and SB202190, which are widely used as p38 MAPK inhibitors, enhanced ODC induction in a way that appeared dependent on p44/42 MAPK activation. By using inhibitors of other key signaling proteins that may lead to activation of p44/42 MAPK, we provide evidence that protein kinase C, but not phosphoinositide 3-kinase, is involved in histamine-stimulated ODC induction. These results show that the p44/42 MAPK pathway, but not p38 MAPK, is essential for ODC induction stimulated either by agonists of G-protein-coupled receptors, phorbol esters, or serum, and suggest that the inhibition of ODC induction may be an important event in the antiproliferative response to p44/42 MAPK pathway inhibitors.

Keywords: Ornithine decarboxylase; p44/42 MAPK; p38 MAPK; U0126; SB203580; Histamine

1. Introduction

Stimulation of cells with mitogens results in the triggering of a variety of signal transduction pathways that control the expression of specific genes, eventually leading to cell proliferation [1]. MAPKs, which comprise the p44/42 MAPK (also called extracellular signal-regulated kinase 1 and 2), stress-activated protein kinase, and p38 MAPK subfamilies, are important signaling proteins and can play a major role in the control of cell proliferation and survival [1, 2]. The MAPK subtypes are preferentially activated by diverse extracellular stimuli and are located in distinct, but structurally similar modules, although cross-talk among these pathways may exist at some levels. Cell-permeant, selective inhibitors of p44/42 MAPK and p38 MAPK have recently been developed; they have facilitated the study of the roles of these kinases in intact cells and may also have therapeutic potential in some pathological conditions. In particular, PD98059, a specific p44/42 MAPK pathway inhibitor, is also known as an angiogenesis inhibitor and is able to impair DNA synthesis and cell migration [3, 4]. The p44/42 MAPK pathway is typically activated by tyrosine kinase-associated receptors, although a number of seven-transmembrane-spanning receptors (G-protein-coupled receptors) are also known to activate this pathway, in some cases in a PKC- or PI3K-dependent manner [2].

ODC, the key enzyme in polyamine biosynthesis, is rapidly induced following growth stimuli [5, 6]. The *ODC* gene is now recognized as a proto-oncogene required for

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Abbreviations: MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; ODC, ornithine decarboxylase; ODQ, 1*H*-[1,2,4]oxadiazole [4,3-*a*]quinoxalin-1-one; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; and PMA, phorbol myristate acetate.

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cell-cycle progression and transformation, and a pivotal role for polyamines in tumor angiogenesis has been proposed [5–7]. Although a large variety of stimuli are able to induce ODC in target cells, the involvement of MAPK subfamilies in ODC induction has scarcely been investigated. Quite recently, we have shown that p44/42 MAPK is required for ODC expression in leukemia cells stimulated to growth by dilution in fresh medium containing serum [8], but it is not known if p44/42 MAPK is important for ODC induction in other cell types or following ligation of G-protein-coupled receptors.

In the present study, we utilized ECV304 cells, a human cell line which exhibits some endothelial characteristics [9, 10]. A number of G-protein-coupled receptors have been found to be expressed in these cells, including H1 histamine receptors and P2U purinoceptors, which are functionally linked to phosphoinositidase C activation [10]. Extracellular histamine and ATP, which can derive from several sources, can stimulate proliferation of various cell types via specific receptors [6, 11–13]. We report here that histamine and ATP can induce ODC in confluent, serum-starved ECV304 cells to levels similar to those elicited by serum. The involvement of p44/42 MAPK, p38 MAPK, and other key regulatory proteins in mediating these signals was investigated by using specific inhibitors.

2. Materials and methods

2.1. Materials

Anti-phospho-specific p44/42 MAPK (Thr202/Tyr204) and p38 MAPK (Thr180/Tyr182) antibodies were purchased from New England Biolabs, Inc. Difluoromethylornithine was kindly provided by the Merrell Dow Research Institute. SB203580 and other inhibitors of signal transduction pathways were purchased from Alexis Corp., except U0126 (from Promega) and manumycin A, SB202190, and SB202474 (from Calbiochem). ATP and histamine were from Sigma.

