Receptor-mediated regulation of peroxisomal motility in CHO and endothelial cells

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This paper is dedicated to Professor Dr Hans Schimassek

The regulation of peroxisomal motility was investigated both in CHO cells and in cells derived from human umbilical vein endothelium (HUE). The cells were transfected with a construct encoding the green fluorescent protein bearing the C-terminal peroxisomal targeting signal 1. Kinetic analysis following time-lapse imaging revealed that CHO cells respond to simultaneous stimulation with ATP and lysophosphatidic acid (LPA) by reducing peroxisomal movements. When Ca^{2+} was omitted from the extracellular medium or the cells were incubated with inhibitors for heterotrimeric G_i/G_o proteins, phospholipase C, classical protein kinase C isoforms (cPKC), mitogen-activated protein kinase kinase (MEK) or phospholipase A_2 (PLA_2), this signal-mediated motility block was abolished. HUE cells grown to confluency on microporous membranes responded similarly to ATP–LPA receptor co-stimulation, but only when the ligands had access to the basolateral membrane region. These data demonstrate that peroxisomal motility is subject to specific modulation from the extracellular environment and suggest a receptor-mediated signaling cascade comprising Ca^{2+} influx, G_i/G_o proteins, phospholipase C, cPKC isoforms, MEK and PLA_2 being involved in the regulation of peroxisomal arrest.

Keywords: ATP/LPA/microtubules/organelle motility/peroxisomes

Introduction

Motility of cellular organelles has fascinated observers for many decades. Among the movements known, some are remarkable with respect to their ability to be regulated directly by the extracellular environment. Chloroplasts in cells of photosynthetic leaves, for example, frequently respond to changes in light intensity by differential distribution. Under intense light, the organelles are packed closely at the cell sides, whereas when little light is available they are dispersed evenly (for a review, see Haupt and Scheuerlein, 1990). Pigment granules of vertebrate chromatophores are distributed differentially in the cell upon neuronal or hormonal stimulation, allowing the animal to change color or pattern. The signal molecules

involved bind to G protein-coupled receptors and subsequently induce granule aggregation or dispersion (Lerner, 1994). The two processes are regulated differentially in that elevated intracellular Ca^{2+} causes granule aggregation, whereas enhanced cAMP is responsible for granule dispersion (Oshima *et al.*, 1986; Kotz and McNiven, 1994). Similarly, mitochondria in the lower Malpighian tubule of the blood-feeding bug *Rhodnius prolixus* have been reported to move from below the cell cortex into the apical microvilli upon feeding *in vivo* or serotonin application *in vitro* (Bradley and Satir, 1979).

Many cellular organelles have been described to be motile (Bereiter-Hahn, 1978; Cole and Lippincott-Schwartz, 1995; Rapp *et al.*, 1996), although the underlying mechanisms of regulation have not been elucidated in detail thus far. Frequently, the organelles exhibit movements in all directions, thus shuttling to and fro (Forman *et al.*, 1987; Murphy *et al.*, 1996; Rapp *et al.*, 1996; Huber *et al.*, 1997; Nilsson and Wallin, 1997; Wacker *et al.*, 1997; Rodionov *et al.*, 1998). However, to ensure either single directed movements, a polarized net transport or a uniform distribution of organelles, motility events have to be regulated. This regulation might, for example, affect the activity of organelle-specific motor proteins, binding of motor proteins to the organelles or, as in the case of organelle arrest, function of specific cytosolic linker proteins (Pierre *et al.*, 1992; de Zeeuw *et al.*, 1997).

As described previously (Rapp *et al.*, 1996; Huber *et al.*, 1997; Wiemer *et al.*, 1997), peroxisomes exhibit three distinct states of motility: saltation, oscillation and arrest. The motility state of a given peroxisome may change with time; however, the percentages of the whole population exhibiting these states remain unaffected. In CHO cells, under control conditions, ~10–20% of the entire peroxisome population are saltating or at rest, presupposing association of the organelles with the cytoskeleton, whereas ~70% are thermally oscillating. The peroxisomal movements were shown to be subject to regulation by a signaling cascade involving a heterotrimeric G protein of the G_i/G_o class, phospholipase A_2 (PLA_2), and arachidonic acid (AA). Activation of these proteins, followed by the release of AA, blocked peroxisomal movements (Huber *et al.*, 1997). Since in these experiments a prolonged incubation period of 20–30 min was necessary to achieve complete block of movements—G proteins are known to operate in fast signal transduction—we characterized this type of regulation further by investigating the involvement of extracellular signals. Various G protein-coupled receptors activating G_i/G_o have been described for CHO wild-type cells, including those activated by ATP (Felder *et al.*, 1991; Traiffort *et al.*, 1992; Iredale and Hill, 1993), serotonin (Berg *et al.*, 1994; Giles *et al.*, 1996), cholecystokinin (CCK) (Freund *et al.*, 1994), thrombin (Winitz *et al.*, 1994) and lysophosphatidic acid (LPA) (Jalink *et al.*,

Table I. Effect of receptor agonists and agonist combinations on the motility of peroxisomes (experiments 1–14) and various other organelles (experiments 15–17) in CHO cells

Treatment	% motility after treatment	Mean translocation before treatment	No. of organelles analyzed
1. PBS (vehicle)	101	0.35	249
2. ATP γ S + LPA	19	0.39	172
3. ATP + LPA	15	0.41	168
4. ATP γ S	90	0.32	114
5. LPA	112	0.43	103
6. LPA + ATP γ S at an interval of 1 min	13	0.39	225
7. LPA + ATP γ S at an interval of 5 min	78	0.32	285
8. ATP γ S + serotonin	96	0.39	284
9. ATP γ S + CCK-8	89	0.42	217
10. ATP γ S + thrombin	93	0.38	163
11. LPA + serotonin	95	0.47	109
12. LPA + CCK-8	88	0.41	173
13. LPA + thrombin	91	0.35	246
14. Serotonin + CCK-8	95	0.31	166
15. Mitochondria; ATP γ S + LPA	97	0.18	115
16. Lysosomes; ATP γ S + LPA	84	0.52	314
17. Endosomes; ATP γ S + LPA	94	0.40	93

Organelle motility was analyzed by time-lapse imaging and evaluated as described in Materials and methods. The change in organelle motility due to treatment is expressed as a percentage, 100% motility representing no change. The mean distance covered by an organelle between two frames is given in micrometers.

1994, 1995). In some instances, as for example the ATP receptor, stimulation of PLA₂ was demonstrated. However, since none of these agonists was able to influence peroxisomal motility, we applied various combinations of agonists. Co-stimulation of two different G protein-coupled receptors previously was shown to enhance the induction of cytoplasmic PLA₂ activity, a process involving phospholipase C (PLC), protein kinase C (PKC), Ca²⁺ influx and mitogen-activated protein kinase (MAPK) (Felder *et al.*, 1991; Lin *et al.*, 1992, 1993; Nemenoff *et al.*, 1993; Piomelli, 1993; Clark *et al.*, 1995; Fukuda *et al.*, 1996). Of all agonist combinations tested, co-stimulation with ATP and LPA (ATP–LPA) specifically inhibited peroxisomal motility both in CHO cells and in a cell line derived from human umbilical vein endothelium (HUE) (Clauss *et al.*, 1996). These data demonstrate that peroxisomal motility is subject to regulation by the extracellular environment. Since ATP and LPA receptors are widely distributed, this type of regulation might not be restricted to CHO and HUE cells.

