Volatile Anesthetics Affect Nutrient Availability in Yeast

Laura K. Palmer,[1] Darren Wolfe,[1,2] Jessica L. Keeley and Ralph L. Keil[3]

Department of Biochemistry and Molecular Biology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

Manuscript received January 28, 2002
Accepted for publication March 8, 2002

ABSTRACT

Volatile anesthetics affect all cells and tissues tested, but their mechanisms and sites of action remain unknown. To gain insight into the cellular activities of anesthetics, we have isolated genes that, when overexpressed, render *Saccharomyces cerevisiae* resistant to the volatile anesthetic isoflurane. One of these genes, *WAK3/TAT1*, encodes a permease that transports amino acids including leucine and tryptophan, for which our wild-type strain is auxotrophic. This suggests that availability of amino acids may play a key role in anesthetic response. Multiple lines of evidence support this proposal: (i) Deletion or overexpression of permeases that transport leucine and/or tryptophan alters anesthetic response; (ii) prototrophic strains are anesthetic resistant; (iii) altered concentrations of leucine and tryptophan in the medium affect anesthetic response; and (iv) uptake of leucine and tryptophan is inhibited during anesthetic exposure. Not all amino acids are critical for this response since we find that overexpression of the lysine permease does not affect anesthetic sensitivity. These findings are consistent with models in which anesthetics have a physiologically important effect on availability of specific amino acids by altering function of their permeases. In addition, we show that there is a relationship between nutrient availability and ubiquitin metabolism in this response.

THE initial public demonstration of general anesthesia in 1846 marked a pivotal event in the history of medicine. Until that time, surgery was performed only as a last, desperate resort because of the unbearable pain and suffering inflicted on the patient. Although volatile anesthetics are essential for modern clinical practice due to their ability to render patients unconscious and insensitive to pain, the mechanisms and sites of action of these drugs remain unknown.

Volatile anesthetics affect all cells and tissues that have been tested, including a wide array of mammalian neuronal and nonneuronal cells, plant cells, yeast, and bacteria (Overton 1901; Keil *et al.* 1996; Batai *et al.* 1999). We are taking a molecular genetic approach to investigate anesthetic action using the yeast *Saccharomyces cerevisiae*. We find that volatile anesthetics arrest yeast cell division in a manner that strikingly parallels the activities of these drugs in mammals (Koblin 1994; Keil *et al.* 1996; Wolfe *et al.* 1998). These parallels include correlation of lipophilicity and potency (the Meyer-Overton rule; Koblin 1994); rapid and reversible effects; a sharp dose-response curve; additivity of partial doses of different anesthetics; and volatile, lipophilic compounds that do not induce anesthesia in mammals do not inhibit growth of yeast. These similarities indicate that the manner in which anesthetics inhibit yeast growth and induce mammalian anesthesia may be closely related, if not identical.

To investigate anesthetic action in *S. cerevisiae*, spontaneous mutants that confer resistance to the growth-inhibitory effects of the volatile anesthetic isoflurane have been isolated and characterized (Keil *et al.* 1996; Wolfe *et al.* 1998, 1999). Two of the genes identified in this analysis have been implicated in ubiquitin metabolism: *ZZZ1* is identical to *BUL1*, which encodes a protein that interacts with yeast ubiquitin ligase (Yashiroda *et al.* 1996), and *ZZZ4* is identical to *DOA1*, which affects degradation of ubiquitinated proteins (Ghislain *et al.* 1996). Finding that mutations in these genes affect anesthetic sensitivity suggests that ubiquitin metabolism plays a critical role in the normal response of yeast to these drugs.

To identify additional proteins involved in yeast anesthetic response, we isolated genes that, when present in multiple copies, confer resistance to the volatile anesthetic isoflurane. Here we report that one of these genes, *WAK3*, is identical to *TAT1*, which encodes a yeast amino acid permease (Schmidt *et al.* 1994). Finding that overexpression of an amino acid permease confers anesthetic resistance suggests that nutrient availability may be a critical factor in yeast anesthetic response. Multiple, mutually supportive experimental findings are

[1]These authors contributed equally to this work.

[2]*Present address:* Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

[3]*Corresponding author:* Department of Biochemistry and Molecular Biology H171, The Milton S. Hershey Medical Center, The Pennsylvania State University, 500 University Dr., Hershey, PA 17033.
E-mail: rkeil@psu.edu

consistent with this proposal. In addition, we find a relationship between ubiquitin metabolism and nutrient availability in the response of yeast to these drugs.

MATERIALS AND METHODS

Strains, media, and anesthetic exposure: Yeast strains used in this study are derivatives of RLK88-3C (Lin and Keil 1991) and are listed in Table 1. Strains P1353, P1337, and P1361 were constructed from RLK88-3C by PCR-directed correction of the chromosomal *leu2-3,112* and/or *trp1-HIII* mutations. Oligonucleotides used to generate the *LEU2* and *TRP1* PCR products from plasmid-borne copies of the wild-type genes were as follows: *LEU2*, 0-230 (5′-GAATACTCAGGTATC-3′) and 0-231 (5′-TCGTAAGGCCGTTTC-3′); and *TRP1*, 0-228 (5′-GTGACTATTGAGCAC-3′) and 0-229 (5′-GTGCACAAAC AATAC-3′). PCR products were transformed into RLK88-3C, and stable Leu^+ or Trp^+ transformants were isolated. In a similar manner, strain P1814 was constructed from P1361 by PCR-directed correction of the chromosomal *his4-260* and *ade2-1* mutations. Oligonucleotides used to generate the *HIS4* and *ADE2* PCR products from plasmid-borne copies of the wild-type genes were as follows: *HIS4*, 0-253 (5′-TCATCGGAA GAGGTGGCATC-3′) and 0-254 (5′-AAACCGTCAGGACGGT CTGT-3′); and *ADE2*, 0-259 (5′-GAAACTTCATGCTCGAAA AAG-3′) and 0-260 (5′-CAAGGGAACATTATAGGGTG-3′). Unless otherwise noted, yeast (Lin and Keil 1991) and bacterial (Sambrook *et al.* 1989) media were prepared as previously described. Isoflurane (Baxter Healthcare Corporation) exposure of yeast grown on solid media was performed as described previously (Keil *et al.* 1996; Wolfe *et al.* 1999).