2.2. Cell culture and treatments

Human ECV304 cells were routinely grown in M199 medium containing 10% heat-inactivated fetal bovine serum and antibiotics. For experiments, confluent cells were kept in a serum-free medium for 48 hr before treatment with 10 μM histamine, 100 μM ATP, 100 ng/mL of PMA, or 10% serum. U0126 and/or other inhibitors were added to quiescent cells 30 min before these treatments (60 min in the case of manumycin A). The inhibitors were added in dimethylsulfoxide (final concentration: 0.1%). Control cells received equal amounts of dimethylsulfoxide. At the time indicated after treatment, cells were washed with PBS and harvested. Unless stated otherwise, the data shown in the figures are from one experiment representative of two or more experiments.

2.3. Determination of ODC activity

Cell extracts were prepared and assayed for ODC activity as previously described [14]. Specific ODC activity is expressed as units/mg protein, where 1 unit corresponds to 1 nmol of CO_2/hr of incubation.

2.4. Western blot analysis

Western blot analysis of phosphorylated p44/42 MAPK and p38 MAPK was performed by using phospho-specific antibodies. Approximately 10^7 cells were resuspended in 0.1 mL of lysis buffer (20 mM Tris/HCl, pH 8, 100 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM benzamidine, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 1 μg/mL of aprotinin, leupeptin, pepstatin), sonicated, and centrifuged. The supernatant was boiled in loading buffer and an aliquot corresponding to 80 μg of protein was analyzed by SDS/PAGE (12% gel). Separated proteins were transferred to a nitrocellulose membrane for 1 hr. The membrane was saturated with 5% powdered milk, 0.05% Tween 20 in 10 mM Tris pH 8, 150 mM NaCl for 1 hr, and then incubated with anti-phospho-specific p44/42 MAPK antibody at 4° overnight. In the case of phosphorylated p38 MAPK, powdered milk was replaced by BSA. Bands were revealed by the Amersham Enhanced Chemiluminescence (ECL) detection system.

3. Results

3.1. ODC induction and p44/42 MAPK activation by histamine, ATP, PMA and serum

The effect on ODC activity of treating serum-starved ECV304 cells with histamine or ATP is shown in Fig. 1A. Both agonists transiently induced ODC activity with a similar time–course and a peak at 4 hr. ODC activity returned to basal levels after 8 hr of treatment. Fig. 1A also reports the effect of PMA, a phorbol ester and known activator of PKC. PMA was an effective inducer of ODC in ECV304 cells, although in this case the induction of ODC appeared more delayed with respect to histamine and ATP, peaking at 8 hr. In general, the magnitude of the ODC induction by these agents (histamine, ATP, and PMA) was similar to that elicited by the re-addition of serum, a universal and powerful ODC inducer.

In order to assess the involvement of MAPKs in the signal transduction of the above-mentioned agents, we evaluated the amount of the active, phosphorylated forms of two subfamilies of MAPKs (p44/42 MAPK and p38 MAPK) by

