Reactive oxygen species are important mediators of taurine release from skeletal muscle cells

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The ability to regulate cell volume is a crucial constituent of mammalian cell homeostasis and has an important impact on cell function (metabolism, secretion) and cell fate (proliferation, apoptosis) (22). Cells exposed to hypotonic solutions respond by rapid cell swelling due to a high water permeability, followed by a regulatory volume decrease due to net loss of intracellular inorganic ions and organic osmolytes, e.g., free amino acids, polyols, and methylamines. Taurine, a sulphur-containing amino acid, is abundant in most mammalian cells, and the cellular taurine concentration in e.g., skeletal muscle is reported to vary between 10 and 60 mM, which is ~500 times higher than the taurine concentration in plasma (1, 4). The cellular taurine concentration is kept high by a Na+-dependent taurine transporter, which is highly expressed in skeletal muscle (37). Osmotic perturbation, as well as hormonal stimulation and pathological factors such as ischemia, have been shown to lead to the release of taurine from various cells, including cardiac myocytes (15), brain neurons (25, 38), and skeletal muscle (19). In skeletal muscle, there is considerable evidence that contractile activity leads to loss of taurine from the muscle cell (36) and increase in plasma taurine (4). Furthermore, we have recently demonstrated that addition of the phospholipase A2 (PLA2) product lysophosphatidyl choline (LPC) leads to taurine loss from muscle cells (20). Although taurine is an important constituent in the volume regulatory mechanism in muscle, it has also been shown to modulate various steps in the excitation-contraction coupling (1) and promote cellular membrane stabilization (12). However, the signal cascades involved in activation of the osmolyte releasing pathways after swelling, ischemia, or contraction remain incompletely understood (20, 22).

The swelling-induced activation of taurine release in various cells involves PLA2-mediated release of arachidonic acid from phospholipids and sequential formation of a lipoxygenase product by the 5-lipoxygenase (5-LO) (17, 21, 40). On the other hand, the LPC-induced taurine release seems to involve a calmodulin-/calmodulin-dependent kinase II (CaMKII)-modulated production of reactive oxygen species (ROS), (18). However, in spite of apparent differences in the signaling cascades, both the swelling-induced, as well as the LPC-induced, pathways comprise PLA2 activity, but the type of fatty acid and lysophospholipids formed by the PLA2 and the subsequent physiological consequences depend on the isoform and the substrate specificity of the PLA2, as well as the subcellular site of action.

An increase in ROS production has been demonstrated under osmotic cell swelling, muscle contraction, anoxia, and sepsis (16, 25, 33). However, it is at present not unequivocal whether ROS production is upstream or downstream of PLA2 activation. One suggested mechanism for ROS production involves an increase in the cytosolic Ca2+ concentration ([Ca2+]i), reduction in ATP, and a subsequent activation of PLA2 (secretory type, sPLA2), whereupon the released fatty acid.

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acids interfere with the electron transport chain in the mitochondria, resulting in increased ROS production (32). On the other hand, anoxia and ROS have been reported to directly increase PLA2 activity, either due to peroxidation of lipids, reduction in membrane fluidity, and/or oxidation of protein side chains or sulfhydryl formation (25, 27). Additionally, ROS may directly modulate the activity of arachidonic acid oxidation by activation of, e.g., 5-LO (42).

It follows that activation of the osmolyte-releasing pathways potentially comprise PLA2 activity, polyunsaturated fatty acids, lysophospholipids, ROS, and [Ca2+]i. In the present study, we have used myotubes, derived from porcine primary satellite cells, and C2C12 myotubes as model systems for skeletal muscle cells to 1) describe the taurine efflux pathways after hypotonicity, anoxia, and exogenous addition of the lipase activator melittin or LPC and 2) to analyze the role of PLA2, ROS, and [Ca2+]i, in the signaling cascades involved in the activation of the taurine efflux. Cell cultures have a number of important advantages in the study of cell signaling, i.e., 1) release of osmolytes can be examined with a relatively high time resolution, 2) quantitative estimation of variations in the intracellular level of free Ca2+ and ROS production are easily obtained, 3) osmotic or ischemic perturbation, or addition of various drugs or metabolites, has nearly instant effects, and 4) relatively pure muscle cell systems can be obtained compared with intact preparations. Based on the present results and previously published data, we present a model (Fig. 7) illustrating the role of PLA2 and ROS in release of the organic osmolyte taurine from muscle.

MATERIALS AND METHODS

Chemicals. Antibiotics, fetal bovine serum (FCS), horse serum (HS), Dulbecco's modified Eagle's medium (DMEM; high glucose) with L-glutamine, and PBS were from Life Technologies (Naperville, IL). [14C]taurine was from NEN Life Science Products, (Boston, MA). Fura 2-AM, 5-(and-6)carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), pluronic F-127, and ionomycin were purchased from Molecular Probes (Eugene, OR). Laminin and collagen were from (Becton Dickinson, Denmark). ETH 615 was provided by Dr. I. Ahnhelt-Rønne (Lavens Kemiske Fabrik, Denmark). LPC (with palmitic acid in sn-1) and all other compounds were from Sigma Chemical (St. Louis, MO). Cholesterol was added from a 10-mM stock solution in EtOH.