Isolation of anesthetic resistance-conferring genes: To identify genes that confer resistance to anesthetics when present in multiple copies, RLK88-3C was transformed (Schiestl and Gietz 1989) with a 2μ-based (YEp; multicopy) yeast genomic plasmid library (Carlson and Botstein 1982) and screened for transformants resistant to isoflurane. Plasmids recovered from these transformants were propagated in *Escherichia coli* strain MC1066 [*leuB trpC pyrF::*Tn5 (Kan^r) *araT lacX74 del strA hsdR hsdM* (obtained from M. Casadaban)].

DNA manipulations, plasmids, and gene deletions: PCR reagents as well as restriction and modification enzymes were purchased from various sources and used according to the instructions of the manufacturers. Standard procedures for the purification of plasmid (Sambrook *et al.* 1989) and yeast genomic (Rose *et al.* 1990) DNA were used.

Plasmid pL3271 contains an 11.1-kb fragment of yeast genomic DNA from chromosome II that includes *WAK3*. This fragment is inserted in the *Bam*HI site of YEp24 (Botstein *et al.* 1979). Oligonucleotides used to sequence into the insert from both ends were 0-73 (5′-GCCAGCAACCGCACC-3′) and 0-74 (5′-GCCACTATCGACTAC-3′), which hybridize to plasmid sequences flanking the insert. DNA sequencing was performed in the Molecular Genetics Core Facility of the M. S. Hershey College of Medicine using an ABI 377 DNA sequencer.

To initially localize the sequences encoding *WAK3*, deletion derivatives of pL3271 were constructed by digestion with convenient restriction enzymes. The restricted DNA was religated to produce plasmids with the various deletions. Among the deletion derivatives constructed were pL3273, which contains the C-terminal portion of *TAT1* and the entire protein-encoding sequence for *BAP2*, and pL3277, which contains the entire *TAT1* gene and the amino-terminal portion of *BAP2*. These plasmids are termed YEp*BAP2* and YEp*TAT1*, respectively.

TAT1 and *TAT2* on the low-copy YCplac33 vector (Gietz and Sugino 1988), named YCp*TAT1* and YCp*TAT2*, respectively (original designations pTAT1 and pTAT2; Schmidt *et al.* 1994), were kindly provided by M. Hall. The 3.7-kb *Eco*RI fragment from YCp*TAT2* containing the *TAT2* open reading frame and flanking genomic sequences was ligated into the *Eco*RI site of the *URA3*-marked YEplac195 vector (Gietz and Sugino 1988). This plasmid was termed YEp*TAT2*. The high-affinity lysine permease, *LYP1*, on the 2μ-based pYX212 vector (original designation pRB165; Rengenberg *et al.* 1999), and *BAP2* on the pYX212 vector (original designation pRB145; Regenberg *et al.* 1999) were obtained from M. C. Kielland-Brandt. These plasmids were termed YEp*LYP1* and YEp*BAP2*-2, respectively.

The entire protein-encoding sequences of *TAT1* or *BAP2* were deleted from RLK88-3C and replaced with *loxP-kanMX-loxP* from pUG6 (Guldener *et al.* 1996) by using appropriate PCR-generated gene disruption cassettes. Oligonucleotides used to generate these cassettes were as follows: *TAT1*, 0-153 (5′-GTAGCTACCTAATATAGTTTCTCGATAAAAAGGCGTAAA ACAGGTCGACAACCCTTATA-3′) and 0-154 (5′-AAGCCCG ATGAAGCCAAGCGGAAAATGAATCGAATTGCTGGTGGA TCTGATATCACCTA-3′); and *BAP2*, 0-216 (5′-CAATTTAT TTAGCTTCAATAAAACTCAAGTGATTTTAGAACAGGTCG ACAACCCTTAAT-3′) and 0-217 (5′-TCTAATGGGTAGTGT CCAGACCTGAGTGGTGTAGTTAAGTGTGGATCTGATAT CACCTA-3′). Correct gene deletions were verified by PCR.

To delete the entire protein-encoding sequence of *TAT2* in RLK88-3C, a vector derived from YCp*TAT2* in which the *TAT2* gene was precisely replaced with a *loxP-kanMX-loxP* fragment was constructed as follows: Inverse PCR was used to create a derivative of YCp*TAT2* in which the *TAT2* gene was precisely deleted and replaced with a *Not*I restriction site. Oligonucleotides 0-384 (5′ATACGATAGCGGCCGCATGAG AGTGTGTTGCGTAATTTGC-3′) and 0-385 (5′-ATAAGATA GCGGCCGCTACCGAAGAAACAAGTTC-3′) were used for this PCR reaction. The resulting PCR product was digested with *Not*I and the 1.6-kb *Not*I fragment of pUG6 containing *loxP-kanMX-loxP* was inserted, producing pL4071. A PCR product was generated from pL4071 using oligonucleotides 0-373 (5′-CATGATATTGCATC TACCTC-3′) and 0-374 (5′-AAATT TGATTCCTACGGCAG-3′) and transformed into RLK88-3C. The *tat2Δ::loxP-kanMX-loxP* transformants created by this process were identified on the basis of resistance to G418, and the occurrence of the appropriate deletion was verified by PCR.

