Journal of Cellular Biochemistry 102:368–376 (2007)

Volatile Anesthetics Affect the Morphology of Rat Glioma C6 Cells via RhoA, ERK, and Akt Activation

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Abstract Treatment of rat glioma C6 cells with the β-receptor agonist isoproterenol induces a massive increase in cAMP. Concomitantly the cells change their morphology from a fibroblast-type to an astrocyte-like (stellated) cell shape. The stellated morphology can be completely reverted by thrombin and sphingosine-1-phosphate (S-1-P) but also to a certain extent by clinical concentrations of volatile anesthetics. The anesthetic-induced reversion of the stellated cell shape seems to be mediated by a number of cellular alterations. Central to the effect is most likely a RhoA/Rho-kinase activation, but also the MAPKK/MEK and the Akt/protein kinase B pathway are activated by the anesthetics. With the use of specific inhibitors we were able to show that activation of the MAPKK/MEK pathway inhibits, whereas activation of the Akt/protein kinase B pathway stimulates the reversal of the stellated cell shape by the anesthetics. In summary, volatile anesthetics affect the morphology of rat glioma C6 cells by activation of the RhoA/Rho kinase, the MAPKK/MEK, and the Akt/protein kinase B signaling pathways. J. Cell. Biochem. 102: 368–376, 2007.

Key words: volatile anesthetics; C6 glioma cells; RhoA; ERK; Akt

Cyclic AMP (cAMP) stimulates the proliferation of various epithelial cells, hepatocytes, keratinocytes, pancreatic islet βcells, Schwann cells, and Swiss 3T3 cells [Dumont et al., 1989]. On the other hand, cAMP inhibits proliferation of normal fibroblast, smooth muscle cells, lymphoid cells, neuronal cells, and glial cells [Hollenberg and Cuatrecasas, 1973; Nilsson and Olsson, 1984; Blomhoff et al., 1988; Mark and Storm, 1997; Dugan et al., 1999]. The growth inhibitory effect of cAMP in C6 glioma cells is probably mediated by inactivation of MAPK, also known as ERK1/2 [Hordijk et al., 1994; Dugan et al., 1999; Schmitt and Stork, 2001] and phosphatidylinositol 3-kinase/Akt pathways [Wang et al., 2001]. The growth inhibition of these cells is accompanied by a morphological alteration of the cell shape [Oey, 1975; Tas and Koschel, 1990]. After treatment with a β-receptor agonist in serum-free medium the cells change from a fibroblast-like flat morphology to a rounded astrocyte-like (stellated) shape with processes. The stellated morphology can be reverted by thrombin [Tas and Koschel, 1990], lysophosphatidic acid [Koschel and Tas, 1993], and sphingosine-1-phosphate (S-1-P) [Tas and Koschel, 1998]. The mechanism of the reversion of the stellated cell shape by these substances has not been explored. It seems however likely that the Rho family of small GTPases (Rac, cdc 42, and Rho) is involved in this reversion [Narumiya, 1996].

In neurons and astrocytes Rho activation leads to retraction of cell processes [Jalink et al., 1994; Katoh et al., 1996; Majumdar et al., 1998], whereas Rac signaling has been implicated primarily in opposing responses such as formation of focal contacts, cell attachment, and neurite extensions [Nobes and Hall, 1995; Rottner et al., 1999]. In this respect, it has been suggested that the integrated effects of Rac and Rho signaling pathways determine the ability of a particular G protein coupled receptor agonist to produce retraction and cell rounding

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Received 6 December 2006; Accepted 17 January 2007
DOI 10.1002/jcb.21294

[Seasholtz et al., 2004]. Identification of the physiological functions of the RhoA pathway is greatly facilitated by the use of the highly selective, cell permeant Rho-kinase inhibitor Y-27632 [Uehata et al., 1997; Fu et al., 1998].