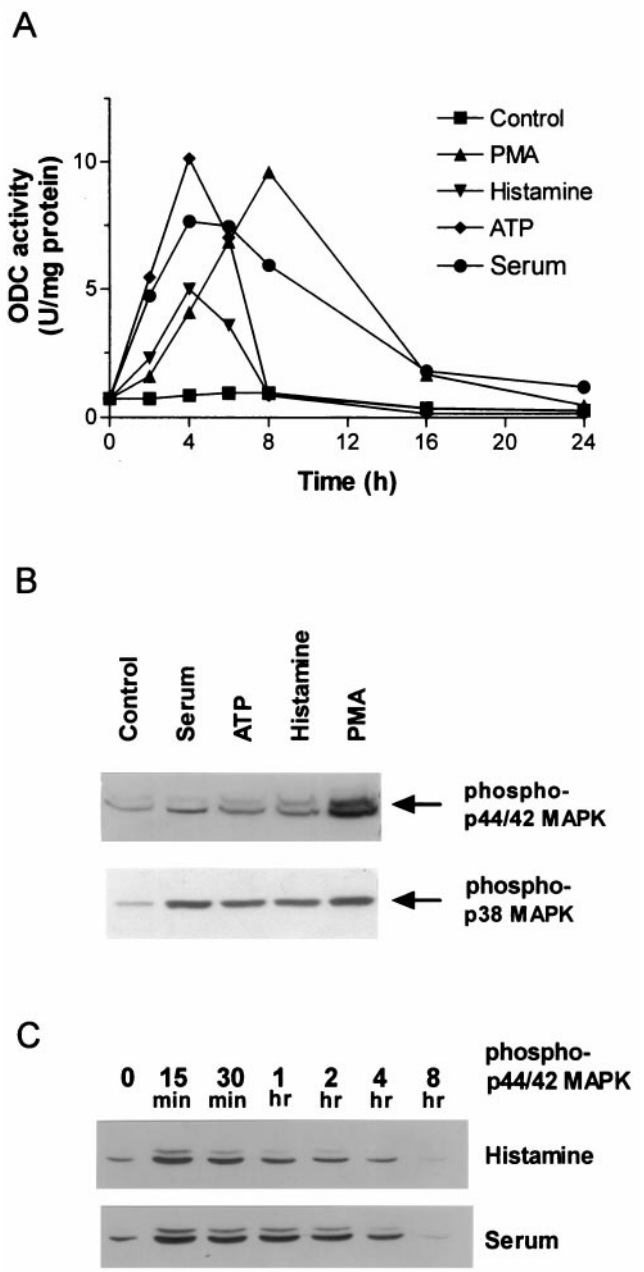

Fig. 1. Histamine, ATP, PMA, and serum induce ODC activity and stimulate the phosphorylation of p44/42 MAPK and p38 MAPK in ECV304 cells. (A) Confluent, serum-starved cells were treated with histamine, ATP, PMA, or 10% serum. At the time indicated, cells were harvested and assayed for ODC activity. (B) Confluent, serum-starved cells were treated with 10% serum, ATP, histamine, or PMA. After 30 min, cells were harvested and cell extracts were analyzed by Western blotting by using specific antibodies. (C) Confluent, serum-starved cells were treated with histamine or 10% serum. At the time indicated, cells were harvested and analyzed for phospho-p44/42 MAPK.

using phospho-specific antibodies (Fig. 1B). A low but detectable level of phosphorylated p44/42 MAPK was present in serum-starved ECV304 cells, and histamine, ATP, PMA, and serum all caused an increase in the active, phosphorylated forms of p44/42 MAPK. The amount of

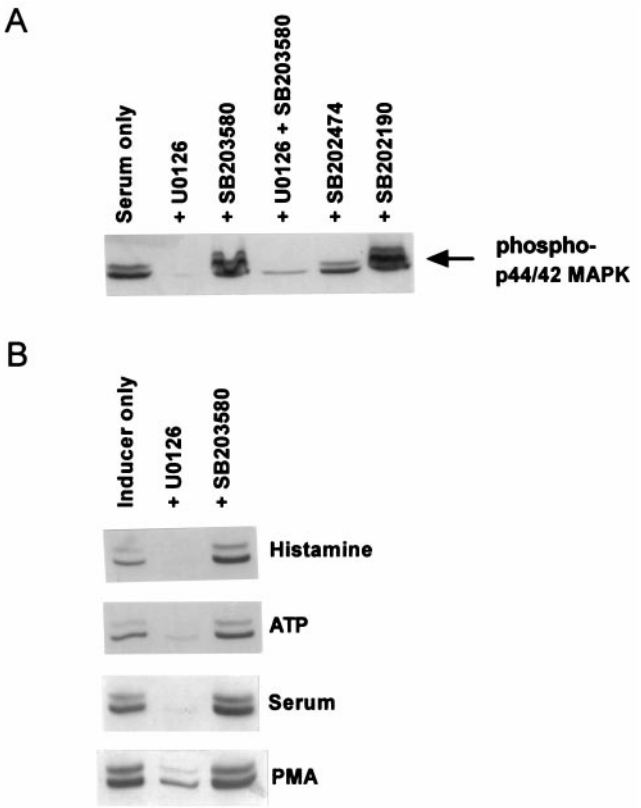

Fig. 2. Effect of p44/42 MAPK and p38 MAPK inhibitors on the phosphorylation state of p44/42 MAPK. The MAPK inhibitors were added at the concentration of 10 μM to confluent, serum-starved cells 30 min before serum (A) or various inducers (B). After a further 30-min incubation, cells were harvested and analyzed by Western blotting by using phospho-specific p44/42 MAPK antibodies.