Amino acid import studies: To measure leucine or tryptophan uptake in the presence or absence of isoflurane, cells were grown to an approximate OD_{600} of 0.4 in synthetic complete (SC) media (Lin and Keil 1991) containing 70 μg/ml leucine and 10 μg/ml tryptophan. Twenty-five-milliliter aliquots of this culture were injected into 250-ml evacuated bottles (Baxter Healthcare Corporation) containing the desired concentration of volatilized anesthetic. Air was admitted into the bottles to achieve 1 atmosphere of pressure. After 15 min of incubation at 30°, 20 μCi of L-[U-^{14}C]leucine (50 μCi/ml; Amersham, Arlington Heights, IL) or 80 μCi of L-[5-^{3}H]tryptophan (1 mCi/ml; Amersham) was added. Triplicate samples of cells were removed at 0- and 10-min time intervals after the addition of the labeled amino acid, collected on glass-fiber filters, and washed extensively with cold liquid medium containing excess unlabeled leucine (20 mg/ml) and tryptophan (4 mg/ml). Washed filters were placed in scintillation vials and treated with 1 M NaOH. This solution was neutralized with 1 M acetic acid and liquid scintillation fluid (Fisher Scientific, Pittsburgh) was added. Uptake of labeled amino acid into the cells was determined by liquid scintillation counting and levels in the presence or absence of isoflurane were compared.

To measure leucine uptake in *tat1Δ*, YEp*TAT1*, *zzz1Δ*, or *zzz4Δ* strains or lysine uptake in RLK88-3C transformed with YEp*LYP1* or the vector control, 25-ml aliquots of cells grown

to an approximate OD_{600} of 0.4 in SC or SC-ura medium were harvested and resuspended in 1.25 ml of fresh media. The cell suspension was added to SC or SC-ura medium containing 1.25 μCi of L-[U-^{14}C]leucine or 5 μCi of L-[4,5-^{3}H]lysine (1 mCi/ml; Amersham) to a total volume of 3.2 ml. Triplicate samples of cells were removed at 0- and 10-min time points after addition of the cells to the medium containing the labeled amino acid and collected on glass-fiber filters. The filters were washed and treated as described above, and radioactivity was quantified by liquid scintillation counting.

RESULTS

Altered levels of *TAT1* affect yeast anesthetic response: To identify novel genes involved in volatile anesthetic action in *S. cerevisiae*, an overexpression library containing random fragments of yeast genomic DNA inserted into the 2μ-based YEp24 vector (Botstein *et al.* 1979) was screened for plasmids that confer resistance to the volatile anesthetic isoflurane. Seven transformants resistant to a normally growth-inhibitory concentration of isoflurane [12%; the *m*inimum *i*nhibitory *c*oncentration (MIC; Keil *et al.* 1996)] were isolated from ~20,000 transformants. Loss of the plasmids from these transformants resulted in reversion to the anesthetic-sensitive phenotype of the wild-type strain, RLK88-3C (Table 1), indicating that the anesthetic resistance was due to plasmid-borne genes. Plasmids from each of these transformants were recovered into *E. coli* and reintroduced into RLK88-3C. All of the transformants tested were anesthetic resistant, further demonstrating that the plasmids were responsible for altering anesthetic response. Genes on these plasmids critical for anesthetic resistance (identified in subsequent analyses) were termed *WAK* (pronounced "wake") genes to indicate their roles in preventing yeast growth inhibition, or "sleep," produced by anesthetic agents.

Restriction mapping of the plasmids indicated that five of the seven plasmids contained a common DNA segment. These plasmids represent five isolates containing the *WAK3* gene. Sequencing into the insert in one of these plasmids, pL3271, revealed that the insert was an 11.1-kb DNA fragment from chromosome II containing the carboxy terminus of *YBR070C*, full-length *TAT1*, *BAP2*, *TIP1*, and *NRG2* genes, and the amino-terminal portion of *ECM2* (Figure 1). Deletion analysis of pL3271 showed that *TAT1* was essential for producing the anesthetic resistance (Figures 1 and 2). *TAT1* encodes an amino acid permease that transports leucine, tryptophan, isoleucine, valine, and tyrosine (Schmidt *et al.* 1994; Regenberg *et al.* 1999). Even when present on a low-copy, centromeric plasmid (YCplac33; Gietz and Sugino 1988), *TAT1* increased the anesthetic resistance of RLK88-3C (Figure 2, compare strains 5 and 6), indicating that a relatively small increase in *TAT1* expression is sufficient to alter anesthetic response. These results suggest that nutrient availability may be a critical determinant in the anesthetic response of yeast.

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype	Designation[a]	Source
RLK88-3C	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R}*	Leu^{-}Trp^{-}	Lin and Keil (1991)
P1353	*MATa his4-260 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R}*	Leu^{+}Trp^{-}	This study
P1337	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 lys2ΔBX can1^{R}*	Leu^{-}Trp^{+}	This study
P1361	*MATa his4-260 ura3-52 ade2-1 lys2ΔBX can1^{R}*	Leu^{+}Trp^{+}	This study
P1814	*MATa ura3-52 lys2ΔBX can1^{R}*	Lys^{-}Ura^{-}	This study
P1198	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R} tat1Δ::loxP-kanMX-loxP*	*tat1Δ*	This study
P1370	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R} bap2Δ::loxP-kanMX-loxP*	*bap2Δ*	This study
P1848	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R} tat2Δ::loxP-kanMX-loxP*	*tat2Δ*	This study
P754	*MATα his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R} zzz1Δ::hisG*	*zzz1Δ*	Wolfe *et al.* (1999)
P1894	*MATa his4-260 leu2-3,112 ura3-52 ade2-1 trp1-HIII lys2ΔBX can1^{R} zzz4Δ::hisG*	*zzz4Δ*	Keil *et al.* (1996)

[a] *TAT1*, *TAT2*, *ZZZ1*, and *ZZZ4* are identical to *VAP1*, *SCM2*, *BUL1*, and *DOA1/UFD3*, respectively.