Inorganic media. Isosmotic KCl solution contained in mM 150 KCl, 1.3 CaCl2, 0.5 MgCl2, and 10 HEPES. Hyposmotic KCl solution was obtained by reduction of the KCl in the isosmotic KCl solution to 95 mM, with the other components remaining unchanged, giving an osmolality of 210 mosM. Krebs-HEPES buffer (KHB) contained in mM 118.0 NaCl, 4.7 KCl, 1.2 KH2PO4, 4.2 NaHCO3, 1.3 CaCl2, 1.2 MgSO4, 10.0 HEPES, and 10 d-glucose. pH was in all solutions adjusted to 7.40.

Myotube cell cultures. Measurements were performed on myotube cultures derived from porcine primary satellite cells, isolated from M. semimembranosus from 6- to 7-week-old pigs using a modified version of Bischof's original method (3), unless otherwise stated. The muscle tissue was excised, placed in ice-cold PBS buffer (Ca2+ free and with 1,000 IU/ml of penicillin), and transferred to a laminar flow bench. The muscle tissue was finely chopped with a pair of scissors and digested (3 × 20 min) in 20 ml of PBS (Ca2+ free) containing 1% glucose, 1.5 mg/ml collagenase II, 0.25% trypsin, and 0.01% DNAs. After digestion, the cells were transferred to a primary growth medium (PGM; DMEM with 10% FCS and 10% HS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate, 3 μg/ml amphotericin B, and 20 μg/ml gentamycin), triturated 10 times (glass pipette), centrifuged at 150 g for 10 min at 4°C, resuspended, and filtered through a 200-μm and then a 50-μm Nytex filter. To enrich the relative proportion of satellite cells in the cell suspensions, we optimized the procedure, before seeding and growth of the cells, with respect to 1) dispase treatment, 2) preplating, and 3) Percoll-gradient centrifugation. After all treatments (except the dispase treatment), the subsequent cell suspensions were seeded at a density of 2,000 cells per cm2 in 25-cm2 Nunc flasks coated with matrigel (1:200 vol/vol) and grown in PGM until near confluence (95% air-5% CO2 at 37°C). Initial studies showed that matrigel was superior to laminin or collagen coating and uncoated Nunc plastic for experimentation. Wells (data not shown) previously (34), control cells were left to fuse in DMEM containing 10% FCS in the absence or presence of 1 μM insulin for 5 days. Fusion percentage was determined as the number of myotube nuclei in relation to the total number of nuclei. A myotube was defined as three or more nuclei within a cell membrane.

From Fig. 1, it is seen that direct seeding of the filtered cell suspension on the matrigel-coated surface (control) resulted in fusion percentages of 36 and 19% in the presence and absence of insulin, respectively. Treatment of cells near confluence with dispase (0.025%), to release/remove nonmyogenic cells (fibroblasts), did not improve the final fusion percentage (Fig. 1). Preplating for 30 min on either primaria or uncoated plates, followed by retrieval of nonattached cells, e.g., myogenic satellite cells, was also not effective (Fig. 1), which is in accordance with the findings of Dounit and Merkel (9). Spinning the cells for 20 min at 1,250 g through a density gradient of 35/50/70% Percoll did not improve the fusion percentage (Fig. 1). However, spinning the cells for 5 min at 20,000 g through a 20% Percoll density gradient layer resulted in populations with fusion percentages of 58 and 50% in the presence and absence of insulin, respectively. In the present investigations, we have accordingly used 20% Percoll centrifugation, seeding density of 15,000 cells per cm2, and insulin (1 μM) in the fusion medium.

For the intracellular Ca2+ and ROS measurements, cells were cultured on matrigel-coated (1:10 vol/vol) glass coverslips (10-45 mm) in four-well multilidishes (Nunc 366148) with two coverslips in each well. For the taurine efflux measurements, cells were seeded in six-well Primaria Multiwell plates (35-mm diameter; Becton Dickinson Labware). For Western blots, cells were grown in Nunc 25-cm2 flasks (Nunc 156340). Rat myotube cultures derived from primary satellite cells were prepared according to the method used for porcine myotubes, with the following exceptions: 1) rat satellite cells were preplated for 30 min before being seeded, 2) fusion medium was DMEM with 10% HS, and 3) insulin was omitted. The myoblast cell line (C2C12), originally derived from the thigh muscle of the mouse (Ref. 44; American Type Culture Collection, Manassas, VA), was prepared, grown, and differentiated for experiments as described previously (34).