phosphorylated p38 MAPK also increased after PMA, ATP, serum as well as histamine treatment. The time–course of p44/42 MAPK activation by histamine or serum is shown in Fig. 1C. The level of phosphorylated enzyme increased rapidly (ie. within 15 min), remained elevated up to 4 hr, and was markedly reduced after 8 hr.

3.2. Effects of p44/42 MAPK and p38 MAPK inhibitors

We tested the involvement of p44/42 MAPK and p38 MAPK pathways in ODC induction by using known, selective inhibitors. First, we ascertained the effects of these inhibitors on the phosphorylation state of p44/42 MAPK (Fig. 2). Treatment with 10 μM U0126, a specific and potent inhibitor of p44/42 MAPK kinase (MEK1/2) [15], abolished p44/42 MAPK phosphorylation in serum-stimulated cells (Fig. 2A). On the contrary, SB203580, a selective inhibitor of p38 MAPK [16, 17], further increased the serum-induced activation of p44/42 MAPK. However, when administered in addition to U0126, SB203580 no longer potentiated p44/42 MAPK activation, which actually vanished almost completely. Interestingly, the level of phosphorylated p44/42 MAPK was not increased by SB202474,

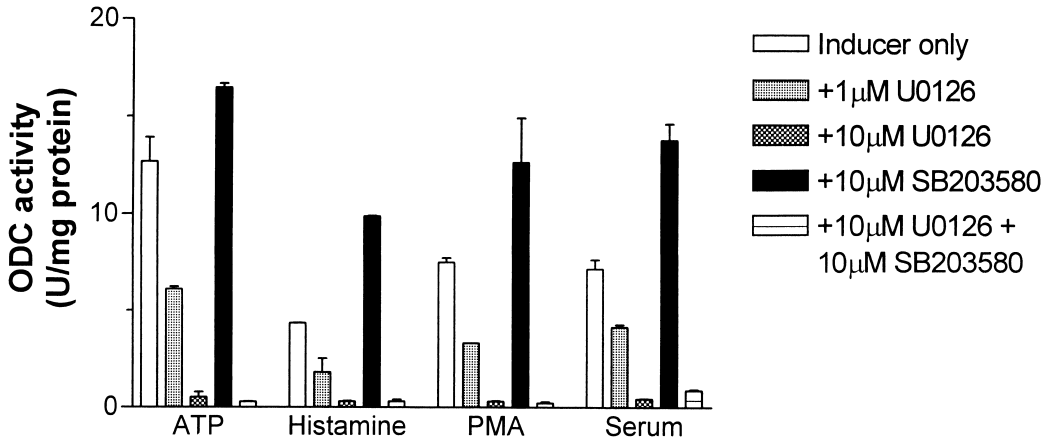

Fig. 3. Effect of U0126 and SB203580 on ODC induction. Confluent, serum-starved cells were treated with U0126 and SB203580 at the indicated concentrations, and after 30 min, with histamine, ATP, PMA, or 10% serum to induce ODC. After a further 4-hr incubation (6 hr in the case of PMA), cells were harvested and assayed for ODC activity. Results represent means ± SEM (N = 3).

an inactive analogue, whereas SB202190, another p38 MAPK inhibitor [16], was effective. Opposite effects of U0126 and SB203580 on p44/42 MAPK activation were found not only in serum-treated cells, but even when cells were stimulated with ATP, PMA, or histamine (Fig. 2B). On the other hand, U0126 treatment did not affect p38 MAPK phosphorylation (not shown).