Results

ATP–LPA co-stimulation induces peroxisomal arrest

In a search for the natural ligand(s) involved in the regulation of peroxisomal motility, we first investigated the activation of ATP receptors known to stimulate AA release in CHO cells (Felder *et al.*, 1991). However, ATP up to a concentration of 100 μ M failed to block peroxisomal motility (Huber *et al.*, 1997). One possible explanation for this result might have been that the amount of AA released was too low. Therefore, we studied co-stimulation of ATP receptors with receptors for serotonin, CCK, thrombin and LPA, known to be present endogenously and to activate G_i/G_o in CHO wild-type cells. The results of these experiments are summarized in Table I. Of the different combinations, only the simultaneous activation of ATP and LPA receptors blocked peroxisomal

motility, inhibiting both long-range saltatory and short-range oscillatory movements (cf. Huber *et al.*, 1997). ATP γ S, known to stimulate ATP receptor subtypes with a potency similar to ATP (Iredale and Hill, 1993; for a review, see Dubyak and El-Moatassim, 1993), could completely replace ATP (Table I; Figure 1). We therefore used ATP γ S to avoid possible interactions with ecto-ATPases. Given alone, LPA, like ATP or ATP γ S, did not influence peroxisomal motility (Table I; Huber *et al.*, 1997). Application of the agonists LPA and ATP γ S within a time interval of 1 min resulted in peroxisomal arrest, whereas almost no decrease in peroxisomal motility was seen when the interval was extended to 5 min (Table I), suggesting specific down-regulation of the LPA signal. Within 20 min after receptor co-stimulation, peroxisomal motility reverted to the control state (not shown).

Blocking of motility upon administration of ATP γ S and LPA was only found for peroxisomes. Mitochondria, lysosomes and endosomes did not change their movements (Table I), and cytoskeletal reorganizations were not detected in CHO cells (Figure 2; cf. Rapp *et al.*, 1996). These observations suggest that peroxisomal movements are blocked by a peroxisome-specific mechanism.

ATP γ S–LPA co-stimulation activates cytosolic PLA₂

Our observation that peroxisomal arrest requires the simultaneous activation of two different receptors may be interpreted in two ways. The receptors either act independently, each receptor activating distinct pathways, or their signaling converges on a certain point leading to synergistic effects. In order to differentiate between these two possibilities, we investigated the main constituents involved in these pathways. In a series of experiments, cells were treated with specific inhibitors for trimeric G proteins of the class G_i/G_o, PLC, classical protein kinase C isoforms (cPKC), PLA₂ and MAPK or were incubated in Ca²⁺-free medium, in order to monitor the effects of Ca²⁺ influx. G_i/G_o was inhibited by pertussis toxin (Tamura *et al.*, 1982), PLC by U-73122 (Smith *et al.*, 1990; Powis

Fig. 1. Effect of receptor activation on peroxisomal motility in CHO cells. Time-lapse imaging was performed prior to (A–C) and after (D–F) ATP–LPA receptor co-stimulation. Two consecutive images (A and B, and D and E) were acquired within a period of 16 s, and difference images (C and F) were obtained by subtracting the intensities of the corresponding pixels (image 2 minus image 1). Peroxisomes on the first image appear black, whereas those on the second image are white, visualizing displaced peroxisomes as black and white spots. Peroxisomes at rest appear gray and are hardly distinguishable from the background. Note that receptor stimulation results in almost complete loss of peroxisomal motility (F). Bar, 25 μ m.

et al., 1992), classical PKC isoforms by 100 nM Gö-6976 (Martiny-Baron *et al.*, 1993), PLA₂ by ONO-RS-082 (Banga *et al.*, 1986) and activation of MAPK was prevented by treating the cells with the MAPK kinase (MEK)-specific inhibitor PD-98059 (Pang *et al.*, 1995). Whereas all these inhibitors, as well as the Ca²⁺-free medium, did not influence peroxisomal motility *per se*, they effectively abolished peroxisomal arrest induced by ATP γ S–LPA co-stimulation (Table II; cf. Huber *et al.*, 1997). To demonstrate that the main signaling components proposed to mediate this block become activated by the agonists, we studied PKC and MAPK activation. cPKC isoforms translocate to cellular membranes upon activation (for a review, see Newton, 1997), whereas MAPK becomes specifically phosphorylated (Sturgill *et al.*, 1988; Payne *et al.*, 1991). Therefore, membrane translocation of PKC α , the only cPKC isoform present in CHO wild-type cells (Clark *et al.*, 1994; Tippmer *et al.*, 1994), and specific phosphorylation of MAPK were assayed following co-stimulation of CHO cells with LPA and ATP γ S. As revealed by immunoblot analysis, both the amount of PKC α present in membrane-enriched fractions and the amount of specifically phosphorylated MAPK increased (Figure 3), suggesting that activation has occurred.

While these results strongly support the idea that signal transduction pathways leading to block of peroxisomal motility involve PLA₂ activation, some observations suggested that additional pathways seem to be required. When we determined the release of [³H]AA as a measure of PLA₂ activation following receptor stimulation, we found

Fig. 2. Visualization of actin (A) and microtubules (B) in CHO cells treated with ATP γ S and LPA. No change in the pattern was observed in comparison with control cells (cf. Rapp *et al.*, 1996). Bar, 20 μ m.

that ATP γ S, serotonin and CCK only weakly increased [³H]AA, whereas LPA markedly enhanced the release (Figure 4). Compared with LPA, ATP γ S–LPA co-stimulation did not further enhance the release of [³H]AA. Moreover, replacing LPA by either dioctanoyl glycerol (DiC₈), an analog of PLC-generated diacylglycerol, or thymeleatoxin, an activator of cPKC (Ryves *et al.*, 1991; Roivainen and Messing, 1993), prior to stimulation with

Table II. Effects of receptor ligands as well as signaling inhibitors and activators on peroxisomal arrest in CHO cells

Pre-treatment	Receptor agonists	% motility after agonist application	Mean translocation before receptor agonist treatment	No. of peroxisomes analyzed
None	None (PBS)	101	0.35	249
None	ATP γ S-LPA	19	0.39	172
Pertussis toxin (100 ng/ml)	ATP γ S-LPA	98	0.43	194
U-73122 (2.5 μ M)	ATP γ S-LPA	93	0.40	184
Medium without Ca ²⁺	ATP γ S-LPA	88	0.35	223
Gö-6976 (100 nM)	ATP γ S-LPA	87	0.40	296
PD-98059 (25 μ M)	ATP γ S-LPA	98	0.38	110
ONO-RS-082 (25 μ M)	ATP γ S-LPA	101	0.39	335
DiC ₈ (0.3 mM)	ATP γ S	52	0.41	431
Thymeleatoxin (1 μ M)	ATP γ S	66	0.29	181
PP1 (20 μ M)	ATP γ S-LPA	91	0.42	151

Peroxisomal motility was analyzed by time-lapse imaging and evaluated as described in Materials and methods. The change in peroxisomal motility in pre-treated cells, stimulated as indicated during time-lapse imaging, is expressed as a percentage, 100% motility representing no change. The mean distance covered per peroxisome between two frames is given in micrometers.

ATP γ S decreased peroxisomal motility far less than ATP γ S-LPA co-stimulation (Table II).

In addition to PLA₂ activation, non-receptor tyrosine kinases are implicated in mediating the physiological effects of LPA receptor activation (for a review, see Moolenaar *et al.*, 1997). We therefore employed the *src* family tyrosine kinase inhibitor PP1 (Hanke *et al.*, 1996) to investigate the possible participation of these enzymes in the ATP-LPA-induced block of peroxisomal motility. Pre-treatment of the cells with PP1 abolished extracellular signal-mediated peroxisomal arrest (Table II), indicating that besides PLA₂ activation, pathways including tyrosine kinase(s) are required for the block of motility.