Estimation of the free cytoplasmic Ca2+ concentration and ROS production. For the Ca2+ measurements, the myotubes were grown on coverslips, washed in KHB, and incubated in KHB containing 3 μM of fura 2-AM and 0.04% pluronic F-127 for 1 h at 25°C, washed three times with KHB, and
ux from the cells was measured at room temperature before use, obtained anoxia. The preincubation solution was experimentally determined using a standard Ca\(^{2+}\)

Perkin-Elmer LS50B fluorimeter (Beaconsfield). For the Ca\(^{2+}\) measurements, the emission wavelength was 510 nm and the subsequent ratiometric data (the ratio R at excitation 340 nm/ excitation 380 nm ratio) were collected every 2 s. For ROS experiments, the excitation and emission wavelengths were 490 and 515 nm, respectively, and data were collected every 2 s. The background signal (cells without fura 2) was subtracted from the fluorescence data before the ratio values were converted to free Ca\(^{2+}\) concentrations according to the equation \[\text{[Ca}^{2+}] = \frac{K_d (R - R_{\text{min}})/(R_{\text{max}} - R)}{(S_{\text{f}} - S_{\text{b}})}\] (10). For each series of eight experiments, the ratio signal was calibrated by adding 5 \(\mu\)M ionomycin (R\(_{\text{max}}\)) or 8 mM EGTA (R\(_{\text{min}}\)), and a K\(_d\) of 224 nM was experimentally determined using a standard Ca\(^{2+}\) calibration buffer kit (Molecular Probes, Eugene, OR). Sequential degassing by suction and bubbling the assay buffer with N\(_2\) before use, obtained anoxia.

**Efflux measurements—estimation of rate constants.** Taurine efflux from the cells was measured at room temperature (\(-20^\circ\)C), as described previously (11, 19). Myotubes plated in six-well dishes were loaded with \(^{14}\)C\)taurine (30 nCi/ml) for 12 h at 37°C. Preliminary loading experiments on porcine myotubes revealed that 12 h were necessary for optimal loading. For the experiments with C2C12 cells and rat myotubes, cells were loaded with \(^{14}\)C\)labeled taurine for 3 h, unless otherwise stated. The preincubation solution was aspirated, and the cells were washed five times with isosmotic KCl solution to remove excess extracellular \(^{14}\)C\)taurine. Experimental solution (1 ml) was added to the dish, left for 2 min, and transferred to a scintillation vial for estimation of \(^{14}\)C\) activity (\(\beta\)-scintillation counting; Ultima Gold). This procedure was repeated for 30 min. At the end of the experiment, the amount of \(^{14}\)C\)taurine remaining inside the cells was estimated by adding 1 ml of 0.5 M NaOH for 1 h, washing the dishes twice with distilled water, and estimating the \(^{14}\)C\) activity in the NaOH, as well as in water washouts. The natural logarithm to the fraction of \(^{14}\)C\)activity remaining in the cells at a given time was plotted vs. time (see Fig. 2A), and the rate constant for the taurine efflux at all time points was estimated as the negative slope of the graph between time point and its proceeding time point and plotted vs. time (see Fig. 2B). Efflux experiments under anoxic conditions were performed with deoxygenated buffer in a closed chamber with a constant flow of N\(_2\) (glove box environmental chamber 34790-10, Cole-Parmer).

**Western immunoblots.** Cells, lysed in 3 ml of buffer containing Tris [50 mM/\(\text{NP}-40\) (1%)/\(\text{SDS}\) (0.1%)/\(\text{NaCl}\) (150 mM)/ Na-azide (0.02%)/Na-deoxycholate (0.5%)], were removed from the flask and sonicated (two times for 10 min) on ice and centrifuged at 600 g for 10 min. The supernatant was transferred to new Eppendorf tubes and centrifuged in two steps at 120,000 g for 45 min at 4°C. The membrane fractions were diluted 1:1 with solubilization buffer containing 62.5 mM Tris, 4% SDS, 18% glycerol, 200 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 \(\mu\)M pepstatin A, 10 \(\mu\)M E-64, and 1 mM leupeptin before being loaded on SDS/ polyacrylamide gels. Twenty five-microgram membrane preparations were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with dry milk and incubated in rabbit polyclonal antibodies raised against native purified human leukocyte 5-LO (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, QC, Canada) (1:250) for 2.5 h. The blot was subsequently incubated in alkaline phospha-

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**Fig. 1.** Validation of preparation procedures to enrich the relative number of satellite cells, isolated from *M. semimembranosus*. After filtration of the digested muscle tissue, the cells were subjected to 1) dispase treatment of confluent cells to release fibroblasts, 2) Percoll-gradient centrifugation, or 3) preplating, as described in MATERIALS AND METHODS. After all treatments, the subsequent cell suspensions were seeded in 25-cm² Nunc flasks coated with matrigel (1:200 vol/vol) and grown in primary growth medium (PGM) until near confluence. At confluence, cells were left to fuse in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% FCS in the absence or presence of 1 \(\mu\)M insulin for 5 days. The fusion percentage was estimated after staining with hematoxilin. There was a significant effect of both treatment (\(P < 0.005\)) and insulin (\(P < 0.001\)).
tase-conjugated anti-rabbit antibody (1:500) for 1 h. Protein bands were detected by the phosphatase activity.