One of the downstream effectors of RhoA is Rho kinase (also known as p160ROK). Activated Rho kinase phosphorylates and thereby inactivates myosin light chain (MLC) phosphatase leading to increased MLC phosphorylation [Fukata et al., 2001]. It has been shown for example that the Rho mediated cytoskeletal response in 1321 N1 astrocytoma cells depends on MLC phosphorylation [Majumdar et al., 1998]. In agreement with this view is the observation that treatment of primary astrocytes with a MLC kinase inhibitor leads to stress fiber dissolution and the formation of processes [Manning et al., 1998].

Besides thrombin and bioactive lipids also small n-alkanols such as ethanol and 1-propanol can revert the cAMP-induced stellation [Koschel and Tas, 1993]. Small n-alkanols have anesthetic properties for example in anesthesizing tadpoles and rats [Alifimoff et al., 1989; Fang et al., 1997]. The anesthetic potency of a particular n-alkanol depends on its lipophilicity which increases with chain length [Meyer, 1899] and the mechanism of action resembles that of conventional inhaled (volatile) anesthetics [Fang et al., 1997; Mihic et al., 1997]. This prompted us to investigate the effect of volatile anesthetics on the reversion of the cAMP-induced stellation. The anesthetics caused a partly reversion of the stellated cell shape by the activation of the RhoA, ERK1/2, and Akt pathways. This is the first observation that volatile anesthetics at clinical relevant concentrations can affect the morphology of cultivated cells.

MATERIALS AND METHODS

Materials

Y27632 dihydrochloride, HA 1077 (Fasudil hydrochloride), and PD98059 were from Biotrend (Köln, Germany). Lysophosphatidic acid (LPA), thrombin, and S-1-P were from Sigma. Exoenzyme C3 was from Merck (Bad Soden, Germany).

Cell Culture

C6 rat glioma cells (ATTC CCL107) were cultured in Dulbecco's modified Eagle's medium (Linaris, Wertheim-Bettingen, Germany) with 10% fetal calf serum (Linaris) in a 5% CO2/95% air atmosphere and were passaged by trypsination.

RhoA Activation Measurement

For the RhoA activation measurements rat glioma C6 cells were preincubated for 3 h in DMEM without serum. After different treatments, RhoA activation was measured with the commercially available G-Lisa RhoA activation assay kit (Fa. Tebu, Offenbach, Germany). The kit contains a RhoA-GTP binding protein linked to the wells of a 96-well plate. Active, GTP-bound RhoA in cell lysates will bind to the wells while inactive GDP-bound RhoA is removed during washing steps. The bound active RhoA is detected with a RhoA-specific antibody and the absorbance measured at 492 nm.

cAMP-Measurement

The cAMP concentration after treatment of the cells with 100 μM isoproterenol was measured with a direct cAMP-Elisa from Biotrend (Köln, Germany).

Incubation Assays With Volatile Anesthetics

In order to maintain the proper concentrations of the volatile anesthetics during the incubation assays we prepared stock solutions of the anesthetics in DMEM by stirring liquid anesthetic in DMEM in a stoppered glas flask for 30 min. After adding DMEM medium with anesthetic to the cells, these were placed in a gastight chamber at 36°C under an air stream containing the desired anesthetic concentration using calibrated vaporizers. Minimum alveolar concentrations were based on MAC values for rats as proposed by Mazze et al. [1985]. Thus we assumed that 1 MAC halothane corresponds to 1.03 Vol.% (290 μM), 1 MAC isoflurane to 1.46 Vol.% (350 μM), and 1 MAC enflurane to 2.21 Vol.% (680 μM). The concentration of halothane was checked by HPLC [Altmayer et al., 1987] and of the other anesthetics by gas chromatography [Tas and Koschel, 1991].

Western Blot Analysis

After incubation with or without agonist or anesthetic rat glioma C6 cells on 3 cm Petri dishes were washed in PBS and harvested in SDS loading buffer (200 mM Tris, 6% SDS, 15% glycerol, and 0.001% bromophenol blue)

and heated for 5 min at 95°C. Samples were analyzed by Western blot using the following antibodies: Phosphor (Ser473) PKB, ERK1/2 (Thr202/Tyr204), MLC 2 (Thr18/Ser19), PKC delta (Ser643), all from Cell Signaling (Frankfurt/Main, Germany).