Polarized stimulation of ATP and LPA receptors in endothelial cells

Various cell lines, including endothelial cells (McLees *et al.*, 1995; Patel *et al.*, 1996a,b), have been demonstrated to share signal transduction pathways similar to those mentioned above for CHO cells. In order to demonstrate that the observed regulation of peroxisomal motility by ATP-LPA is not restricted to CHO cells, we analyzed movement of peroxisomes in HUE cells. Under control conditions, we observed essentially the same motility characteristics as for CHO cells. However, in contrast to CHO cells, a confluent layer of HUE cells plated on glass-bottom microwell dishes did not respond to ATP γ S-LPA co-stimulation (Table III). Since this observation might be the result of polarized receptor distribution, we selectively stimulated the basolateral membrane region with ATP γ S-LPA. This was made possible by plating the cells on microporous membranes and inverting the membrane prior to mounting on the microscope stage as depicted in Figure 5. Under these conditions, we observed complete peroxisomal arrest, which could not be achieved by application of each of the agonists alone (Table III). These data demonstrate that ATP and LPA receptors mediate peroxisomal arrest in HUE cells and suggest a basolateral membrane localization of at least one of these receptors.

Discussion

Extracellular signals affect peroxisomal motility

ATP and LPA receptors are widely distributed in various tissues and cell types, and were described to activate a

Fig. 3. Activation of PKC α and MAPK in CHO wild-type cells following co-stimulation with LPA and ATP γ S for 2 min. (A) Cytosolic (Cyt) and membrane-enriched (Mem) fractions of control and stimulated cells were immunoblotted with an antibody recognizing PKC α . (B) Cytosolic fractions were also subjected to immunoblot analysis with an antibody directed against specifically phosphorylated (activated) MAPK.

Fig. 4. Release of [³H]AA from pre-labeled CHO cells following receptor stimulation by a 20 min incubation in the presence of various receptor agonists. (A) Control treatment; (B) ATP γ S; (C) LPA; (D) ATP γ S plus LPA; (E) CCK-8; (F) serotonin; (G) CCK-8 plus serotonin. Agonist concentrations were essentially the same as those used in the motility assay. Values per well are expressed in c.p.m. and represent the mean $\pm$ SD of four independent experiments.

broad spectrum of physiological responses. As reviewed by Moolenaar *et al.* (1997), the LPA receptor may activate three distinct G proteins, G_i, G_q and G_{12/13}. G_i may activate *ras* via non-receptor tyrosine kinases and inhibit adenylyl cyclase, whereas G_q and G_{12/13} may lead to activation of PLC and *rho*, respectively. The biological responses to LPA are diverse, and include, for example, cell proliferation, stress fiber formation in serum-starved cells, neuro-

transmitter release or transient inhibition of cell communication via connexin 43. ATP, which may be either secreted or released from damaged cells, was shown to activate various cell surface receptors that could also act in concert. They include both G protein-coupled receptors (P_{2Y} subclass) and ligand-gated ion channels (P_{2X} subclass). The physiological effects of P_{2X} receptors were studied frequently in excitable cells such as neurons or muscle cells and include, for example, membrane depolarization, calcium influx, contraction or secretion. Activation of P_{2Y} receptors was found to induce, for example, calcium mobilization, phosphoinositol hydrolysis and AA release, depending on the cell type. These events frequently may serve to modulate the biological responses to other stimuli (for reviews, see Dubyak and El-Moatassim, 1993; Brake and Julius, 1996).

CHO wild-type cells contain receptors for ATP, LPA, CCK, serotonin and thrombin, known to activate trimeric G_i proteins. However, stimulation of one of these receptors did not influence peroxisomal motility. Searching for the reasons for this inability, we noticed that various cellular processes, for example pigment granule aggregation (Kumazawa *et al.*, 1984), adenylyl cyclase activation (Federman *et al.*, 1992) or tissue factor expression on the surface of endothelial cells (Clauss *et al.*, 1996), were shown to be induced by receptor co-stimulation. Similarly

to this, Felder *et al.* (1991) reported that co-stimulation by receptor ligands and extracellular ATP of CHO cells transfected with various neuronal G protein-coupled receptors potentiated the ATP-mediated release of AA. Investigating the effect of co-stimulation with dual ligand combinations, we found that co-stimulation with ATP and LPA specifically induces arrest of peroxisomes, neither affecting the movement of various other cell organelles nor triggering of cytoskeletal reorganizations, which have been observed upon LPA administration (Moolenaar *et al.*, 1997).

Signaling pathways involved in peroxisomal arrest following receptor co-stimulation of CHO cells

Surveying the literature on the signaling pathways activated by ATP and LPA (for reviews, see Dubyak and El-Moatassim, 1993; Moolenaar *et al.*, 1997) allowed us to hypothesize on the signaling events participating in ATP–LPA receptor co-stimulation, particularly the activation of PLA_2 . Following receptor-mediated Ca^{2+} influx, PLA_2 may translocate to cellular membranes, e.g. the plasma membrane (Felder *et al.*, 1990; J.D.Clark *et al.*, 1991; Lin *et al.*, 1992; Schievella *et al.*, 1995), and could be activated by phosphorylation mediated by a cascade involving PLC, Ca^{2+} influx, PKC (Felder *et al.*, 1990, 1991; for a review, see Clark *et al.*, 1995) and MAPK (Lin *et al.*, 1993; Nemenoff *et al.*, 1993). Ca^{2+} influx as well as activation of MEK as a result of PKC activation (Sozeri *et al.*, 1992; Schaap *et al.*, 1993; Hawes *et al.*, 1995; Yamaguchi *et al.*, 1995; Ueda *et al.*, 1996; van Biesen *et al.*, 1996) both might be triggered by heterotrimeric G proteins, which also have been suggested to interact directly with PLA_2 (Jelsema and Axelrod, 1987; for reviews, see Axelrod *et al.*, 1988; Clark *et al.*, 1995). Participation of trimeric G proteins of class G_i/G_o in the regulation of peroxisomal motility was documented in this study by analyzing the effects of pertussis toxin. Involvement of the other PLA_2 -activating components, PLC, cPKC, Ca^{2+} influx and MEK, was verified by using specific inhibitors that effectively prevented the ATP–LPA-induced peroxisomal arrest. In addition, activation of PKC α and MAPK following ATP–LPA treatment was shown by immunoblot analysis, demonstrating the increase in both membrane localization of PKC α and specific phosphorylation of MAPK.

While these observations underline the important role of PLA_2 in the process of regulating peroxisomal motility, some results obviously indicated the participation of additional signaling components. In particular, co-stimulation of cells with ATP γ S and DiC $_8$ or thymeleatoxin

Fig. 5. Polarized cell culturing and imaging of peroxisomal motility in HUE cells (not to scale). Cells were plated on the outside of the microporous snapwell membrane and subsequently transfected by microinjection of GFP–PTS1 cDNA (Huber *et al.*, 1997). For time-lapse imaging, the snapwell was inverted and mounted on a 24×60 mm coverslip where it adhered due to the surface tension of the Hanks' solution, allowing focusing of the cells with immersion objectives ($f = 0.17$ mm). Basolateral receptor stimulation is achieved by adding the drugs to the interior of the snapwell.