Statistical analysis. Comparison of measurements made on a single parameter (i.e., taurine release or ROS production) during two or more experimental conditions (i.e., different agents or release taurine modes) was made by using one-way analysis of variance (ANOVA). When the ANOVA resulted in a significant F value (P < 0.05), the significant difference between means was located by the Fisher’s protected least significant difference post hoc test. Statistical comparisons of Ca$^{2+}$ content and ROS production during anoxia were evaluated with a Student’s t-test. For all statistical evaluations, significance was established at the 0.05 level; n is the number of experiments. All values are given as means ± SE.

RESULTS

Osmotic cell swelling and exogenous addition of melittin or LPC stimulate taurine release from porcine myotubes. We have recently demonstrated that exposing myotube cultures derived from porcine satellite cells to hypotonic KCl medium induces taurine release via a transport pathway that requires 5-LO activity for activation and that is inhibited by the anion channel blocker DIDS (20). This is confirmed in Fig. 2, A and B, and now quantified in Table 1. Dilution of the isotonic solution to 66% of the normal osmolarity (210 mosM) leads to a 20-fold increase in the rate constant for taurine efflux, and the maximal rate constant for taurine efflux, obtained by the osmotic cell swelling, is significantly decreased by 80 and 70% in the presence of the 5-LO inhibitor ETH 615–139 and DIDS, respectively (Table 1). It is noted that taurine efflux is carried out in KCl medium to avoid the regulatory volume reduction after the osmotic cell swelling and the concomitant inactivation of the volume-sensitive taurine efflux pathway (14). However, cells depolarize when exposed to KCl medium and as the volume-sensitive taurine efflux from, e.g., HeLa cells is reduced under depolarized conditions (40), it seems reasonable to as-

Fig. 2. Swelling-, lysophosphatidyl choline (LPC)-, and melittin-induced taurine release from porcine myotubes. Cells were loaded with 14C-labeled taurine for 12 h. A: the natural logarithm to the fraction of 14C activity remaining in the cells was estimated at 2-min time intervals under isotonic conditions (KCl medium, 300 mosM) and after a shift to hypotonic conditions (KCl medium, 210 mosM), as indicated by an arrow. B: the rate constant for the taurine efflux, estimated as the negative slope between one time point and its proceeding time point in A (see MATERIALS AND METHODS), is plotted vs. time. C and D: rate constants for the taurine efflux under isotonic conditions (300 mosM) after addition of melittin (1 µg/ml) (C) or 5 µM of LPC (D), as indicated by an arrow. The efflux measurements were performed in the absence and the presence of cholesterol (10 µM), which was present from the time of initiation of the efflux experiment (0 min). Mean values for the maximal rate constants are given in Table 1.
Table 1. Effect of DIDS, cholesterol, and ETH 615–139 on taurine release in porcine myotube cultures

<table>
<thead>
<tr>
<th></th>
<th>Hypotonic</th>
<th>Melittin</th>
<th>LPC</th>
</tr>
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<tbody>
<tr>
<td><strong>Maximal rate constant absolute value, min⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.059 ± 0.006(6)</td>
<td>0.038 ± 0.005(4)</td>
<td>0.043 ± 0.003(7)</td>
</tr>
<tr>
<td>DIDS</td>
<td>28 ± 2⁺(6)</td>
<td>16 ± 7⁺(4)</td>
<td>207 ± 24⁺(6)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>84 ± 2⁺(3)</td>
<td>107 ± 4(3)</td>
<td>12 ± 2⁺(3)</td>
</tr>
<tr>
<td>ETH 615–139</td>
<td>18 ± 3⁺(3)</td>
<td>21 ± 6⁺(3)</td>
<td></td>
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</table>

The maximal rate constant for taurine release following osmotic cell swelling in hypotonic solution or stimulation with melittin (1 μg/ml) or lysophosphatidyl choline (LPC) (5 μM) under isotonic conditions was estimated as indicated in Fig 2. DIDS (100 μM), cholesterol (10 μM), and ETH 615–139 (10 μM) were present during the whole experimental period. Values for taurine release in the presence of drugs are the maximal rate constants given relative to the respective control with no drug present. Number of experiments is given in parentheses. Asterisks indicate significant difference from the control value.

assume that the maximal values for the rate constants in Table 1 are slightly underestimated. Subjecting whole cell lysates from pig myotubes to SDS/PAGE and using polyclonal antibodies, raised against human leukocyte 5-LO, revealed two protein bands in the molecular range of 55–60 kDa (data not shown). Thus the 5-LO is present in the porcine myotubes and required for activation of the volume-sensitive taurine efflux pathway.