The effects of the p44/42 MAPK and p38 MAPK inhibitors on ODC induction are shown in Fig. 3. U0126 caused a dose-dependent inhibition of ODC induction and at 10 μM completely abolished the induction of the enzyme elicited by any of the stimuli employed. PD98059, a widely used and different MEK1/2 inhibitor, was also effective, but to a lesser degree than U0126 (not shown), in accordance with its lower affinity for MEK1/2 [15]. Instead, SB203580 enhanced the induction of ODC (Fig. 3), and the simultaneous treatment with U0126 abolished even this action of SB203580, preventing the induction of the enzyme. The ODC-increasing effect of SB203580 was further characterized in serum-treated cells, and SB202190 was also considered. SB203580 enhanced serum-induced ODC activity particularly at 2 and 4 hr, and increased basal ODC activity only slightly and transiently (Fig. 4A). The effect of SB202190 was similar to that of SB203580 (Fig. 4B) and was abolished by U0126 as well. In conclusion, the results with these inhibitors indicate that p44/42 MAPK, but not p38 MAPK, activation is essential for ODC induction following these stimuli and that the ODC increasing effect of p38 MAPK inhibitors is mediated by p44/42 MAPK as well.

3.3. Involvement of PKC in ODC induction by histamine

The signaling pathways involved in ODC induction by histamine were investigated further. It has been reported that H1 receptors are expressed in ECV304 cells and coupled to the activation of phosphoinositidase C [10], which in turn may utilize PKC as a mediator. Thus, in order to ascertain if PKC is involved in the ODC-inducing effect of histamine in this model, we treated the cells with Go6850 [18] or chelerythrine [19], two selective PKC inhibitors, or provoked PKC down-regulation by a long pretreatment (24 hr) with PMA. All these treatments were effective in reducing ODC induction by histamine (Fig. 5). Figure 5 also reports the effects of other inhibitors of key signaling proteins, which may have an impact on the p44/42 MAPK pathway: manumycin A, a Ras farnesylation inhibitor [20], caused some reduction in ODC induction, whereas LY294002, a specific PI3K inhibitor [21], and ODQ, a guanylate cyclase inhibitor [22], were ineffective. Although PKC is considered the main target of PMA, other possible targets have been proposed recently [23]. Figure 6 shows that PKC inhibitors reduced PMA-induced ODC activity and p44/42 MAPK activation, confirming the involvement of PKC in the effect of PMA as well as histamine.

4. Discussion

It has been reported that ECV304 cells express P2U and H1 receptors functionally linked to phosphoinositidase C activation [10]. The present study shows that histamine and ATP could induce ODC in serum-starved ECV304 cells to an extent similar to that elicited by serum re-addition, although serum-induced ODC levels persisted longer. The experiments with U0126 and PD98059, two highly specific MEK inhibitors, indicate that the p44/42 MAPK pathway is required for ODC induction by any stimulus employed. According to Shin *et al.* [24], p44/42 MAPK activity is rather elevated in ECV304 cells, which may be consistent with the high levels of ODC activity observed in these cells. In fact, we found a detectable, but low level of phosphorylated p44/42 MAPK after a 2-day serum starvation. However, all the stimuli able to induce ODC rapidly increased the amount of phosphorylated p44/42 MAPK, suggesting