Table III. Motility of HUE cell peroxisomes following apical (cells plated on coverslips) and basolateral (cells plated on snapwells; Figure 5) stimulation of ATP and LPA receptors

Cell culturing	Receptor agonists	% motility after treatment	Mean translocation before treatment	No. of peroxisomes analyzed
Cells on coverslip	ATP γ S–LPA	78	0.35	355
Cells on snapwell	ATP γ S–LPA	9	0.30	88
Cells on snapwell	ATP γ S	99	0.38	76
Cells on snapwell	LPA	95	0.45	104

Peroxisomal motility was analyzed by time-lapse imaging and evaluated as described in Materials and methods. The change in motility due to the drug treatment is expressed as a percentage, 100% motility representing no change. The mean distance covered by an organelle between two frames is given in micrometers.

decreased peroxisomal motility to a far lesser extent than ATP γ S-LPA, suggesting that it is not only the activation of a PKC α -triggered cascade by the LPA receptor that mediates peroxisomal arrest. The observations that LPA alone does not affect peroxisomal motility and ATP-LPA co-stimulation does not increase the LPA-induced AA release further also argue for additional pathways triggered by ATP.

This view is compatible with our previous findings that PLA $_2$ inhibitors were not able to prevent peroxisomal arrest completely following direct stimulation of G proteins (Huber *et al.*, 1997). On the other hand, it seems to be contradictory to the observation that adding 1 mM AA to the medium or microinjecting PLA $_2$ -activating protein (PLAP) peptide caused peroxisomal arrest. To explain these discrepancies, we analyzed the concentration dependence of the AA-mediated block by microinjecting AA in concentrations up to 0.5 mM. These concentrations become ~10-fold diluted within the cell. No change in peroxisomal movements was noticed following this treatment (not shown), suggesting that at high concentrations AA may perturb, for example, Ca $^{2+}$ homeostasis leading to peroxisomal arrest. On the other hand, the mechanism of cytosolic PLA $_2$ activation by PLAP is not yet understood and might involve signaling cascades rather than direct interaction with the enzyme. Indications for this may be derived from the close homology of PLAP to G β proteins (cf. M.A. Clark *et al.*, 1991) and the fact that PLAP fails to activate a purified high-molecular-weight PLA $_2$ (Steiner *et al.*, 1993). Thus, the signaling cascades activated by microinjecting a PLAP peptide might well interfere with those acting on peroxisomal arrest.

Indications for the involvement of additional signaling components were also obtained by using the specific *src* family tyrosine kinase inhibitor PP1, which effectively prevented the ATP-LPA-mediated block of peroxisomal motility. *src* family tyrosine kinases might be part of the signaling machinery that is triggered by LPA receptors and otherwise comprises, for example, small GTPases of the *ras* or *rho* family (cf. Moolenaar *et al.*, 1997) or phosphoinositol-3-kinase (Takeda *et al.*, 1999). Thus, peroxisomal motility might be regulated by a network of signaling components.

Similarly to peroxisomes, movements of pigment granules in melanophores recently were demonstrated to be regulated by complex signaling pathways. These include protein kinase A and PKC, which both activate different pathways leading to granule dispersion, as well as protein phosphatase 2A, which has been shown to be involved in granule aggregation (Reilein *et al.*, 1998).

ATP-LPA co-stimulation is not restricted to CHO cells

Co-stimulatory regulation of peroxisomal motility is not unique to CHO cells, but may be of more general character. In addition to CHO cells, endothelium-derived HUE cells also respond to receptor co-stimulation by ATP γ S-LPA. Moreover, regulation of peroxisomal motility in these cells depends on their polarity in that only stimulation of the basolateral membrane region affected peroxisomal motility. This observation seems to be physiologically reasonable, since apical LPA receptors might be permanently occupied by LPA released from platelets (Eichholtz

et al., 1993; for reviews, see Moolenaar, 1995; Moolenaar *et al.*, 1997). Specific basolateral stimulation could be mediated by LPA generated by the activity of secreted PLA $_2$, acting as a distal mediator of inflammation (Vadas *et al.*, 1993), or by secreting the ligands from surrounding cells in a paracrine mode.

Physiological role of peroxisomal movements

Intracellular movements have been reported for most if not all cellular organelles, but only in a very few cases, such as axonal transport or pigment granule redistribution, is it obvious that these movements are essential for the functioning of the cell or the whole organism. However, since high amounts of energy are consumed by the cellular motors, we expect organellar movements to serve important functions. This idea is consistent with the observation that the machineries regulating these movements seem to be quite complex. Many high-copy-number organelles including peroxisomes (Rapp *et al.*, 1996; Huber *et al.*, 1997), mitochondria (Forman *et al.*, 1987), secretory vesicles (Wacker *et al.*, 1997) or endosomes (Murphy *et al.*, 1996) frequently exhibit a to-and-fro type of shuttling in any direction at any given time point, resulting either in a net transport of the organelles (cf. Murphy *et al.*, 1996; Wacker *et al.*, 1997) or in a particularly even distribution. The latter has been demonstrated for the steady-state distribution of both aggregated and dispersed pigment granules (Nilsson and Wallin, 1997; Rodionov *et al.*, 1998) and is most likely also the case for peroxisomes (Schrader *et al.*, 1996; Huber *et al.*, 1997). Thus, we assume that keeping the organelles evenly distributed or precisely driving their net transport are important functions of organelle motility, including that of peroxisomes. Among the processes most easily affected by moving organelles are, for example, the shortening of metabolite diffusion distances, the inheritance of equal organelle populations in cell division or the continuous post-translational import of proteins into organelles such as peroxisomes, mitochondria or chloroplasts. Correct net transport and positioning of organelles, ensuring the maintenance of the evolutionarily optimized cellular organization and functioning, therefore might considerably improve both the effectivity and selectivity of cellular processes.

For mitochondria, molecular components of a machinery have been described that enable the cell to organize organelle morphology and distribution. Microtubules are the heart of this machinery, and components of it include the kinesin homologs KIF1B and KIF5B, the dynamin-related proteins Drp1 and Dnm1p, an intermediate filament-like protein (Mdm1p) and various integral polypeptides of the outer mitochondrial membrane (McConnell and Yaffe, 1993; Nangaku *et al.*, 1994; Otsuga *et al.*, 1998; Smirnova *et al.*, 1998; Tanaka *et al.*, 1998; for a review, see Yaffe, 1999). Most of these components have also been identified in yeast, and a common phenotype of mutants lacking one of them is mitochondrial aggregation and a defect in transmission of mitochondria to daughter cells. KIF1B and KIF5B were localized to mitochondria isolated from mouse cells. Targeted disruption of KIF5B resulted in embryonic death, and clusters of mislocalized mitochondria were found near the nucleus of cells derived from the visceral yolk sac of the mutant embryos (Tanaka

et al., 1998). Similarly to mitochondria, peroxisomes display striking differences in shape and distribution (Gorgas, 1984, 1987). Peroxisomal morphology may vary between spherical and extended tubular structures, the latter of which may form dynamic networks. In various polarized cells, peroxisomes frequently are localized to specific cytoplasmic regions (Zaar *et al.*, 1984; Bradke and Dotti, 1997), raising the question as to the molecular components mediating and regulating the morphology, distribution and dynamics of the organelles. As with mitochondria, interaction of peroxisomes with microtubules may play a key role in these processes, a view that is supported by the observation of locally aggregating peroxisomes following either depletion of microtubules (Schrader *et al.*, 1996) or impairment of biogenesis (Passreiter *et al.*, 1998).

As discussed above, cells respond to a specific signal by peroxisomal arrest, demonstrating that peroxisomal function can be modulated directly by physiological signals from the extracellular environment. Following a decrease in peroxisomal motility that is expected to interfere with metabolite diffusion, both the clearance of peroxisomal substrates and the evenness of distribution of peroxisomal products might be reduced. One example might be AA which is involved in inducing peroxisomal arrest; it is also a substrate for peroxisomal β -oxidation (Jedlitschky *et al.*, 1991) and therefore might be part of a feedback loop regulating its distribution. Similarly to AA, both ATP and LPA can serve as locally active messengers, involved for example in inflammatory responses, and both may function as mitogens (Ishikawa *et al.*, 1997; Neary *et al.*, 1998; for reviews, see Dubyak and El-Moatassim, 1993; Piomelli, 1993; Clark *et al.*, 1995; Moolenaar, 1995; Moolenaar *et al.*, 1997). Related to the physiological role of ATP-LPA-mediated peroxisomal arrest, we thus might speculate that this cellular response is involved in the induction of inflammation and subsequent regeneration upon injury.