Melittin is reported to activate various lipases including sPLA₂ isoform (24). From Fig. 2C and Table 1, it can be seen that exposing myotubes to melittin (1 μg/ml) under isotonic conditions leads to a release of taurine and that the rate constant for the melittin-induced taurine efflux within 15 min of stimulation becomes similar in magnitude to the rate constant for the swelling-induced taurine efflux. Furthermore, the maximal rate constant for the melittin-induced taurine release is decreased to the same degree as the maximal rate constant for the swelling-induced taurine efflux in the presence of DIDS and ETH 615–139 (Table 1). Thus stimulation of PLA₂ activity in porcine myotubes, either indirectly by osmotic swelling or directly under isotonic conditions by addition of melittin, induces taurine release via activation of signaling cascades which involve 5-LO activity, and DIDS-sensitive efflux pathways.

From Fig. 2D and Table 1, it can be seen that exposure of porcine myotube cultures to isotonic medium containing LPC at a low concentration (5 μM) induces taurine release. The maximal rate constant for the LPC-induced taurine efflux is decreased by 90% in the presence of cholesterol and increased by DIDS. Cholesterol, on the other hand, only causes a 5-min delay of the onset of the melittin-induced taurine release (Fig. 2C and Table 1) and a minor reduction in the maximal rate constant for the swelling-induced taurine efflux (Fig. 2B and Table 1). From the data presented in Fig. 2 and Table 1, it is suggested that myotubes have two separate taurine efflux pathways, i.e., a pathway that is activated by osmotic cell swelling or exposure to melittin and a pathway that is activated by exogenous addition of LPC. The two pathways are distinguished by their opposing sensitivity toward the anion channel blocker DIDS and cholesterol.

ROS production increases after osmotic cell swelling and after exposure to melittin and LPC. DCFH is rapidly oxidized to the fluorescent compound dehydrochlorofluorescein (DCF) by various ROS, as well as peroxynitrite (23). An increase in the DCF fluorescence is accordingly taken as an indication of formation of the entire group of ROS. With the fluorescent probe DCFH/DCF used as an indicator of ROS production, it is demonstrated that after an initial lag phase of about 3–4 min, the ROS production in porcine myotubes increases linearly with time after osmotic cell swelling and after treatment with melittin (Fig. 3A). The ROS production, estimated from the linear increase in the fluorescence between 5–30 min in Fig. 3A, indicates that there is a small background ROS production in myotubes incubated in isotonic KHB (0.03 ± 0.02 s⁻¹, n = 19, Fig. 3, A and B) and that the ROS production increases significantly after hypotonic exposure (0.21 ± 0.01 s⁻¹, n = 7, Fig. 3B) and by the addition of melittin (0.24 ± 0.01 s⁻¹, n = 6, Fig. 3B).

Exogenous addition of LPC also increases ROS production in the porcine myotube cultures. This is seen in Fig. 3, C and D, where it is demonstrated that the fluorescence of DCFH/DCF after a 1–3-min lag phase increases linearly with time. The ROS production after LPC addition (5 μM) is estimated at 0.36 ± 0.03 s⁻¹ (n = 6, Fig. 3D), which is in the same range as the ROS production after melittin addition (Fig. 3B). There is a modest 19% decrease (P < 0.05) in the LPC-induced ROS production in the presence of 10 μM cholesterol (0.29 ± 0.01 s⁻¹, n = 6, Fig. 3, C and D). Consequently, the inhibitory effect of cholesterol on the LPC-induced taurine release (Table 1) can only to a minor extent be due to an antioxidative or substrate inhibitory effect of the cholesterol.

Effect of antioxidants on the swelling-induced taurine efflux. Cell swelling induces taurine release, as well as an increased ROS production (Figs. 2B and 3, A and B); however, it is not known whether there is a causal relationship between the two events. Both the swelling-induced increase in ROS production in porcine myotubes (data not shown), as well as the concomitant taurine efflux (Fig. 4A), are inhibited in the presence of the antioxidant BHT (butylated hydroxytoluene,
In three sets of experiments, it was estimated that 0.5 mM BHT reduced the maximal rate constant for the swelling-induced taurine efflux from the porcine myotubes to 18 ± 3% of the value in control cells. In C2C12 and in rat myotube cultures, the equivalent value was estimated at 48 ± 10% (n = 5, 0.5 mM BHT) and 50% (n = 2, 0.5 mM BHT), respectively. Short-term exposure to the antioxidant vitamin E (100 µg/ml) decreases the maximal rate constant for the LPC-induced taurine efflux and the melittin-induced taurine release to 42 ± 7% (n = 3) and 41 ± 6% (n = 3) of the control values, respectively. Furthermore, preexposure for 24 h with vitamin E (100 µg/ml) reduced the swelling-induced taurine efflux from C2C12 cells to 58 ± 5% (n = 6) of the control efflux. Notable is the observation that short-term exposure to vitamin E does not affect the efflux significantly; hence, vitamin E has to be incorporated in the membrane to scavenge the swelling-induced ROS production. Accordingly, swelling of skeletal muscle cells increases the ROS production, and ROS seem to interfere with the signal cascade involved in the concomitant activation of the taurine release.