PKA activity was evaluated by the phosphorylation of the well-known PKA substrate vasodilator-stimulated phosphoprotein (VASP) detected by monoclonal anti-phospho (Ser239)VASP mouse antibody [Smolenski et al., 1998]. Equal loading was controlled by Ponceau-S-staining of the membrane and incubation of the stripped membranes with corresponding non-phospho antibodies. For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase were used as secondary antibodies, followed by enhanced chemiluminescence detection (Amersham, Pharmacia Biotech, Freiburg, Germany).

Statistics

Data are presented as mean ± SE. Statistical evaluation was made using Student's *t*-test—$P < 0.05$ was considered to be statistically significant.

RESULTS

Volatile Anesthetics Reverse the Isoproterenol-Induced Morphological Alteration of C6 Glioma Cells

C6 glioma cells were seeded at low density on 3 cm Petri dishes. In order to study the morphological alteration, the cells were washed twice with DMEM without FCS. Isoproterenol, a β-receptor agonist was then added to a final concentration of 100 μM. Usually 10–20 min after isoproterenol addition the cytoplasm of the cells started retracting around the nucleus. This process was fully completed in 30–45 min (Fig. 1b). Reversion of this morphological alteration is obtained by the addition of 0.1 U/ml thrombin (Fig. 1d), but also by the bioactive lipids S-1-P (200 nM) and lysophosphatidic acid [not shown, Koschel and Tas, 1993; Tas and Koschel, 1998]. Interestingly clinical concentrations of volatile anesthetics, here shown for 1 Vol.% isoflurane (Fig. 1c) partly reverse the morphological alteration.

The isoproterenol addition strongly activates the cAMP production in the C6 glioma cells. The cAMP increase is maximal after about 20 min and then slowly declines. A typical experiment

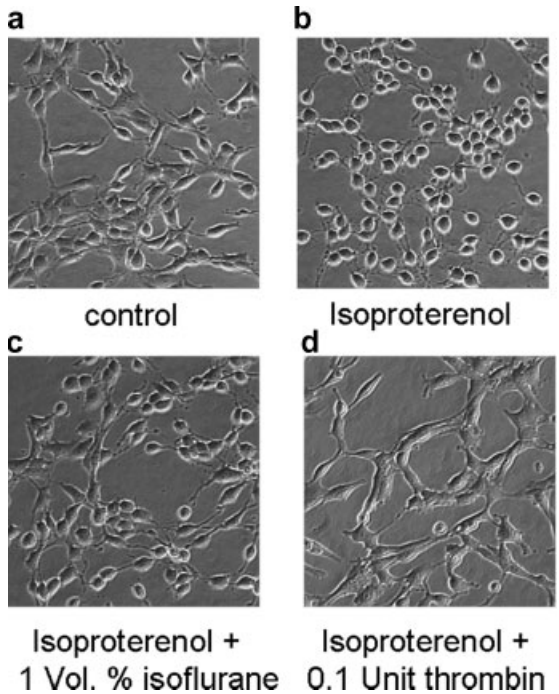

Fig. 1. Reversion of the isoproterenol-induced stellation of rat glioma C6 cells by isoflurane and thrombin. C6 glioma cells were incubated in DMEM without FCS. The β-agonist isoproterenol was added to b, c, and d to a final concentration of 100 μM. The dish without isoproterenol addition served as control (a). After 30 min isoflurane was added to a final concentration of 1 Vol.% to c and thrombin to a final concentration of 0.1 U/ml to d. The effect of the different treatments was analyzed 20 min later (magnification 200×).

is shown in Figure 2. A comparable morphological alteration of the cells is also obtained with 10 μM forskolin with causes a receptor-independent increase in the cAMP concentration of the cells (not shown).