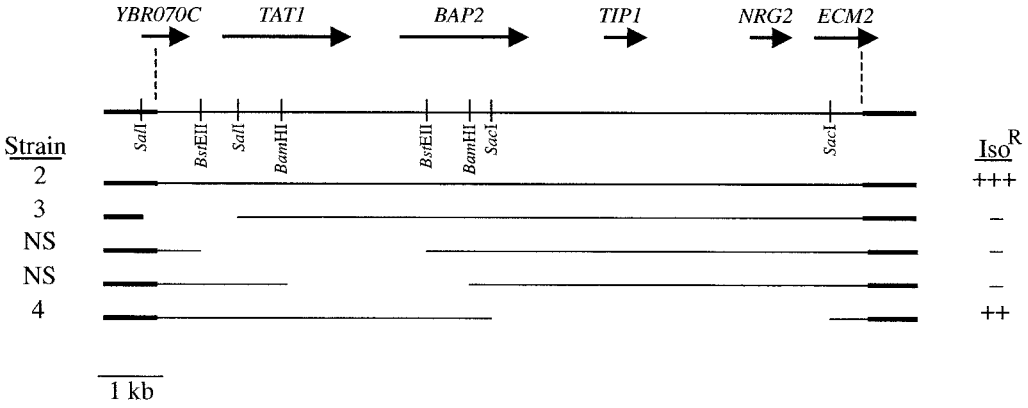

FIGURE 1.—*WAK3* and flanking genomic DNA. *WAK3* was identified on the basis of deletion analysis of an 11.1-kb fragment of yeast genomic DNA contained in plasmid pL3271. Thick lines represent multicopy vector (YEp24) sequences and thin lines represent the fragment of yeast genomic DNA containing *WAK3*. The horizontal arrows indicate the open reading frames contained within this fragment and their direction of transcription, with the arrowhead at the 3′ terminus. The short dashed vertical lines indicate the positions at which the *YBR070C* and *ECM2* genes are truncated in pL3271. The sequences present in several deletion derivatives of this plasmid are shown below the restriction map of the fragment, with the lines indicating the DNA present in the deletion derivative. Strain numbers refer to strains shown in Figure 2 that contain the indicated plasmid derivatives; NS, not shown in Figure 2. The level of isoflurane resistance (Iso^R) of each derivative as compared to the wild-type strain (RLK88-3C; Table 1) is indicated: −, same level of resistance as the wild-type strain; ++, intermediate level of isoflurane resistance; +++, higher level of isoflurane resistance.

In addition to Tat1p, pL3271 also contains the protein-encoding sequence for Bap2p, a branched-chain amino acid permease that transports leucine, isoleucine, and valine (GRAUSLUND *et al.* 1995). Although deletion analysis clearly showed that *TAT1* was indispensable for the anesthetic resistance, a plasmid containing both *TAT1* and *BAP2* rendered RLK88-3C more resistant to isoflurane than did a plasmid overexpressing only *TAT1* (Figure 2, compare strains 2 and 4). Overexpression of *BAP2* alone did not render cells anesthetic resistant

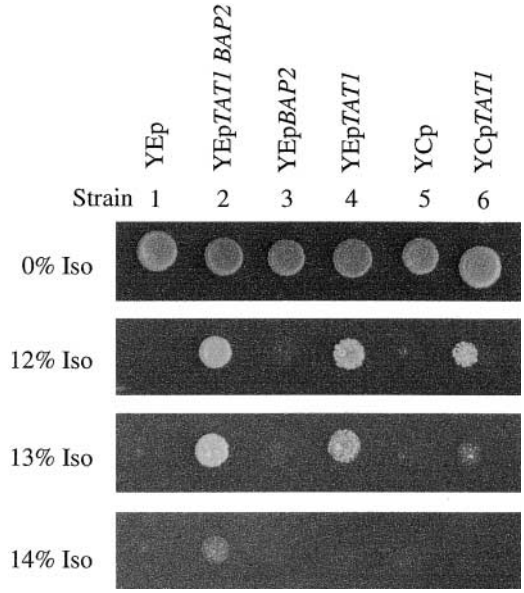

FIGURE 2.—Increased levels of *TAT1* affect isoflurane MIC. Approximately 10^4 cells from freshly saturated cultures of the indicated strains were spotted on SC-ura medium and incubated for 3 days at 30° in the presence or absence of various concentrations of isoflurane (Iso). YEp, multicopy plasmid containing the indicated permease gene(s); YCp, low-copy-number plasmid containing the indicated permease gene.

(Figure 2, compare strains 1 and 3), suggesting that while increasing the availability of only branched-chain amino acids is not sufficient to impart anesthetic resistance to RLK88-3C, it can augment the level of resistance provided by overexpression of *TAT1*.

If overexpression of *TAT1* confers anesthetic resistance by increasing amino acid availability, decreased levels of this permease might render cells hypersensitive to anesthetics due to reduced ability of the cells to transport one or more critical amino acids. To test this possibility, the anesthetic response of cells containing a precise deletion of the protein-encoding sequence of *TAT1* was examined. We find that deletion of *TAT1* increases the sensitivity of our wild-type strain (Figure 3, compare strain 1 *TAT1* to strain 1 *tat1*Δ). This provides further evidence that *TAT1* plays a critical role in anesthetic response.

Other permeases can affect anesthetic response: Of the amino acids transported by Tat1p, our wild-type strain is auxotrophic for only leucine and tryptophan (Table 1), suggesting that the availability of one or both of these amino acids affects anesthetic response. The finding that *BAP2* does not confer anesthetic resistance to RLK88-3C when overexpressed alone raises the possibility that *TAT1* is unique in its ability to alter anesthetic response. Alternatively, the ability of *TAT1* to transport both leucine and tryptophan may be the critical property, and permeases that transport only one of these amino acids may have the ability to alter anesthetic response if overexpressed in appropriately prototrophic strains. To distinguish between these possibilities, anesthetic response was assessed when *BAP2* was overexpressed in yeast strains with varying leucine or tryptophan prototrophies. Overexpression of *BAP2* in a Leu^-Trp^- strain had no effect on the isoflurane MIC (Figure 2, compare strains 1 and 3) and only very slightly increased resistance in a Leu^+Trp^- strain (Figure 4,