Taurine is released from porcine myotubes during anoxia. Previous reports have shown that ischemia causes osmotic cell swelling (25), as well as release of large amounts of taurine from, e.g., mouse hippocampal slices (38) and striatum of hypertensive rats (31). Taurine is also released from porcine myotubes during anoxia. This is seen in Fig. 4B, where it is shown that the rate constant for taurine efflux increases almost at the time of oxygen deprivation and reaches a plateau within 5 min.

**Effect of anoxia on [Ca\textsuperscript{2+}]\textsubscript{i} and ROS production.** Because anoxia leads to cell swelling and anoxic conditions lead to an increased PLA\textsubscript{2} activity, we investigated whether known activators of the various PLA\textsubscript{2}, i.e., Ca\textsuperscript{2+} or ROS, are elevated during anoxia. From Fig. 5A, it can be seen that after an initial 4-min lag
phase, \([\text{Ca}^{2+}]_i\); increases linearly with time in porcine myotubes during anoxic exposure. The anoxia-induced increase in \([\text{Ca}^{2+}]_i\), estimated from the linear part in the time range of 5–30 min after the onset of the experiments, is 28 ± 1 nM/min (Fig. 5C). A similar time course for increase in \([\text{Ca}^{2+}]_i\), under anoxic conditions has recently been reported for C2C12 myotubes (20). From Fig. 5B, it can be seen that the ROS production in porcine myotube cultures after a short time lag increases linearly with time under anoxic conditions. The increase in ROS production, estimated from the linear part, is 0.24 ± 0.01 s\(^{-1}\) (Fig. 5D) and, thus, similar in magnitude to the ROS production seen after cell swelling and stimulation with melittin (Fig. 3). The anoxia-induced increase in ROS production was completely inhibited by addition of the antioxidant BHT (0.5 mM, Fig. 5, B and D). Taking the experimental setup for estimation of \([\text{Ca}^{2+}]_i\) and ROS production into account, it is not possible to deduce when the cells become anoxic, and we are accordingly not able to deduce whether the increase in \([\text{Ca}^{2+}]_i\) and ROS precedes the increase in taurine release during anoxia seen in Fig. 4.

**Effects of cholesterol and antioxidants on anoxia-induced taurine efflux.** Cell swelling increases the ROS production (Fig. 3, A and B) and ROS modulate the concomitant taurine release (Fig. 4A). Furthermore, the signaling pathway leading to swelling-induced taurine release can be distinguished from the LPC-induced taurine release by the sensitivity to cholesterol (Table 1). To investigate the pathway that leads to taurine release during anoxia, taurine release was followed in myotubes (C2C12 cells) during anoxia in the presence and absence of either cholesterol or the antioxidant BHT. One hour of anoxia induced a substantial taurine release, and from Fig. 6 it can be seen that the taurine release during anoxia is inhibited by 23 ± 6% by the antioxidant BHT (0.5 mM), demonstrating a significant role of ROS in the anoxia-induced signal cascade leading to taurine efflux from the myotube. Cholesterol (10 μM), on the other hand, does not inhibit the taurine release during anoxia (120 ± 5%, Fig. 6), which could be taken to indicate that LPC plays a minor role in anoxia-induced taurine loss.

**DISCUSSION**

The present study was initiated to characterize the role of PLA2 and ROS in the signal cascades leading to taurine efflux in skeletal muscle cells. The model presented in Fig. 7 and commented on in the following summarizes the present and previously published data. With myotube cultures derived from porcine satellite cells and C2C12 myotubes used as a cellular model for a skeletal muscle, it is indicated that myotubes have two separate taurine efflux pathways, i.e., a pathway that is activated by osmotic cell swelling and a pathway that is activated by exogenous exposure to LPC. The two pathways are distinguished by 1) their opposing sensitivity toward the anion channel blocker DIDS and cholesterol and 2) the second messengers involved in their activation. Swelling-induced and LPC-induced taurine efflux pathways with opposing sensitivity to anion channel blockers and serum starvation have previously been demonstrated in HeLa cells (21). Both cell swelling and exogenous addition of LPC significantly augments the ROS production in porcine myotubes, and because antioxidants inhibit the concomitant taurine efflux, there are strong indications that ROS modulate taurine release from muscle. Exposing myotubes to anoxia increases both \([\text{Ca}^{2+}]_i\) and the ROS production, as well as the taurine release from the myotubes, which is sensitive to antioxidants but not cholesterol. In addition, skeletal muscle contractile activity leads to elevated \([\text{Ca}^{2+}]_i\), swelling, increased ROS production (32, 33), and a
concomitant loss of taurine from the muscle cell (36) and increase in plasma taurine (4).

Role of PLA2 in taurine release. Because all the regiments used to induce taurine release from the myotubes, i.e., osmotic cell swelling, anoxia, isotonic exposure to the lipase activator melittin and to LPC, stimulate PLA2 activity either directly or indirectly via an increased ROS production or an increase in [Ca\(^{2+}\)], it is suggested that PLA2 is an essential upstream element in the signaling cascade involved in the regulation of the cellular content of organic osmolytes in myotubes.