The Rho-Kinase Inhibitor Y27632 Inhibits the Anesthetic-Induced Reversion of the Stellated Cell Shape

Thrombin causes cell shape changes by activating the RhoA/Rho-kinase pathway in platelets [Bodie et al., 2001], vascular smooth muscle cells [Pang et al., 2005], and endothelial cells [Ohkawara et al., 2005]. It was therefore of interest to study whether Y27632, a specific Rho-kinase inhibitor, can prevent the reversion of the morphological response by isoflurane. Figure 3a shows the morphological alteration of the cells after addition of 100 μM isoproterenol and the subsequent reversion by 1 Vol.% isoflurane is shown in Figure 3b. The specific Rho-kinase inhibitor Y27632 does not have an effect on isoproterenol treated cells (Fig. 3c), but

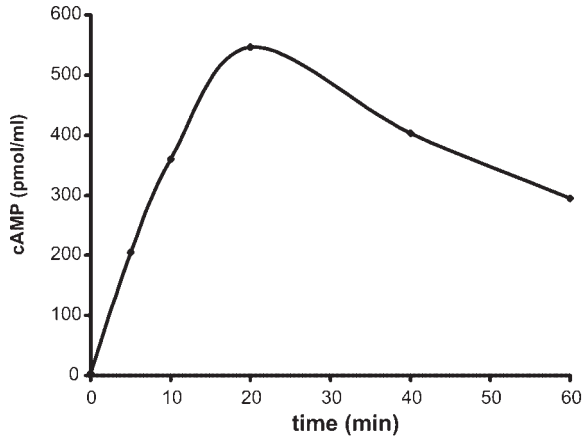

Fig. 2. Isoproterenol induces a massive cAMP response in rat glioma C6 cells. C6 glioma cells on 3 cm Petri dishes were preincubated for 10 min in DMEM without FCS. Isoproterenol was then added to a final concentration of 100 μM. At the indicated timepoints the supernatants of the cells were removed and 0.1 M HCl was added to the dishes. The cells were then homogenized and the supernatants collected after low speed centrifugation and frozen at −70°C. The cAMP concentration of the samples was measured with a direct cAMP-Elisa from Biotrend. Shown is a typical experiment performed in duplicate.

completely inhibits the reversion by 1 Vol.% isoflurane (Fig. 3d). The IC50 for the inhibition of the reversal of the morphological response by isoflurane and thrombin were similar (IC50 = 1 μM).

A different Rho-kinase inhibitor HA1077 at a concentration of 10 μM also inhibited the reversal of the morphological response by isoflurane and thrombin (not shown). Unfortunately, we were not able to test the C3 toxin from clostridium botulinum on the reversion of the isoproterenol-induced morphological alteration since the method to introduce the C3 toxin into the cells using optimem I medium + lipofectamine as described by Borbiev et al. [2000] already caused stellation of the cells.

PD98059 Potentiates the Isoflurane-Induced Reversion of the Stellated Cell Shape

To study the potential involvement of the ERK1/2 signaling pathway in the reversion of the stellated cells' shape by isoflurane, we tested PD98059 a specific inhibitor of the mitogen-activated protein kinase kinase (MAPKK/MEK). Figure 4 shows that 20 μM PD98059