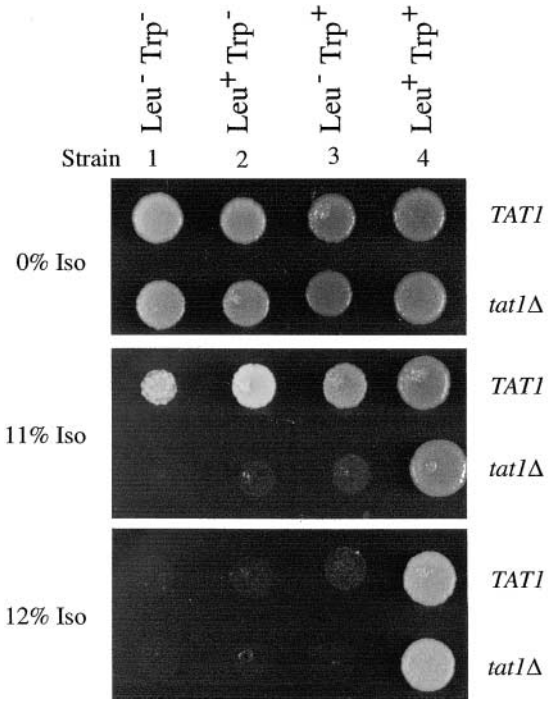

FIGURE 3.—Deletion of *TAT1* (*tat1*Δ) decreases isoflurane resistance of Leu^- or Trp^- strains but not of a Leu^+Trp^+ strain. Approximately 10^4 cells from freshly saturated cultures of the indicated strains were spotted on SC medium and tested for response to isoflurane (Iso).

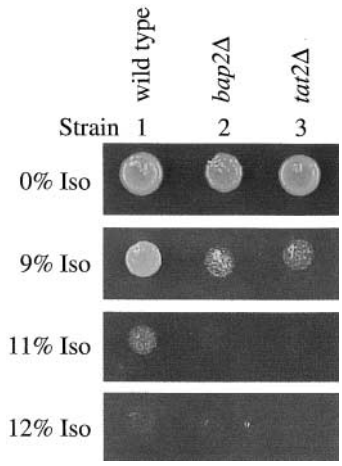

FIGURE 5.—Deletion of *BAP2* (*bap2*Δ) or *TAT2* (*tat2*Δ) from the Leu^-Trp^- wild-type strain (RLK88-3C; Table 1) decreases MIC. Approximately 10^4 cells from freshly saturated cultures of the indicated strains were spotted on SC medium and tested for response to isoflurane (Iso).

compare strains 1 and 2). However, *BAP2* overexpression in a Leu^-Trp^+ derivative, which is slightly more resistant to isoflurane than the Leu^-Trp^- strain (Figure 3, compare strains 1 and 3), increased the resistance of this strain to the same level as that of overexpression of Tat1p (Figure 4, compare strains 4 and 5 to strain 3). YEp*BAP2* contains open reading frames in addition to *BAP2* (Figure 1, strain 3 plasmid). To ensure that the altered anesthetic response required only overexpression of *BAP2*, we tested a plasmid containing only *BAP2* (YEp*BAP2*-2). We observed a similar level of resistance with this plasmid as with YEp*BAP2* in the Leu^-Trp^+ strain (data not shown). These results indicate that overexpression of *BAP2*, which encodes a high affinity leucine transporter, can increase anesthetic resistance when overexpressed in a strain that is prototrophic for tryptophan but auxotrophic for leucine. In a similar manner, overexpression of *TAT2*, which encodes the high affinity yeast tryptophan transporter, in Leu^-Trp^- or Leu^-Trp^+ strains did not increase anesthetic resistance (Figure 4, compare strains 6 and 7 or strains 8 and 9), while overexpression of this permease in a Leu^+Trp^- strain resulted in increased resistance to isoflurane (Figure 4, compare strains 10 and 11).

Analogous to *TAT1*, deletion of *BAP2* or *TAT2* increases the anesthetic sensitivity of RLK88-3C (Figure 5). Taken together, these results indicate that the avail-

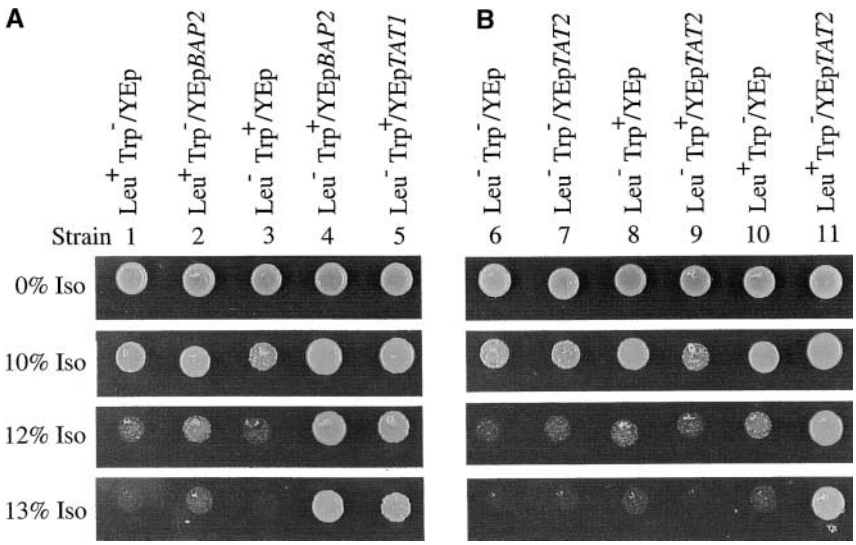

FIGURE 4.—Overexpression of (A) *BAP2* or (B) *TAT2* can alter anesthetic response when expressed in strains with appropriate prototrophies. Approximately 10^4 cells from freshly saturated cultures of strains transformed with the indicated plasmids were spotted on SC-ura medium and examined for growth in the presence of various concentrations of isoflurane (Iso). YEp, multicopy plasmid containing the indicated permease gene.