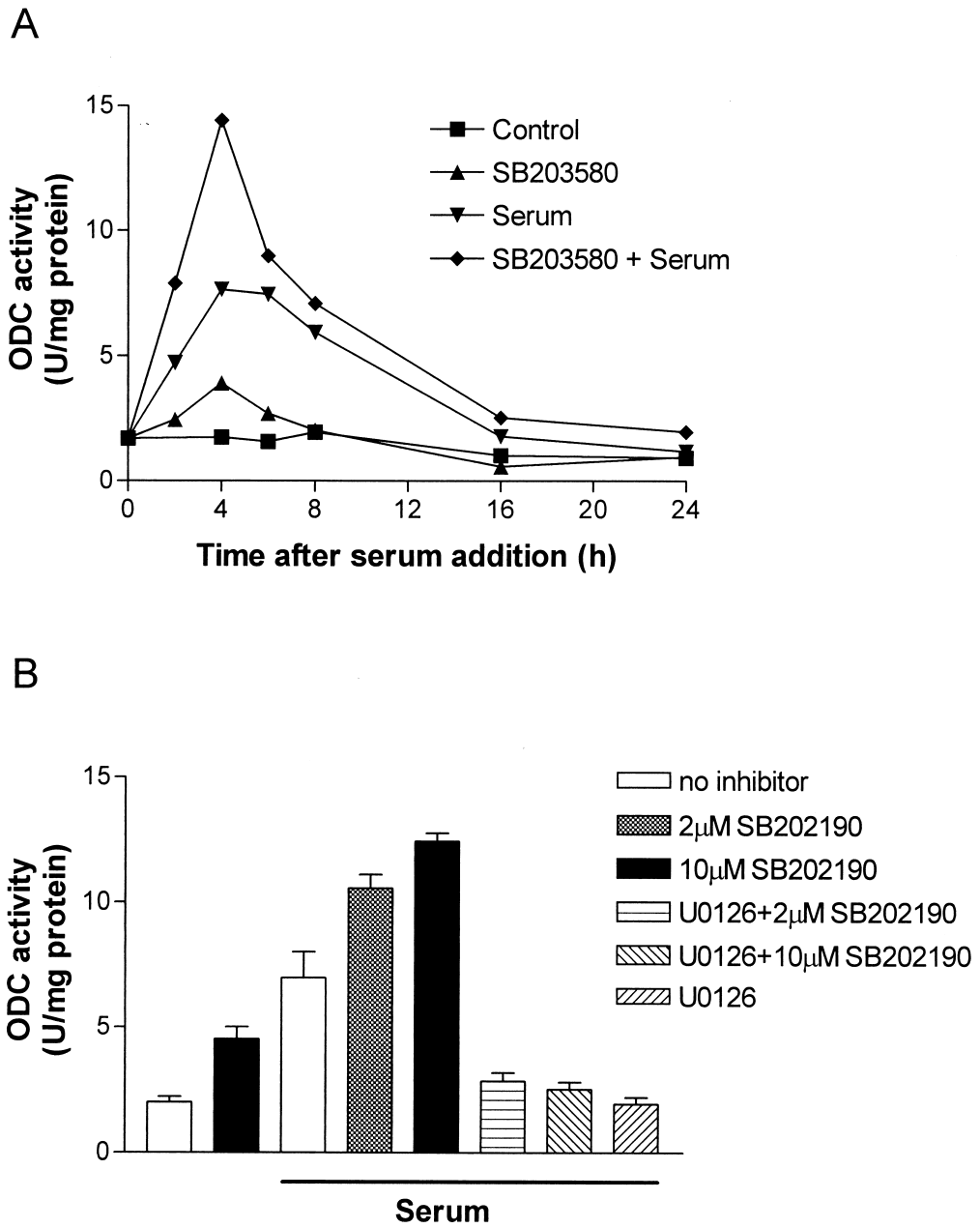

Fig. 4. (A) Effect of SB203580 on the time–course of serum-induced ODC activity. SB203580 (10 μM) was administered to confluent, serum-starved cells 30 min before treatment with 10% serum. At the time indicated after serum re-addition, cells were harvested and assayed for ODC activity. (B) Effect of SB202190 on serum-induced ODC activity. U0126 (10 μM) and/or SB202190 at the indicated concentration were added to confluent, serum-starved cells. After 30 min, cells were treated with 10% serum and further incubated for 4 hr. Then, cells were harvested and assayed for ODC activity. Results represent means ± SEM (N = 3).

that p44/42 MAPK activation may play an important role in the signal transduction of these agonists. Shin *et al*. [24] also reported that ECV304 cells are resistant to the inhibitory effect of PD98059. It is known, however, that PD98059 may not completely inhibit p44/42 MAPK activity in intact cells [3, 8]. In our hands, ECV304 cell growth was almost completely blocked by incubation of cells with either U0126 (10 μM), which is more potent than PD98059, or the ODC inhibitor difluoromethylornithine (data not shown). Therefore, the induction of ODC, being located downstream of p44/42 MAPK, may be an important event in the antiproliferative response to p44/42 MAPK pathway inhibitors.