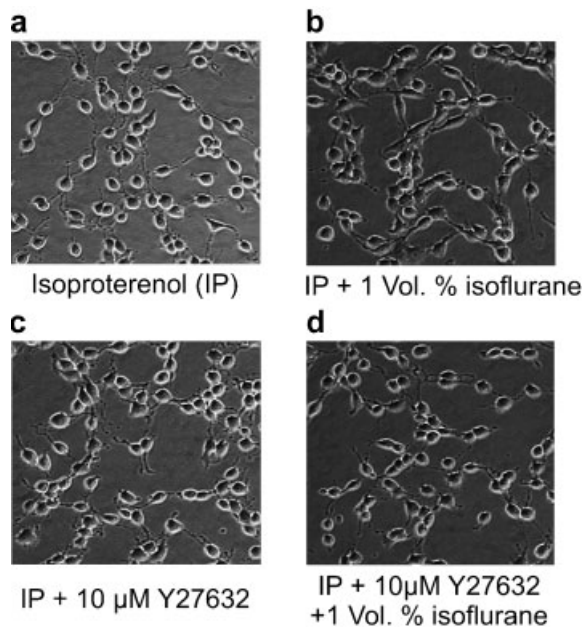

Fig. 3. The anesthetic-induced reversion of the isoproterenol-induced stellation is inhibited by Y27632. C6 glioma cells were treated with 100 μM isoproterenol. After 30 min isoflurane was added to a final concentration of 1 Vol.% in the presence (d) or absence (b) of the specific RhoA/Rho-kinase inhibitor Y27632. The inhibitor was also tested in the absence of anesthetic (c). The dish treated with isoproterenol alone served as control (a). The effect of the different treatments was evaluated 20 min later (magnification 200×).

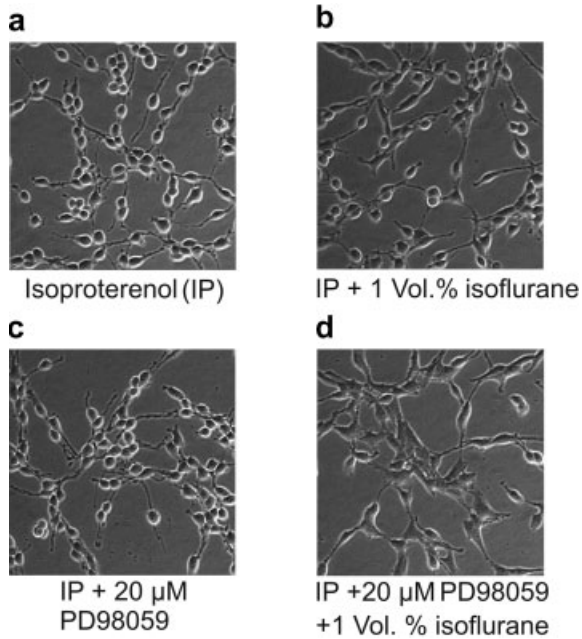

Fig. 4. The anesthetic-induced reversion of the isoproterenol-induced stellation is stimulated by PD98059. C6 glioma cells were treated with 100 μM isoproterenol. After 30 min isoflurane was added to a final concentration of 1 Vol.% in the presence (d) or absence (b) of 20 μM of the ERK1/2 inhibitor PD98059. The inhibitor was also tested in the absence of anesthetic (c). The dish treated with isoproterenol alone served as control (a). The effect of the different treatments was evaluated 20 min later (magnification 200 ×).

does not affect the IP-induced morphological alteration (compare a and c) but strongly potentiates the reversion of the stellated cells' shape by isoflurane (compare b and d). This suggests that activation of the ERK1/2 signaling pathway inhibits the reversion of the stellated cell shape by isoflurane. Interestingly the Rho-kinase inhibitor Y27632 completely inhibits the combined effect of isoflurane and PD 98059 indicating that Rho-kinase activation is central to the effect (not shown).

Wortmannin Inhibits the Isoflurane-Induced Reversion of the Stellated Cell Shape

In order to find out whether Akt/protein kinase B is involved in the reversion of the stellated cell shape by isoflurane, we tested wortmannin in a concentration of 10 μM. Figure 5 shows that a preincubation for 20 min with 10 μM wortmannin completely prevented the reversion of the stellated cell shape by 1 Vol.% isoflurane. However, wortmannin did not inhibit the reversion of the stellated cell shape by thrombin or S-1-P (not shown).

Volatile Anesthetics Cause RhoA Activation in C6 Glioma Cells

To directly demonstrate the involvement of the RhoA/Rho-kinase pathway in the anesthetic-induced reversion of the IP-induced stellation, we used a RhoA activation assay, which measures active GTP-bound RhoA in lysates of untreated and treated cells. RhoA activation by thrombin and S-1-P reached a maximum after 2 resp. 4 min whereas the anesthetics more slowly activated RhoA reaching a maximum after 4–8 min (not shown). Figure 6a,b shows the RhoA activation by clinical concentrations of halothane and isoflurane in comparison with the RhoA activation by thrombin and S-1-P. The halothane activation of RhoA reached 37% of the S-1-P-induced RhoA activation and 46.3% of the thrombin-induced RhoA activation. The isoflurane activation of RhoA reached 51.7% of the S-1-P and 70% of the thrombin-induced RhoA activation.