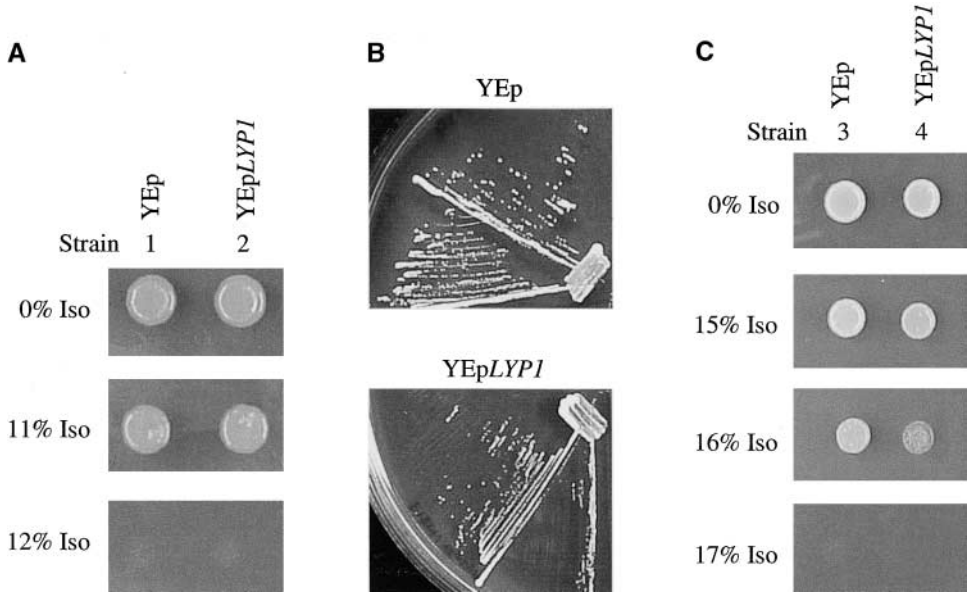

FIGURE 6.—Lysine availability does not affect anesthetic sensitivity. (A) Overexpression of the lysine-specific permease, *LYP1*, does not affect the anesthetic response of RLK88-3C. Approximately 10^4 cells from freshly saturated cultures of RLK88-3C (*his4 leu2 ura3 ade2 trp1 lys2*) transformed with YEp or YEp*LYP1* plasmids were spotted on selective media and tested for the ability to grow in various concentrations of isoflurane (Iso). (B) Overexpression of *LYP1* inhibits growth of P1814 (*HIS4 LEU2 ura3 ADE2 TRP1 lys2*). P1814 strains transformed with YEp or YEp*LYP1* plasmids were streaked on selective medium and incubated at 30° in the absence of anesthetic for 48 hr. (C) *LYP1* overexpression does not increase the anesthetic resistance of P1814. Approximately 10^4 cells from freshly saturated cultures of P1814 transformed with YEp or YEp*LYP1* plasmids were spotted on selective media and tested for response to isoflurane (Iso).

ability of leucine and tryptophan together plays a role in anesthetic response. They also demonstrate that Tat1p is not unique in its ability to alter anesthetic response in yeast.

Overexpression of *LYP1* does not affect anesthetic response: To test whether all amino acid permeases are capable of affecting anesthetic response, we examined the behavior of a derivative of our wild-type strain that contained a multicopy plasmid encoding the high affinity lysine-specific permease, *LYP1* (SYCHROVA and CHEVALLIER 1993). Although our wild-type strain is auxotrophic for lysine, *LYP1* overexpression had no effect on the anesthetic response of this strain (Figure 6A). To verify that overexpression of *LYP1* increased lysine import in this strain, uptake of radiolabeled lysine was measured. An ~2.5-fold increase in lysine uptake was observed in cells overexpressing *LYP1* (YEp*LYP1*) compared with cells containing the vector alone (YEp*LYP1*, 249 ± 23%; YEp, 100%).

It is possible that *LYP1* overexpression may alter anesthetic response in a strain with appropriate prototrophies, similar to the finding that overexpression of *BAP2* or *TAT2* only increases anesthetic response in strains that are Trp^+ or Leu^+, respectively. To test this possibility, a derivative of the wild-type strain that is auxotrophic only for lysine and uracil (Lys^-Ura^-) was constructed (Table 1). This strain was transformed with the *URA3*-marked multicopy *LYP1* plasmid or the vector control and anesthetic response was assessed. Although overexpression of *LYP1* decreased growth of this strain for an unknown reason even in the absence of isoflurane (Figure 6B), we find that overexpression of *LYP1* does not increase anesthetic resistance (Figure 6C). Thus, lysine availability from the medium does not affect anesthetic response even when the strain is auxotrophic for only this amino acid. This indicates that Lyp1p levels are not a critical factor in yeast anesthetic response.

Leucine and tryptophan phenotypes affect anesthetic response: To verify that the ability of some amino acid permeases to alter anesthetic response is due to their ability to transport nutrients into the cell and not some other function of the permease, anesthetic response was assessed in a series of Leu^+ and/or Trp^+ derivatives (Table 1) of the Leu^-Trp^- strain. We find slight increases in the level of isoflurane resistance of the Leu^+Trp^- and Leu^-Trp^+ derivatives as compared to the Leu^-Trp^- strain (Figure 7A, compare strains 2 and 3 to strain 1). In contrast, the isoflurane MIC of a Leu^+Trp^+ derivative increased from 12% to >14% (Figure 7A, compare strains 1 and 4), indicating that cells that can synthesize both leucine and tryptophan are more anesthetic resistant than cells that are auxotrophic for one or both amino acids. Deletion of *TAT1* did not affect the isoflurane MIC of the Leu^+Trp^+ strain (Figure 3, compare strain 4 *TAT1* to strain 4 *tat1*Δ). This provides additional evidence that the role of Tat1p in anesthetic response is likely due to its ability to import leucine and tryptophan from the external environment.