Materials and methods

Cell culture and transfection

CHO-K1 cells stably expressing the green fluorescent protein containing the peroxisomal targeting signal 1 (GFP-PTS1) were maintained as described (Huber *et al.*, 1997). HUE cells kindly provided by Dr W. Risau (Max-Planck-Institut für Physiologische und Klinische Forschung, Bad Nauheim, Germany) were cultured in MCDB-131 medium (Sigma, Deisenhofen, Germany) to which was added 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin (Biochrom KG, Berlin, Germany) and 1700 IU/l heparin (Liquemin®; Hoffmann-La Roche, Grenzach-Wyhlen, Germany). For fluorescence microscopy, cells were plated on 32 mm culture dishes containing a central glass coverslip (MatTek Corp., Ashland, MA). Polarized receptor stimulation was investigated in HUE cells grown to confluency on the outer surface of a microporous membrane (snapwell™; Corning Costar, Bodenheim, Germany). The snapwell was inverted, mounted on a 60 $\times$ 24 mm glass coverslip and installed on the microscope stage. Basolateral receptor stimulation was performed during image acquisition by adding the drugs to the upper snapwell reservoir (Figure 5). HUE cells were transfected by microinjecting the GFP-PTS1 vector dissolved in microinjection buffer (Rapp *et al.*, 1996) 1 day prior to analysis.

Receptor activation and drug treatment

Stock solutions [10 mM in phosphate-buffered saline (PBS)] of ATP (Boehringer Mannheim, Mannheim, Germany) and ATP γ S (Sigma, Deisenhofen, Germany) were added to the cells at final concentrations of 75–125 μ M. LPA (C18:1; Sigma, Deisenhofen, Germany) was bound

Fig. 6. Histogram showing the distances covered by peroxisomes in CHO cells before (white bars) and after (black bars) stimulation of ATP and LPA receptors. The x-axis shows translocation between two subsequent frames, given in pixels (one pixel corresponding to 108 nm), and the y-axis shows the numbers of peroxisomes. Before treatment, the total translocation of the 172 peroxisomes counted was 628 pixels (67.8 μ m), resulting in a mean translocation of $0.39 \pm 0.38 \mu$ m. Following receptor stimulation, the total translocation of the 166 peroxisomes counted was 118 pixels (12.7 μ m), yielding a mean translocation of $0.08 \pm 0.17 \mu$ m.

to fatty acid-free bovine serum albumin (BSA) (Boehringer Mannheim, Mannheim, Germany) at a weight ratio of 1:10 and applied at concentrations of 37.5–50 μ M. Serotonin (ICN, Eschwege, Germany) was dissolved in PBS and applied at concentrations of 100–150 μ M. The sulfated C-terminal octapeptide of cholecystokinin (CCK-8; Peptides International, Bad Homburg, Germany) was dissolved in 1% (w/v) NaHCO₃ according to the manufacturer's recommendation and used at concentrations between 5 and 7.5 μ M, whereas thrombin (Boehringer Mannheim, Mannheim, Germany) was added to the cells at a concentration of 1 U/ml from a 100-fold stock. U-73122 (Biomol, Hamburg, Germany), Gö-6976 (Alexis, Grünberg, Germany), PD-98059 (Biomol, Hamburg, Germany), ONO-RS-082 (Biomol, Hamburg, Germany) and PP1 (Calbiochem, Bad Soden, Germany) were dissolved in dimethyl sulfoxide (DMSO) and added to the cells at final concentrations of 2.5–5 μ M, 100 nM, 20–25 μ M, 15–25 μ M and 20 μ M, respectively. DiC₈ (Biomol, Hamburg, Germany) and the PKC activator thymeleatoxin (Biomol, Hamburg, Germany) were dissolved in ethanol and added to the medium at final concentrations of 0.3 mM and 1 μ M, respectively. Final concentrations of DMSO and ethanol did not exceed 0.4%, which did not influence the motility of peroxisomes. Pertussis toxin (100 ng/ml) (Biomol, Hamburg, Germany) was added to the cells 24 h prior to analysis. Whereas receptor agonists were given simultaneously to the cells during time-lapse imaging, the signaling inhibitors and activators were applied 30 min (U-73122 and ONO-RS-082) or 10–20 min (Gö-6976, PD-98059, PP1, DiC₈ and thymeleatoxin) prior to video analysis and receptor stimulation. Immediately before fluorescence microscopy or drug treatment, the culture medium was replaced by Hank's buffered salt solution (Sigma, Deisenhofen, Germany) or Ca²⁺-free PBS. Mitochondria and lysosomes were labeled by pre-treatment of the cells according to the manufacturer's recommendation with MitoTracker™ and LysoTracker™ (Molecular Probes, Leiden, The Netherlands), respectively. Endosomes were visualized by fluorescein isothiocyanate (FITC)-dextran (mol. wt 70 000; Sigma, Deisenhofen, Germany) which was added to the medium for 15 min. Following washing, cells were incubated for a further 45 min prior to analysis.

Imaging and quantitation

Time-lapse imaging was performed on a Zeiss Axiovert 10 inverted microscope (Zeiss, Oberkochen, Germany). Images were acquired with a Photometrics CH250 CCD camera (1317 $\times$ 1035 pixels) using the KHOROS software package (Rasure *et al.*, 1990) as described by Herr *et al.* (1993). A 63-fold magnification objective (Zeiss Plan-Achromat, $\times$ 63, N.A. 1.4) was used, each pixel thus corresponding to a 108 $\times$ 108 nm area. The series usually consisted of 31 pictures taken every 16 s, including exposure time ($\sim$ 1 s), which was adjusted according to the fluorescence intensity. (Videos of the time-lapse imaging may be viewed at: <http://www.rzuser.uni-heidelberg.de/~C14/movies/index.html>)

Changes in peroxisomal motility were determined by the following procedure: first, the time series were evaluated by animation. Most

assays were repeated three times; some experiments were performed up to six times. Secondly, two subsequent frames of a time series suitable for further evaluation (i.e. exhibiting low background, no movements of the coverslip out of the focus plane or of the microscope stage, etc.) were selected to generate difference images, one showing the cells prior to and the other after agonist application (Figure 1). Thirdly, by measuring the translocation of peroxisomes visible on both frames using the PMIS software (Photometrics, Tucson, AZ), histograms were generated, showing the number of peroxisomes covering certain distances (Figure 6). Finally, total translocation was normalized to the number of peroxisomes. By dividing the mean translocation determined after by that before agonist application, the percentages demonstrating the effects of receptor agonists were obtained (Tables I–III). Thus, a value of 100% represents no inhibition, whereas 0% indicates complete inhibition of organelle motility. As revealed by repeated measurements on a single time series, an error of up to 20% could be inherent in this method of evaluation.

Immunofluorescence

Cells stimulated with ATP γ S–LPA for 5 min were washed by quickly bathing the coverslips in PBS pH 7.4, and after fixation for 20 min in 3.7% formaldehyde in PBS they were permeabilized by 1% Triton X-100 in PBS for 4 min. Subsequently, cells were washed again with PBS containing 25 mM glycine and incubated at 37°C for 30 min with the primary antibody (anti- α -tubulin; Amersham Buchler, Braunschweig, Germany) diluted in PBS. A FITC-coupled secondary antibody was used to decorate the first antibody. F-actin was visualized by FITC-phalloidin, which was kindly provided by Dr H. Faulstich (Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany).