The PLA2 family has been divided into four groups, i.e., 1) the Ca\(^{2+}\)-dependent sPLA2, 2) Ca\(^{2+}\)-dependent, cytosolic PLA2 (cPLA2), 3) Ca\(^{2+}\)-independent, cellular PLA2 (iPLA2), and 4) PLA2 with high preference for substrates with a short chain in the sn-2 position (2, 8). The cPLA2, which requires Ca\(^{2+}\) for binding to the membrane substrate and phosphorylation to gain full activity (6), has been assigned a permissive role in the initiation of the signal cascade that is required for activation of the volume-sensitive osmolyte channels in Ehrlich cells (40). In NIH3T3 mouse fibroblasts, this role is governed by iPLA2 (16). ROS have been reported to directly increase PLA2 activity, either due to peroxidation of lipids, reduction in membrane fluidity, and/or oxidation of protein side chains or sulfhydryl formation (25, 27). The swelling-induced ROS production, as well

Fig. 5. Effect of anoxia on cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and ROS production in porcine myotube cultures. Cells, raised on coverslips and loaded with fura 2-AM (3 \(\mu\)M for 1 h at 25\(^\circ\)C) or dichlorodihydrofluorescein diacetate (DCFH-DA; 10 \(\mu\)M, for 2 h at 25\(^\circ\)C), were washed in Krebs-HEPES buffer (KHB) and mounted vertically in a 10-mm path length cuvette. Anoxia was obtained by degassing and bubbling the assay buffer with N\(_2\) before use. A and B: the [Ca\(^{2+}\)]\(_i\) - and ROS-dependent fluorescence intensity in oxygenated (control) and anoxic cells was followed with time. C: increase in the [Ca\(^{2+}\)]\(_i\) (nM/min) after anoxia was estimated from the linear slope in the time range of 5 to 30 min after anoxia (see A). The numbers of independent experiments were 5 and 7 for control and anoxia, respectively. D: ROS production during anoxia, with and without 0.5 mM BHT, was estimated from the slopes of the linear increase in fluorescence intensity in the time frame of 5 to 30 min (see B). The number of experiments was 19 (control), 6 (anoxia), and 6 (anoxia + BHT). *Significantly different from the oxygenated value.
as the taurine efflux in NIH3T3 cells, is inhibited in the presence of the iPLA2 inhibitor bromoenol lactone, which has been taken to indicate that ROS production is downstream to PLA2 activation (16). This assumption is supported by the observation that the PLA2 products LPC (Fig. 3), as well as arachidonic acid (7), stimulate ROS production.

Swelling-induced taurine efflux pathway in myotubes. The 5-LO is previously demonstrated to be involved in the swelling-induced activation of taurine efflux in a variety of cell lines (16, 17, 21). However, it is only in Ehrlich cells that a 5-LO product, leukotriene D4 (LTD4), has been directly demonstrated to induce taurine release under isotonic conditions at a physiological concentration (EC50/5 nM; Ref. 17). The data in the present paper indicate that 5-LO is present in the myotube cultures and that cell swelling, as well as direct stimulation of PLA2 activity with melittin, leads to taurine release via a DIDS-sensitive transport pathway that requires 5-LO activity for activation (Table 1). It is noted that several inhibitors of arachidonic acid metabolism nonspecifically block volume-dependent anion channels, which are permeable to taurine (28). However, 5-LO inhibition in the case of HeLa cells was obtained by different types of inhibition, i.e., substrate inhibition, inhibition of the 5-LO binding to the 5-LO activating protein (FLAP), direct inhibition, and ROS scavenging by addition of antioxidants (21). Therefore, it seems reasonable to suggest that the signaling cascades and taurine efflux pathways activated in myotube cultures by cell swelling are most probably similar to the volume-sensitive, 5-LO-dependent signal pathways described in Ehrlich cells, HeLa cells, and NIH3T3 cells. The 5-LO product that acts as a second messenger in the porcine myotubes is yet to be identified.

The LPC-induced taurine efflux pathway. LPC is produced after osmotic cell swelling (41) and during ischemia (39). Shaik and Downar (39) reported a 60% increase in LPC levels, from 14 to 23 μM, after 8 min of ischemia in the myocardium. LPC constitutes an amplification system by increasing cPLA2 activity (43) and, thereby, enhancing the availability of lysophospholipids (5). We have previously shown that exposing porcine myotube cultures to isotonic media containing low concentrations of LPC (5 μM) induces efflux of taurine (20). These observations, now confirmed and quantified in Fig. 2 and Table 1, are in accordance with previously reported data on Ehrlich cells (13), HeLa cells, (18, 19), and C2C12 myotubes (20). It is noted that the swelling-induced taurine release in Ehrlich cells, HeLa cells, and fibroblasts comprises PLA2 activation (16, 21) and consequently the release of arachi-
Role of PLA2 and ROS on taurine release