Activation of ERK1/2, Akt/Protein Kinase B, and Myosin Light Chain Phosphorylation by Halothane

In view of the potentiation of the reversion by PD98059, an inhibitor of the mitogen activated protein kinase kinase (MAPKK/MEK) and the inhibition of the reversion by wortmannin, an inhibitor of Akt/protein kinase B signaling pathway, it was of interest to study by Western blot analysis the activation of these pathways under our experimental conditions.

Figure 7 shows that halothane mimicked S-1-P in terms of ERK phosphorylation. However, the anesthetic clearly differed from S-1-P concerning Akt/protein kinase B phosphorylation, which was not observed in S-1-P treated cells and in MLC-phosphorylation which was much stronger in S-1-P treated cells. PKA activity was evaluated by activation of P-VASP. Interestingly PKA activity depressed the phosphorylation of P-ERK, P-MLC, and P-PKB induced by S-1-P or halothane.

DISCUSSION

Addition of the β-receptor agonist isoproterenol to rat glioma C6 cells in serum-free medium

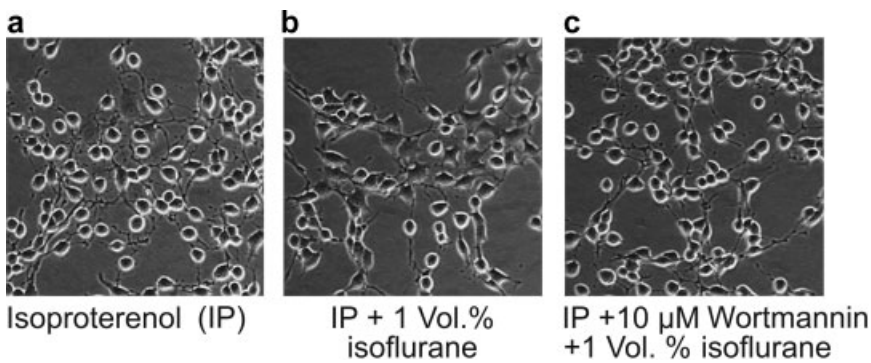

Fig. 5. The anesthetic-induced reversion of the isoproterenol-induced stellation is inhibited by wortmannin. C6 glioma cells were incubated with 100 μM isoproterenol. After 20 min wortmannin (10 μM) was added to c and at 30 min isoflurane was added to a final concentration of 1 Vol.% to b and c. The dish treated with isoproterenol alone served as control (a). The effect of the different treatments was evaluated 20 min later (magnification 200×).

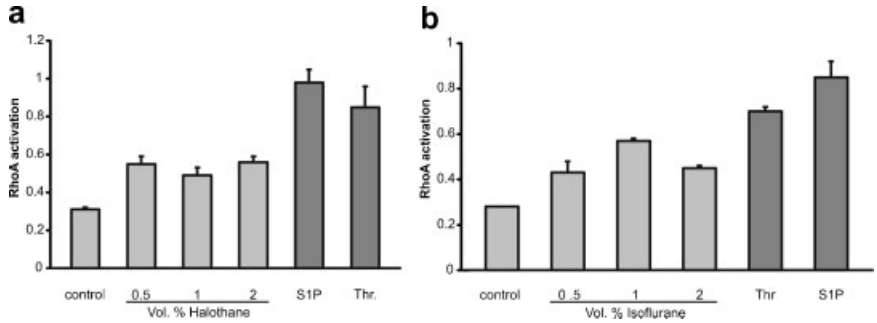

Fig. 6. RhoA activation by thrombin, S-1-P and clinical concentrations of halothane and isoflurane. Rat glioma C6 cells cultured on 3 cm Petri dishes were preincubated for 3 h in DMEM (without FCS) followed by incubation with either 0.1 U/ml thrombin for 2 min or with 200 nM S-1-P for 4 min Other dishes were incubated for 8 min with clinical concentrations of halothane (**a**) or isoflurane (**b**). The cells were then lysed in lysis buffer supplemented with a protease inhibitor cocktail and centrifuged for 2 min at 4°C. The G-LisaTM RhoA activation assay was then performed according to the manufacturer's procedure. Equal amounts of protein were applied to the wells. Absorbance was measured at 490 nm Shown is a representative experiment. Data are mean ± SE of duplicate determinations.