If the ability of a cell to import amino acids is affected by volatile anesthetics, increasing the concentrations of these nutrients in the medium may alleviate the growth-inhibitory effects of these drugs. A similar approach was employed to demonstrate that excess tryptophan in the growth medium can mitigate the toxicity of the immunosuppressive drug FK506, which inhibits amino acid import in yeast (HEITMAN *et al.* 1993). To test whether excess amino acids increase anesthetic resistance, the isoflurane MICs of the Leu^-Trp^-, Leu^+Trp^-, Leu^-Trp^+,

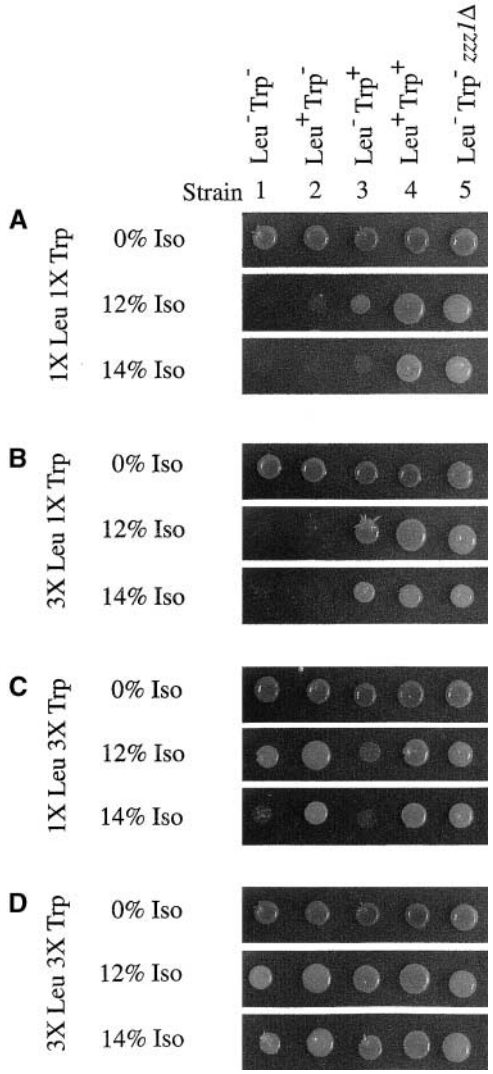

FIGURE 7.—Excess leucine and tryptophan in the growth medium render auxotrophic strains resistant to isoflurane. Approximately 10^4 cells from freshly saturated cultures of the indicated strains were spotted on media containing (A) our normal concentrations of leucine and tryptophan (1× Leu 1× Trp), (B) a threefold excess of leucine (3× Leu 1× Trp), (C) a threefold excess of tryptophan (1× Leu 3× Trp), or (D) a threefold excess of both amino acids (3× Leu 3× Trp) and incubated for 3 days at 30° in the presence or absence of various concentrations of isoflurane (Iso). A strain containing a genomic deletion of *ZZZ1/BUL1* (Leu^-Trp^- *zzz1*Δ) was included as an anesthetic-resistant control.

and Leu^+Trp^+ strains grown on SC medium containing a threefold excess of leucine (3× Leu 1× Trp), tryptophan (1× Leu 3× Trp), or both amino acids (3× Leu 3× Trp) were compared to those of strains grown on media containing our normal concentrations of these amino acids (1× Leu 1× Trp). While increasing the leucine concentration threefold in the medium had no effect on the isoflurane MIC of the Leu^-Trp^- or Leu^+Trp^- strain (Figure 7B, strains 1 and 2), the MIC of the Leu^-Trp^+ strain increased to levels similar to the prototrophic Leu^+Trp^+ strain (Figure 7B, compare strains 3 and 4). Increasing the tryptophan concentration threefold in the medium moderately increased the isoflurane resistance of the Leu^-Trp^- and Leu^-Trp^+ strains, although these strains are still more sensitive than the Leu^+Trp^+ strain (Figure 7C, compare strains 1 and 3 to strain 4). The increased tryptophan concentration rendered the Leu^+Trp^- strain extremely isoflurane resistant, to a level similar to that of the Leu^+Trp^+ strain (Figure 7C, compare strains 2 and 4). Increasing the concentrations of both leucine and tryptophan rendered all strains extremely resistant to isoflurane regardless of their leucine or tryptophan phenotypes (Figure 7D). These results further emphasize that availability of both leucine and tryptophan plays a critical role in anesthetic response and that high levels of amino acids in the medium are able to alleviate anesthetic-induced growth inhibition of strains with appropriate auxotrophies.

TABLE 2

Amino acid uptake in the absence of isoflurane

Strain[a]	Uptake (%)
YEp*TAT1*	135 ± 14 (3)[b]
*tat1*Δ	81 ± 6 (4)
*zzz1*Δ	203 ± 19 (3)
*zzz4*Δ	111 ± 3 (2)

Uptake of radiolabeled leucine was measured in the indicated strains as described in MATERIALS AND METHODS. Uptake values were calculated relative to the uptake in the appropriate wild-type control, which was given a value of 100%. All values are expressed as the average percentage of uptake ± SEM.

[a] YEp*TAT1* is RLK88-3C (Table 1) with a multicopy plasmid containing *TAT1*. All other strain designations are as given in Table 1.

[b] The numbers in parentheses indicate the number of trials that were averaged to obtain the experimental value shown.

Anesthetics inhibit amino acid import: The results described above suggest that anesthetics may inhibit yeast cell division by decreasing amino acid import. Thus, a reasonable explanation for the increased isoflurane resistance conferred by *TAT1* overexpression is that it increases uptake of amino acids from the external environment, allowing the cell to grow in the presence of a normally inhibitory concentration of anesthetic. Indeed, we find that overexpression of *TAT1* increases leucine uptake in our wild-type strain ~35% (Table 2). In addition, we find an ~20% decrease in leucine uptake in an anesthetic-supersensitive *tat1*Δ strain as compared to its wild-type counterpart (Table 2). Taken together, these results suggest a correlation between the level of amino acid import and the level of isoflurane resistance in yeast.