HeLa cells and NIH3T3 cells potentiates the swelling-induced taurine efflux (Fig. 2D and Table 1), whereas cholesterol has only a minor effect on the swelling-induced taurine efflux (Fig. 2B and Table 1). The structural requirement for the lysophospholipids is, in the case of the HeLa cells, strict, and only lyso-phospholipids with choline as the polar head group and oleic, palmitic, or stearic acid in the sn-1 position are able to elicit taurine release (19). Furthermore, LPC binding receptors have recently been identified (13, 46). On the other hand, LPC at high concentrations (>10 μM) induces the release of creatine kinase from cardiomyocytes (5), as well as the release of nucleotides from HeLa cells (19). Thus LPC at high concentrations seems to break down the membrane permeability barrier. It is not possible to deduce whether the effects of 5 μM LPC, used in the present paper, reflect activation of specific osmolyte transporting systems or a general permeabilization of the plasma membrane. Cholesterol is an integral component of the eukaryotic cell membranes, with a highly nonuniform distribution among the membranes, and it is estimated that about 80–90% of the total cell membrane cholesterol is in the surface membrane (45). As cholesterol has only a minor effect on the LPC-induced ROS production (Fig. 3), whereas it decreases the LPC-induced taurine efflux (Fig. 2C), it seems reasonable to exclude the possibility that the effect of cholesterol on the flux reflects an antioxidative effect. However, cholesterol affects membrane fluidity and it is possible that cholesterol affects either LPC binding to a putative receptor or the interaction between the plasma membrane and the cone-shaped LPC (cross-sectional area of the polar head group is larger than that of the area of the acyl chain) and, thereby, the action of LPC.

Role of ROS in the regulation of osmolyte-releasing systems. Cell swelling and stimulation with melittin is accompanied by an increase in the taurine efflux, which is evident after about 2 min (Fig. 2) and an increase in ROS production that appears after a 3- to 4-min delay (Fig. 3). Thus measurable increase in ROS production appears after the onset of the taurine efflux. Swelling-induced ROS production in NIH3T3 cells involves the NAD(P)H oxidase (16), oxidation of polyunsaturated fatty acids (arachidonic acid), and formation of lipoxygenase products by the 5-LO. H2O2 is required for 5-LO activity in mammalian cells (30), and because the 5-LO is present in the myotube cultures (see RESULTS), it seems plausible to assume that ROS, produced by cell swelling, modulate the activity of the volume-sensitive taurine efflux pathway via interference with the 5-LO. This suggestion is supported by the observation that exogenous addition of H2O2 to HeLa cells and NIH3T3 cells potentiates the swelling-induced taurine efflux and that this effect of H2O2 is impaired in the presence of the 5-LO inhibitor ETH 615–139 (16, 19). H2O2 inhibits protein tyrosine phosphatases and, because the swelling-induced taurine efflux in, e.g., NIH3T3 cells is regulated by tyrosine phosphorylation, it has alternatively been suggested that the potentiating effect of ROS on swelling induced taurine efflux reflect oxidation and a subsequent inhibition of protein tyrosine phosphatase activity (16). ROS are also produced after LPC exposure (Fig. 3), and because the LPC-induced taurine efflux from porcine myotubes is inhibited by antioxidants (16), it seems reasonable to assume that ROS production is required for the LPC-induced activation of taurine efflux. The subcellular site of production and action of ROS in the LPC-induced signaling cascade is at present not known; however, it seems to be modulated by a calmodulin/CaMKII (18).
hydrolyze phosphatidyl choline to LPC in the outer leaflet of the plasma membrane (29). Activation of cPLA2 (osmotic swelling) and sPLA2 (melittin addition) stimulate ROS production (Fig. 3). As DIDS depress taurine release after anoxia (data not shown) and the ROS production is concomitantly increased, it is suggested that) PLA2 activity increases during anoxia in muscle and 2) the 5-LO-dependent taurine efflux pathway contributes to the anoxia-induced release of taurine. There seems to be a functional cooperation between cPLA2 and signaling through sPLA2 (IIA), whereas sPLA2 (X)-mediated release is independent of cPLA2 activity (29). It is noted that the volume-sensitive taurine efflux pathways in C2C12 myoblasts are downregulated during differentiation (26), i.e., differentiation most probably add to the complexity of the activation of the taurine-releasing systems.

In conclusion, the results of this study indicate that ROS is produced during regiments that induce taurine release in myotubes, i.e., swelling, anoxia, and addition of LPC or melittin. Furthermore, the present findings indicate that ROS potentiate, whereas antioxidants attenuate, the taurine efflux after swelling, anoxia, and the addition of LPC. PLA2 is a key mediator of the taurine efflux because its products provide upstream elements for two separate taurine efflux pathways, i.e., a swelling-induced pathway that requires arachidonic acid and 5-LO activity for activation and an LPC-induced pathway. The two pathways are distinguished by their opposing sensitivity toward the anion channel blocker DIDS and cholesterol. It appears that the identity, substrate specificity, and subcellular localization of the PLA2 determine the second messengers produced by the PLA2 and, subsequently, the type of taurine efflux pathway to be activated.

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