Immunoblot analysis

Subconfluent (~60%) CHO wild-type cells (8 cm plates) were deprived of serum by culture in serum-free α -minimal essential medium (MEM) (Biochrom, Berlin, Germany) supplemented with 0.2% fatty acid-free BSA for 40 h. Following co-stimulation with LPA and ATP γ S for 2 min, the cells were washed once in ice-cold PBS, scraped in 0.22 ml ice-cold homogenization buffer (20 mM Tris–HCl pH 7.5, 125 mM NaCl, 0.5 mM CaCl $_2$, 0.6 mM MgCl $_2$, 0.33 mM Na $_2$ VO $_4$, 1 mM phenylmethylsulfonyl fluoride, 6.7 μ g/ml leupeptin) and were homogenized in a Teflon pestle tissue grinder. After centrifugation at 17 000 g for 10 min at 0°C, the supernatants were collected and used as a source for MAPK and cytosolic PKC α . The pellets were dissolved in homogenization buffer containing 0.5% Triton X-100, sonicated for 5 min and centrifuged at 17 000 g for 10 min, thus yielding the membrane (solubilized particulate) fraction.

Equal amounts of protein per lane were subjected to SDS–PAGE as described by Laemmli (1970). Following transfer of the separated proteins to polyvinylidene difluoride membranes, PKC α and activated MAPK were immunodecorated using an isoform-specific rabbit polyclonal antibody against PKC α (Sigma, Deisenhofen, Germany) or the mouse antibody (clone 12D4; Biomol, Hamburg, Germany) specifically recognizing the pThr–Glu–pTyr motif of activated p42/p44 MAPK. Immunoreactive bands were detected by the enhanced chemiluminescence detection system (Amersham-Buchler, Braunschweig, Germany).

Measurement of [3 H]AA release

CHO cells were plated onto 24-well cluster dishes at 4×10^4 cells per well. [3 H]AA release as a measure of PLA $_2$ activation was determined after labeling of the cells for 2 h at 37°C with 0.5 μ Ci of [3 H]AA (Hartmann Analytic, Braunschweig, Germany). Radioactivity was added in 0.5 ml of bicarbonate and serum-free Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES pH 7.4 and 0.2% fatty acid-free BSA. Cells were washed twice with 1 ml of DMEM supplemented as above and incubated for 20 min in 0.5 ml of the same medium containing the receptor agonists. [3 H]AA release into the medium of each well was determined by liquid scintillation counting.

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References

- Axelrod, J., Burch, R.M. and Jelsema, C.L. (1988) Receptor-mediated activation of phospholipase A $_2$ via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.*, **11**, 117–123.
- Banga, H.S., Simons, E.R., Brass, L.F. and Rittenhouse, S.E. (1986) Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. *Proc. Natl Acad. Sci. USA*, **83**, 9197–9201.
- Bereiter-Hahn, J. (1978) Intracellular motility of mitochondria: role of the inner compartment in migration and shape changes of mitochondria in XTH cells. *J. Cell Sci.*, **30**, 99–115.
- Berg, K.A., Clarke, W.P., Sailstad, C., Saltzman, A. and Maayani, S. (1994) Signal transduction differences between 5-hydroxytryptamine type 2A and type 2C receptor systems. *Mol. Pharmacol.*, **46**, 477–484.
- Bradke, F. and Dotti, C.G. (1997) Neuronal polarity: vectorial cytoplasmic flow precedes axon formation. *Neuron*, **19**, 1175–1186.
- Bradley, T.J. and Satir, P. (1979) Evidence of microfilament-associated mitochondrial movement. *J. Supramol. Struct.*, **12**, 165–175.
- Brake, A.J. and Julius, D. (1996) Signaling by extracellular nucleotides. *Annu. Rev. Cell. Dev. Biol.*, **12**, 519–541.
- Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N. and Knopf, J.L. (1991) A novel arachidonic acid-selective cytosolic PLA $_2$ contains a Ca $^{2+}$ -dependent translocation domain with homology to PKC and GAP. *Cell*, **65**, 1043–1051.
- Clark, J.D., Schievella, A.R., Nalefski, E.A. and Lin, L.-L. (1995) Cytosolic phospholipase A $_2$. *J. Lipid Med. Cell Signal.*, **12**, 83–117.
- Clark, M.A., Ozgur, L.E., Conway, T.M., Dispoto, J., Crooke, S.T. and Bomalaski, J.S. (1991) Cloning of a phospholipase A $_2$ -activating protein. *Proc. Natl Acad. Sci. USA*, **88**, 5418–5422.
- Clark, S., Keogh, R. and Dunlop, M. (1994) The role of protein kinase C in arachidonic acid release and prostaglandin E production from CHO cells transfected with EGF receptors. *Biochim. Biophys. Acta*, **1224**, 221–227.
- Clauss, M., Grell, M., Fangmann, C., Fiers, W., Scheurich, P. and Risau, W. (1996) Synergistic induction of endothelial tissue factor by tumour necrosis factor and vascular endothelial growth factor: functional analysis of the tumour necrosis factor receptors. *FEBS Lett.*, **390**, 334–338.
- Cole, N.D. and Lippincott-Schwartz, J. (1995) Organization of organelles and membrane traffic by microtubules. *Curr. Opin. Cell Biol.*, **7**, 55–64.
- De Zeeuw, C.I., Hoogenraad, C.C., Goedknegt, E., Hertzberg, E., Neubauer, A., Grosveld, F. and Galjart, N. (1997) CLIP-115, a novel brain-specific cytoplasmic linker protein, mediates the localization of dendritic lamellar bodies. *Neuron*, **19**, 1187–1199.
- Dubyak, G.S. and El-Moatassim, C. (1993) Signal transduction via P $_2$ -purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265**(3 Part 1), C577–C606.
- Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W.H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.*, **291**, 677–680.
- Federman, A.D., Conklin, B.R., Schrader, K.A., Reed, R.R. and Bourne, H.R. (1992) Hormonal stimulation of adenylyl cyclase through G $_i$ -protein $\beta\gamma$ subunits. *Nature*, **356**, 159–161.
- Felder, C.C., Dieter, P., Kinsella, J., Tamura, K., Kanterman, R.Y. and Axelrod, J. (1990) A transfected m $_5$ muscarinic acetylcholine receptor stimulates phospholipase A $_2$ by inducing both calcium influx and activation of protein kinase C. *J. Pharmacol. Exp. Ther.*, **255**, 1140–1147.
- Felder, C.C., Williams, H.L. and Axelrod, J. (1991) A transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding protein G $_i$ that amplifies ATP-mediated arachidonic acid release. *Proc. Natl Acad. Sci. USA*, **88**, 6477–6480.
- Forman, D.S., Lynch, K.J. and Smith, R.S. (1987) Organelle dynamics in lobster axons: anterograde, retrograde and stationary mitochondria. *Brain Res.*, **412**, 96–106.
- Freund, S., Ungerer, M. and Lohse, M.J. (1994) A $_1$ adenosine receptors expressed in CHO cells couple to adenylyl cyclase and to phospholipase C. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **350**, 49–56.