A number of agonists acting on G-protein receptors can stimulate the p44/42 MAPK cascade through the involvement of either Gα or G$\beta\gamma$ subunits, although the intermediate steps have not been completely defined [2, 25]. In some cases, p44/42 MAPK activation appeared mediated by PKC, although routes independent of changes in PKC acti-

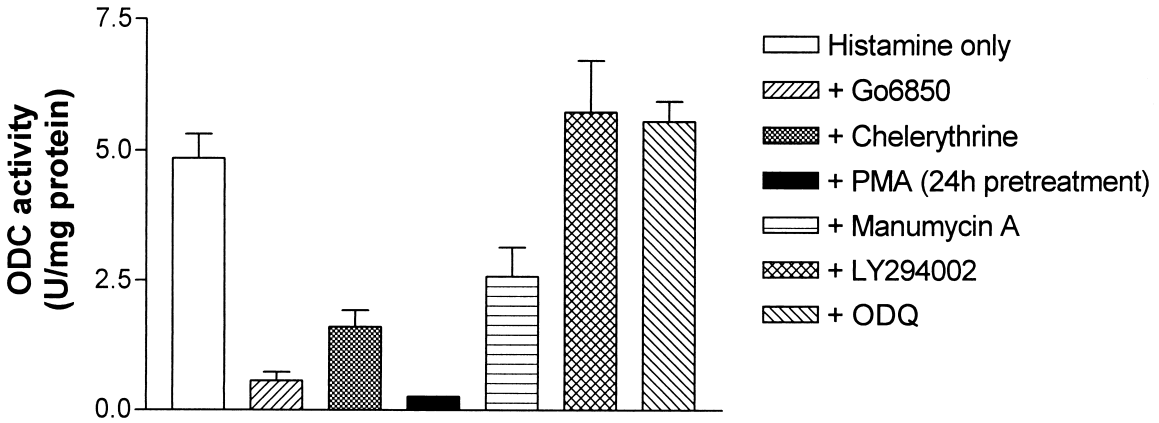

Fig. 5. Effect of various signaling inhibitors on histamine-induced ODC activity. Confluent, serum-starved cells were treated with the indicated inhibitors 30 min before histamine (24 hr before histamine in the case of PMA to induce down-regulation of PKC). After a further 4-hr incubation with histamine, cells were harvested and assayed for ODC activity. Concentration of the inhibitors was as follows: 1 μM Go6850, 5 μM chelerythrine, 100 ng/mL of PMA, 10 μM manumycin A, 20 μM LY294002, and 10 μM ODQ. Results represent means ± SEM (N = 3).

vation have also been observed. PKC may activate the p44/42 MAPK pathway through two potential mechanisms: direct activation and phosphorylation of Raf-1 or, according to more recent findings, in a Ras-dependent manner [2, 25]. Besides, G$\beta\gamma$-dependent activation of p44/42 MAPK may be activated by PI3K activation [2]. In ECV304 cells, PKC appears to be involved in ODC induction provoked by histamine, as judged by the experiments with PKC down-regulation or with PKC inhibitors. This conclusion is also supported by the finding that PMA, a direct activator of classical and novel PKC isoforms, induced ODC activity and p44/42 MAPK activation in these cells and PKC inhibition prevented these effects. The route may also involve Ras, since manumycin A reduced the induction of ODC caused by histamine. Instead, PI3K does not seem to be implicated, on the basis of the lack of efficacy of LY294002. Thus, this finding differs from our previous results with L1210 mouse leukemia cells, where ODC expression was strongly reduced by both MEK and PI3K inhibitors [8, 26], and restricts the importance of PI3K for ODC induction to particular experimental models. It has also been reported that cGMP can play an important role in vascular endothelial growth factor-stimulated p44/42 MAPK activation in endothelial cells [22], although our results with ODQ, a guanylate cyclase inhibitor, argue against a similar role for cGMP in histamine-stimulated ECV304 cells.

The present study also shows that p38 MAPK may be activated by ATP, PMA, serum, and histamine in ECV304 cells. The possibility that p38 MAPK is activated in some cell types by muscarinic and β-adrenergic receptors [2] or by phorbol esters [27] has been reported. However, the pyridinylimidazole compounds SB203580 and SB202190, widely used as specific inhibitors of both p38 MAPK α- and β-isoforms, enhanced ODC induction in ECV304 cells, and this effect appeared to be mediated by p44/42 MAPK. Interestingly, SB203580 has been reported to reverse the antiproliferative action of interleukin-1 and the associated down-regulation of ODC activity in melanoma A375 cells [28] and to enhance the proliferation of umbilical vein endothelial cells stimulated by vascular endothelial growth factor [29]. The present results may contribute to explain these findings.