induces a massive increase in cAMP (Fig. 2) and an alteration in cell shape. In 20–45 min the cells change from a spindle type to an astrocyte-like cell shape (Fig. 1).

Previously we have shown that thrombin [Tas and Koschel, 1990], S-1-P [Tas and Koschel, 1998], and LPA [Koschel and Tas, 1993] cause a reversion of the cAMP-induced stellation. Here we make the interesting observation that clinical concentrations of volatile anesthetics mimic the above agents in that they partly reverse the cAMP-induced stellation (Fig. 1)

The RhoA/Rho-kinase signaling pathway seems to play a central role in the anesthetic-induced reversion of the stellated cell shape since two different specific Rho-kinase inhibitors Y27632 [Uehata et al., 1997; Fu et al., 1998], and HA 1077 strongly inhibited the anesthetic-induced shape change.

With the use of an Elisa specific for activated RhoA we could directly show that RhoA was activated by clinical concentrations of halothane and isoflurane (Fig. 6). The RhoA activation by the anesthetics was approximately 35–70% of the activation of RhoA obtained by thrombin and S-1-P and in agreement with the partly reversal of the cAMP-induced stellation by the anesthetics.

The reversion of the cAMP-induced stellation by thrombin, S-1-P, and volatile anesthetics is most likely mediated by an increase in myosin light chain phosphorylation (MLC-P), which regulates contraction. In smooth muscle and non-muscle cells it has been observed that activated Rho-kinase inhibits myosin phosphatase, which in consequence increases MLC phosphorylation [for review see Somlyo and Somlyo, 2000]. In agreement with this model is our observation that inhibition of MLC phosphatase by the phosphatase inhibitor calyculin A [Ishihara et al., 1989; Kimura et al. 1996] slowly reverses the isoproterenol-induced stellation. This effect was not inhibited by Y27632, indicating that calyculin does not mediate its effect by activation of Rho-kinase.

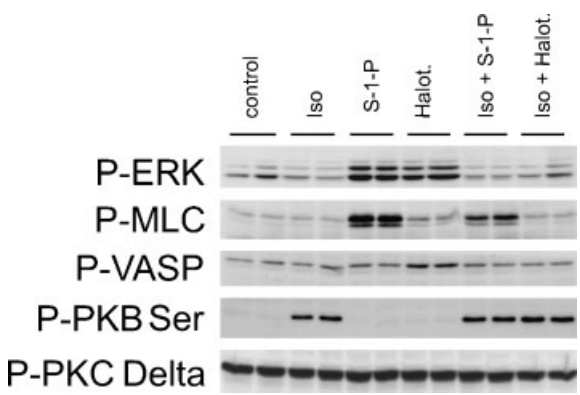

Fig. 7. Activation of different intracellular signaling pathways by halothane, isoproterenol, and S-1-P. Rat glioma C6 cells were preincubated for 3 h in DMEM without FCS and analyzed without stimulation (control) or stimulated for 5 min with resp. 100 μM isoproterenol (Iso), S-1-P, or 1 Vol.% halothane (Halot.). Other cells were stimulated for 5 min with 100 μM isoproterenol and then for 5 min with 200 nM S-1-P (Iso + S-1-P) or for 5 min with 100 μM isoproterenol followed by incubation for 5 min with 1 Vol.% halothane (Iso + Halot). After the incubation the cells were lysed and the activation of the different proteins was detected by Western blotting with phosphospecific antibodies (see Materials and Methods).