- Fukuda,K., Kato,S., Morikawa,H., Shoda,T. and Mori,K. (1996) Functional coupling of the δ -, μ - and κ -opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. *J. Neurochem.*, **67**, 1309–1316.
- Giles,H., Lansdell,S.J., Bolfo,M.-L., Wilson,H.L. and Martin,G.R. (1996) Characterization of a 5-HT_{1B} receptor on CHO cells: functional responses in the absence of radioligand binding. *Br. J. Pharmacol.*, **117**, 1119–1126.
- Gorgas,K. (1984) Peroxisomes in sebaceous glands. V. Complex peroxisomes in the mouse preputial gland: serial sectioning and three-dimensional reconstruction studies. *Anat. Embryol. Berl.*, **169**, 261–270.
- Gorgas,K. (1987) Morphogenesis of peroxisomes in lipid synthesizing epithelia. In Fahimi,H.D. and Sies,H. (eds), *Peroxisomes in Biology and Medicine*. Springer-Verlag, Heidelberg, Germany, pp. 3–17.
- Hanke,J.H., Gardner,J.P., Dow,R.L., Changelian,P.S., Brissette,W.H., Weringer,E.J., Pollok,B.A. and Connelly,P.A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem.*, **271**, 695–701.
- Haupt,W. and Scheuerlein,R. (1990) Chloroplast movement. *Plant Cell Environ.*, **13**, 595–614.
- Hawes,B.E., van Biesen,T., Koch,W.J., Luttrell,L.M. and Lefkowitz,R.J. (1995) Distinct pathways of G_i- and G_q-mediated mitogen-activated protein kinase activation. *J. Biol. Chem.*, **270**, 17148–17153.
- Herr,S., Bastian,T., Pepperkok,R., Boulin,C. and Ansorge,W. (1993) A fully automated image acquisition and analysis system for low light level fluorescence microscopy. *Methods Mol. Cell Biol.*, **4**, 164–170.
- Huber,C.M., Saffrich,R., Anton,M., Paßreiter,M., Ansorge,W., Gorgas,K. and Just,W.W. (1997) A heterotrimeric G protein-phospholipase A₂ signaling cascade is involved in the regulation of peroxisomal motility in CHO cells. *J. Cell Sci.*, **110**, 2955–2968.
- Iredale,P.A. and Hill,S.J. (1993) Increases in intracellular calcium via activation of an endogenous P₂-purinoceptor in cultured CHO-K1 cells. *Br. J. Pharmacol.*, **110**, 1305–1310.
- Ishikawa,S., Higashiyama,M., Kusaka,I., Saito,T., Nagasaka,S., Fukuda,S. and Saito,T. (1997) Extracellular ATP promotes cellular growth of renal inner medullary collecting duct cells mediated via P₂U receptors. *Nephron*, **76**, 208–214.
- Jalink,K., Hordijk,P.L. and Moolenaar,W.H. (1994) Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim. Biophys. Acta*, **1198**, 185–196.
- Jalink,K. *et al.* (1995) Lysophosphatidic acid-induced Ca²⁺ mobilization in human A431 cells: structure–activity analysis. *Biochem. J.*, **307**, 609–616.
- Jedlitschky,G., Huber,M., Völkl,A., Müller,M., Leier,I., Müller,J., Lehmann,W.D., Fahimi,H.D. and Keppler,D. (1991) Peroxisomal degradation of leukotrienes by beta-oxidation from the omega-end. *J. Biol. Chem.*, **266**, 24763–24772.
- Jelsema,C.L. and Axelrod,J. (1987) Stimulation of phospholipase A₂ activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit. *Proc. Natl Acad. Sci. USA*, **84**, 3623–3627.
- Kotz,K.J. and McNiven,M.A. (1994) Intracellular calcium and cAMP regulate directional pigment movements in teleost erythrophores. *J. Cell Biol.*, **124**, 463–474.
- Kumazawa,T., Oshima,N., Fujii,R. and Miyashita,Y. (1984) Release of ATP from adrenergic nerves controlling pigment aggregation in *Tilapia* melanophores. *Comp. Biochem. Physiol. C*, **78**, 1–4.
- Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lerner,M.R. (1994) Tools for investigating functional interactions between ligands and G-protein-coupled receptors. *Trends Neurosci.*, **17**, 142–146.
- Lin,L.-L., Lin,A.Y. and Knopf,J.L. (1992) Cytosolic phospholipase A₂ is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl Acad. Sci. USA*, **89**, 6147–6151.
- Lin,L.-L., Wartmann,M., Lin,A.Y., Knopf,J.L., Seth,A. and Davis,R.J. (1993) cPLA₂ is phosphorylated and activated by MAP kinase. *Cell*, **72**, 269–278.
- Martiny-Baron,G., Kazanietz,M.G., Mischak,H., Blumberg,P.M., Kochs,G., Hug,H., Marme,D. and Schachtele,C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.*, **268**, 9194–9197.
- McConnell,S.J. and Yaffe,M.P. (1993) Intermediate filament formation by a yeast protein essential for organelle inheritance. *Science*, **260**, 687–689.
- McLees,A., Graham,A., Malarkey,K., Gould,G.W. and Plevin,R. (1995) Regulation of lysophosphatidic acid-stimulated tyrosine phosphorylation of mitogen-activated protein kinase by protein kinase C- and pertussis toxin-dependent pathways in the endothelial cell line EAhy 926. *Biochem. J.*, **307**, 743–748.
- Moolenaar,W.H. (1995) Lysophosphatidic acid signalling. *Curr. Opin. Cell Biol.*, **7**, 203–210.
- Moolenaar,W.H., Kranenburg,O., Postma,F.R. and Zondag,G.C.M. (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.*, **9**, 168–173.
- Murphy,C. *et al.* (1996) Endosome dynamics regulated by a rho protein. *Nature*, **384**, 427–432.
- Nangaku,M., Sato-Yoshitake,R., Okada,Y., Noda,Y., Takemura,R., Yamazaki,H. and Hirokawa,N. (1994) KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria. *Cell*, **79**, 1209–1220.
- Neary,J.T., McCarthy,M., Kang,Y. and Zuniga,S. (1998) Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures. *Neurosci. Lett.*, **242**, 159–162.
- Nemenoff,R.A., Winitz,S., Qian,N.-X., van Putten,V., Johnson,G.L. and Heasley,L.E. (1993) Phosphorylation and activation of a high molecular weight form of phospholipase A₂ by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.*, **268**, 1960–1964.
- Newton,A.C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell Biol.*, **9**, 161–167.
- Nilsson,H. and Wallin,M. (1997) Evidence for several roles of dynein in pigment transport in melanophores. *Cell Motil. Cytoskel.*, **38**, 397–409.
- Oshima,N., Yamaji,N. and Fujii,R. (1986) Adenosine receptors mediate pigment dispersion in leucophores of the medaka, *Oryzias latipes*. *Comp. Biochem. Physiol. C*, **85**, 245–248.
- Otsuga,D., Keegan,B.R., Brisch,E., Thatcher,J.W., Hermann,G.J., Bleazard,W. and Shaw,J.M. (1998) The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J. Cell Biol.*, **143**, 333–349.
- Pang,L., Sawada,T., Decker,S.J. and Saltiel,A.R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.*, **270**, 13585–13588.
- Passreiter,M., Anton,M., Lay,D., Frank,R., Harter,C., Wieland,F.T., Gorgas,K. and Just,W.W. (1998) Peroxisome biogenesis: involvement of ARF and coatamer. *J. Cell Biol.*, **141**, 373–383.
- Patel,V., Brown,C. and Boarder,M.R. (1996a) Protein kinase C isoforms in bovine aortic endothelial cells: role in regulation of P_{2Y}- and P_{2U}-purinoceptor-stimulated prostacyclin release. *Br. J. Pharmacol.*, **118**, 123–130.
- Patel,V., Brown,C., Goodwin,A., Wilkie,N. and Boarder,M.R. (1996b) Phosphorylation and activation of p42 and p44 mitogen-activated protein kinase are required for the P₂ purinoceptor stimulation of endothelial prostacyclin production. *Biochem. J.*, **320**, 221–226.
- Payne,D.M., Rossomando,A.J., Martino,P., Erickson,A.K., Her,J.H., Shabanowitz,J., Hunt,D.F., Weber,M.J. and Sturgill,T.W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.*, **10**, 885–892.
- Pierre,P., Scheel,J., Rickard,J.E. and Kreis,T.E. (1992) CLIP-170 links endocytic vesicles to microtubules. *Cell*, **70**, 887–900.
- Piomelli,D. (1993) Arachidonic acid in cell signaling. *Curr. Opin. Cell Biol.*, **5**, 274–280.
- Powis,G., Seewald,M.J., Gratas,C., Melder,D., Riebow,J. and Modest,E.J. (1992) Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. *Cancer Res.*, **52**, 2835–2840.
- Rapp,S., Saffrich,R., Anton,M., Jäkle,U., Ansorge,W., Gorgas,K. and Just,W.W. (1996) Microtubule-based peroxisomal movement. *J. Cell Sci.*, **109**, 837–849.
- Rasure,J., Williams,C., Argiro,D. and Sauer,T. (1990) A visual language and software development environment for image processing. *Int. J. Imaging Systems Technol.*, **2**, 183–199.
- Reilein,A.S., Tint,I.S., Peunova,N.I., Enikolopov,G.N. and Gelfand,V.I. (1998) Regulation of organelle movement in melanophores by a protein kinase A (PKA), protein kinase C (PKC), and protein phosphatase 2A (PP2A). *J. Cell Biol.*, **142**, 803–813.
- Rodionov,V.I., Hope,A.J., Svitkina,T.M. and Borisy,G.G. (1998) Functional coordination of microtubule and actin based motility in melanophores. *Curr. Biol.*, **8**, 165–168.