Initial studies by Cuenda *et al*. [17] had shown that SB203580 (even at 100 μM) did not significantly affect Raf, MEK, or p42 MAPK activities *in vitro*. In addition, SB203580 does not affect the activation of the p44/42 MAPK cascade by a variety of agonists [17, 30]. However, quite recently, Singh *et al*. [31] have reported that SB203580 and SB202190 (but not the inactive analogue SB202474) induced LDL (low-density lipoprotein) receptor expression through activation of the p44/42 MAPK cascade in HepG2 cells and, conversely, selective activation of the p38 MAPK pathway reduced promoter activity of low-density lipoprotein receptor gene, suggesting the existence of a cross-talk between p38 MAPK and p44/42 MAPK in these cells. Thus, it is possible to speculate that in ECV304 cells stimuli such as ATP, PMA, serum, or histamine may limit p44/42 MAPK activation and thus ODC induction by increasing p38 MAPK activity.

On the other hand, according to other recent reports [30, 32], SB203580 can activate Raf in intact cells in a p38 MAPK-independent way, albeit without affecting MEK and p44/42 MAPK activities appreciably. Thus, we cannot exclude the possibility that SB203580 and SB202190 may interfere with the p44/42 MAPK cascade through a p38 MAPK-independent mechanism, although the lack of effect of SB202474 on p44/42 MAPK does not seem to support this hypothesis.

In conclusion, this research shows that the p44/42 MAPK pathway is essential for ODC induction stimulated either by agonists of G-protein-coupled receptors, phorbol esters, or serum in ECV304 cells, and proves to be involved even in the "superinduction" of ODC by p38 MAPK inhibitors.

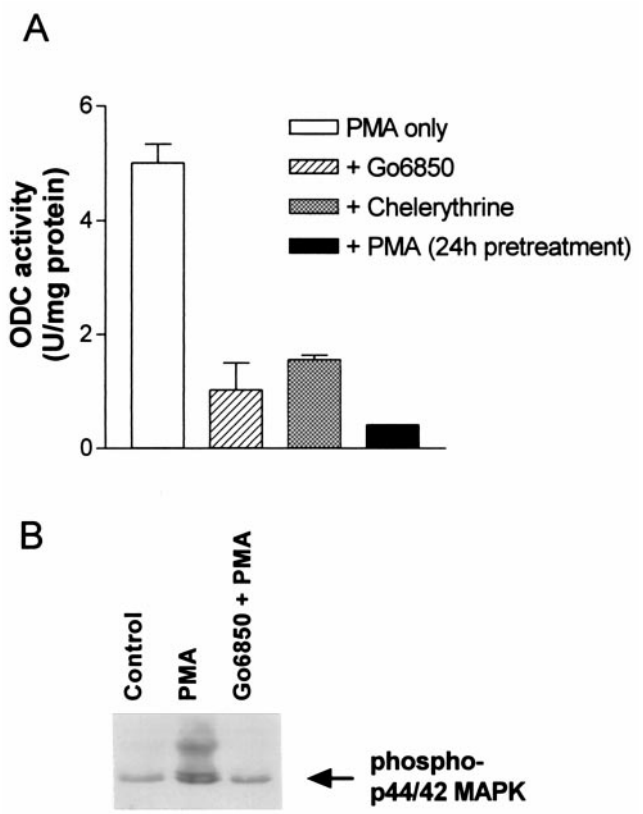

Fig. 6. Effect of PKC inhibition on PMA-induced ODC activity and p44/42 MAPK activation. (A) Cells were treated with the indicated inhibitors as described in the legend to Fig. 5, but then treated with PMA for a further 6 hr. Then, cells were harvested and assayed for ODC activity. Results represent means ± SEM (N = 3). (B) Cells were treated with Go6850, and after 30 min with PMA. After a further 30-min incubation, cells were harvested and the amount of phosphorylated p44/42 MAPK was evaluated by Western blotting.

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