Are Other Pathways Involved in the Reversion of the Isoproterenol-Induced Stellated Cell Shape by Volatile Anesthetics

Interestingly PD98059, a specific inhibitor of the mitogen-activated protein kinase kinase (MAPKK/MEK) strongly potentiated the reversion of the stellated cell shape by volatile anesthetics (Fig. 4). In the presence of PD98059 the reversion of the stellated cell shape by the anesthetics was comparable to the reversion by thrombin and S-1-P (compare Fig. 1d with Fig. 4d). On the other hand the Akt/protein kinase B inhibitor wortmannin prevented the reversion of the stellated cell shape by the anesthetics (Fig. 5). Apparently the volatile anesthetics activate multiple pathways for example the RhoA/Rho-kinase and the Akt/protein kinase B pathways, which stimulate the reversion of the stellated cell shape and the MAPKK/MEK pathway, which inhibits the reversion of the stellated cell shape. In agreement with this model is the fact that the anesthetics strongly activate P-ERK (Fig. 7). The MAPKK/MEK pathway is active upstream of Rho-kinase since its effects can be completely inhibited by Y27632. Western blot analysis also showed that S-1-P strongly activated P-ERK (Fig. 7). This does not seem to affect the reversion of the cAMP-induced stellation by S-1-P. An explanation for this contradictory result could be that the activation kinetics of the MAPKK/MEK pathway in the two systems is different or that the MAPKK/MEK pathway can only inhibit the reversion of the stellated cell shape when the RhoA activation is submaximal. Interestingly the ERK phosphorylation is decreased under conditions where cAMP is increased (Fig. 6). This has also been observed by Wang et al. [2001].

How do Anesthetics Interfere With RhoA and ERK Signaling?

Since thrombin, S-1-P, and LPA signal through G-protein coupled receptors [Jalink and Moolenaar, 1992; Sörensen et al., 2003], the possibility exists that the G proteins are direct targets for the volatile anesthetics. An alternative possibility is that the anesthetics interfere with the RhoGTPase–GDI complexes [Dermardirossian and Bokoch, 2005], which are regulated by various proteins and lipids that exert GDI displacement activity or act in a similar way as certain phospholipids, that change the substrate preference of GTPase activating proteins [Ligeti et al., 2004]. Clearly more research is necessary to clarify the reversion of the cAMP-induced stellation by volatile anesthetics.

This is the first observation that volatile anesthetics mimic thrombin and bioactive lipids such as S-1-P and LPA with respect to their reversal of the cAMP-induced stellation in rat glioma C6 cells. Interestingly the anesthetics mediate this morphological change by activating the RhoA/Rho-kinase, the Akt/protein kinase B, and MAPKK/MEK pathways. The anesthetic-induced changes in these pathways may be of relevance in a number of situations. Volatile anesthetics may stimulate astrogliosis since sustained ERK activation has been associated with glial scar formation after brain injury [Nicole et al., 2005]. It has further been observed that ERK and RhoA are distinct pathways that control pseudopodia extension and retraction increasing cellular motility and migration [Sahai et al., 2001; Jo et al., 2002; Brahmbhatt and Klemke, 2003; Pan et al., 2006].

The anesthetic-induced RhoA activation may be of great relevance in neuronal cells. In several neuronal cell lines it has been observed that LPA, thrombin, and S-1-P trigger growth cone collapse, retraction of developing neurites, and transient rounding of the cell body [Gebbink et al., 1997]. These effects are mediated by RhoA/Rho-kinase activation [Kranenburg et al., 1999] and can be prevented by the Rho-kinase inhibitor Y27632 [Hirose et al., 1998] or by treatment of the cells with clostridium botulinum C3 exoenzyme, which ADP-ribosylates and thereby inactivates RhoA [Jalink et al., 1994]. The study of the effect of volatile anesthetics in the above-mentioned systems will provide an interesting area of future research.

ACKNOWLEDGMENTS

We thank Andrea Jahn for excellent technical assistance. This work was supported by departmental funding.

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