- Roivainen, R. and Messing, R.O. (1993) The phorbol derivatives thymeleatoxin and 12-deoxyphorbol-13-*O*-phenylacetate-10-acetate cause translocation and down-regulation of multiple protein kinase C isozymes. *FEBS Lett.*, **319**, 31–34.
- Ryves, W.J., Evans, A.T., Olivier, A.R., Parker, P.J. and Evans, F.J. (1991) Activation of the PKC-isotypes α , β 1, γ , δ and ϵ by phorbol esters of different biological activities. *FEBS Lett.*, **288**, 5–9.
- Schaap, D., van der Wal, J., Howe, L.R., Marshall, C.J. and van Blitterswijk, W.J. (1993) A dominant-negative mutant of raf blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21ras. *J. Biol. Chem.*, **268**, 20232–20236.
- Schievella, A., Regier, M.K., Smith, W.L. and Lin, L.-L. (1995) Calcium-mediated translocation of cytosolic phospholipase A₂ to the nuclear envelope and endoplasmic reticulum. *J. Biol. Chem.*, **270**, 30749–30754.
- Schrader, M., Burkhardt, J.K., Baumgart, E., Lüers, G., Spring, H., Völkl, A. and Fahimi, H.D. (1996) Interaction of microtubules with peroxisomes: tubular and spherical peroxisomes in HepG2 cells and their alterations induced by microtubule active drugs. *Eur. J. Cell Biol.*, **69**, 24–35.
- Smirnova, E., Shurland, D.L., Ryazantsev, S.N. and van der Bliek, A.M. (1998) A human dynamin-related protein controls the distribution of mitochondria. *J. Cell Biol.*, **143**, 351–358.
- Smith, R.J., Sam, L.M., Justen, J.M., Bundy, G.L., Bala, G.A. and Bleasdale, J.E. (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J. Pharmacol. Exp. Ther.*, **253**, 688–697.
- Sozeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G.E. and Stabel, S. (1992) Activation of the c-raf protein kinase by protein kinase C phosphorylation. *Oncogene*, **7**, 2259–2262.
- Steiner, M.R., Bomalaski, J.S. and Clark, M.A. (1993) Responses of purified phospholipases A₂ to phospholipase A₂ activating protein (PLAP) and melittin. *Biochim. Biophys. Acta*, **1166**, 124–130.
- Sturgill, T.W., Ray, L.B., Erikson, E. and Maller, J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, **334**, 715–718.
- Takeda, H. *et al.* (1999) PI 3-kinase γ and protein kinase C- ζ mediate RAS-independent activation of MAP kinase by a G_i protein-coupled receptor. *EMBO J.*, **18**, 386–395.
- Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A–B model. *Biochemistry*, **21**, 5516–5522.
- Tanaka, Y., Kanai, Y., Okada, Y., Nonaka, S., Takeda, S., Harada, A. and Hirokawa, N. (1998) Targeted disruption of mouse conventional kinesin heavy chain, KIF5B, results in abnormal perinuclear clustering of mitochondria. *Cell*, **93**, 1147–1158.
- Tippmer, S., Quitterer, U., Kolm, V., Faussner, A., Roscher, A., Mosthaf, L., Müller-Esterl, W. and Häring, H. (1994) Bradykinin induces translocation of the protein kinase C isoforms α , ϵ , and ζ . *Eur. J. Biochem.*, **225**, 297–304.
- Traiffort, E., Ruat, M., Arrang, J., Leurs, R., Piomelli, D. and Schwartz, J. (1992) Expression of a cloned rat histamine H₂ receptor mediating inhibition of arachidonic acid release and activation of cAMP accumulation. *Proc. Natl Acad. Sci. USA*, **89**, 2649–2653.
- Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K. and Ohno, S. (1996) Protein kinase C activates the MEK–ERK pathway in a manner independent of ras and dependent on raf. *J. Biol. Chem.*, **271**, 23512–23519.
- Vadas, P., Browning, J., Edelson, J. and Pruzanski, W. (1993) Extracellular phospholipase A₂ expression and inflammation: the relationship with associated disease states. *J. Lipid Mediat.*, **8**, 1–30.
- van Biesen, T., Hawes, B., Raymond, J.R., Luttrell, L.M., Koch, W.J. and Lefkowitz, R.L. (1996) G_o-protein α subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. *J. Biol. Chem.*, **271**, 1266–1269.
- Wacker, I., Kaether, C., Krömer, A., Migala, A., Almers, W. and Gerdes, H.-H. (1997) Microtubule-dependent transport of secretory vesicles visualized in real time with a GFP-tagged secretory protein. *J. Cell Sci.*, **110**, 1453–1463.
- Wiemer, E.A.C., Wenzel, T., Deerinck, T.J., Ellisman, M.H. and Subramani, S. (1997) Visualization of the peroxisomal compartment in living mammalian cells: dynamic behaviour and association with microtubules. *J. Cell Biol.*, **136**, 71–80.
- Winitz, S., Gupta, S.K., Qian, N.-X., Heasley, L.E., Nemenoff, R.A. and Johnson, G.L. (1994) Expression of a mutant G_{i2} α subunit inhibits ATP and thrombin stimulation of cytoplasmic phospholipase A₂-mediated arachidonic acid release independent of Ca²⁺ and mitogen-activated protein kinase regulation. *J. Biol. Chem.*, **269**, 1889–1896.
- Yaffe, M.P. (1999) The machinery of mitochondrial inheritance and behavior. *Science*, **283**, 1493–1497.
- Yamaguchi, K., Ogita, K., Nakamura, S. and Nishizuka, Y. (1995) The protein kinase C isoforms leading to MAP-kinase activation in CHO cells. *Biochem. Biophys. Res. Commun.*, **210**, 639–647.
- Zaar, K., Hartig, F., Fahimi, H.D. and Gorgas, K. (1984) Peroxisomal aggregates forming large stacks in the lipid segment of the canine kidney. *Acta Histochem. Suppl.*, **29**, 165–168.

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