To directly test the effect of isoflurane exposure on amino acid import, transport of radiolabeled leucine or tryptophan was measured in the presence and ab-

TABLE 3

Amino acid uptake in the presence of isoflurane

	Uptake (%)	
Amino acid	15 min	30 min
	A. Wild type[a]	
Leucine	45 ± 4 (5)[b]	ND[c]
Tryptophan	53 ± 6 (5)	39 ± 6 (4)
Amino Acid	Uptake (%)	
	B. *zzz1*Δ	
Leucine	47 ± 2[d] (109 ± 21)[e] (2)	

[a] Uptake of radiolabeled leucine or tryptophan was measured in the wild-type strain (RLK88-3C; Table 1) after exposure to isoflurane for the indicated amount of time. Uptake values were calculated relative to uptake measured in the unexposed wild-type control, which was given a value of 100%.

[b] The numbers in parentheses indicate the number of trials that were averaged to obtain the experimental value shown.

[c] ND, not determined.

[d] Uptake of radiolabeled leucine was measured in strain P754 (Table 1) after 15 min of isoflurane exposure. Uptake values were calculated relative to uptake in the unexposed *zzz1*Δ control, which was given a value of 100%.

[e] Uptake value of the *zzz1*Δ strain after 15 min of isoflurane exposure calculated relative to uptake in the unexposed wild-type control, which was given a value of 100%. All uptake values are expressed as the average percentage of uptake ± SEM.

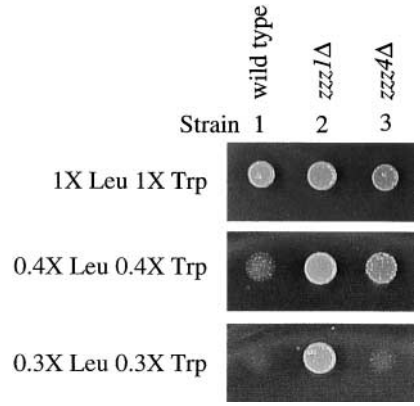

FIGURE 8.—Anesthetic-resistant mutants grow better than the isogenic wild-type strain on media containing low levels of leucine and tryptophan. Approximately 10^4 cells from freshly saturated cultures of wild-type, *zzz1*Δ, and *zzz4*Δ strains were spotted on media containing the indicated concentrations of leucine and tryptophan. Growth was evaluated following incubation at 30° for ~40 hr.

sence of isoflurane. As shown in Table 3A, leucine import decreased ~55% in anesthetic-exposed wild-type cells after 15 min of exposure to isoflurane. This rapid inhibition also corresponds to the time frame in which anesthetic-induced growth inhibition occurs (WOLFE *et al.* 1998). The observed decrease in leucine import as a result of anesthetic exposure (Table 3A) is substantially larger than the decrease observed in the *tat1*Δ strain (Table 2), which is hypersensitive to isoflurane. This is consistent with our finding that leucine permeases, such as Bap2p, in addition to Tat1p play a role in anesthetic response. Similar results were observed for tryptophan import. After 15 min of isoflurane exposure, import of this amino acid decreased ~50% (Table 3A), and an even greater decrease (~60%) was observed after 30 min of exposure (Table 3A), indicating that incubation for extended periods of time in isoflurane further decreases amino acid import.

Nutrient availability and ubiquitin metabolism are related in the anesthetic response of yeast: The results from this study indicate that nutrient availability plays a critical role in the cellular response of yeast to volatile anesthetics. Our previous investigations have shown that ubiquitin metabolism also affects anesthetic response. Mutations in *ZZZ1*, which is identical to *BUL1* (*b*inds *u*biquitin *l*igase), or *ZZZ4*, which is identical to *DOA1/UFD3* and was previously identified on the basis of altered degradation of ubiquitinated proteins, render cells resistant to isoflurane (KEIL *et al.* 1996; WOLFE *et al.* 1999). Ubiquitination has been implicated in regulating the turnover and cellular distribution of amino acid permeases in yeast (HEIN *et al.* 1995; HICKE 1997; BECK *et al.* 1999; HELLIWELL *et al.* 2001; OMURA *et al.* 2001). These findings raise the possibility that the role of ubiquitin metabolism in anesthetic response is related to nutrient availability through regulation of amino acid permeases. To test this possibility, we measured leucine import in *zzz1*Δ and *zzz4*Δ strains. In the absence of anesthetic, we find an approximately twofold increase in the amount of leucine import in a *zzz1*Δ strain compared to its isogenic wild-type counterpart (Table 2). The ability of this anesthetic-resistant mutant to import higher levels of amino acids is further supported by the finding that it is able to grow much better than the wild-type strain on media containing decreased levels of leucine and tryptophan (Figure 8, compare strains 1 and 2). Although only a small increase in leucine import was detected in the *zzz4*Δ strain as compared to the wild-type strain (Table 2), *zzz4*Δ mutants had a slight growth advantage compared to wild-type cells when grown on media with low levels of leucine and tryptophan (Figure 8, compare strains 1 and 3 at 0.4× Leu 0.4× Trp). This suggests that only a slight increase in amino acid import is sufficient to affect anesthetic response and is reminiscent of the finding that even a single extra copy of *TAT1* (YCp*TAT1)* can increase anesthetic resistance (Figure 2, strain 6).

We also tested the effect of isoflurane exposure on amino acid import in *zzz1*Δ cells. Leucine import decreased ~50% when these cells were exposed for 15 min to a concentration of isoflurane that inhibits growth of wild-type but not *zzz1*Δ strains (Table 3B). This anesthetic-induced decrease of leucine import in the *zzz1*Δ

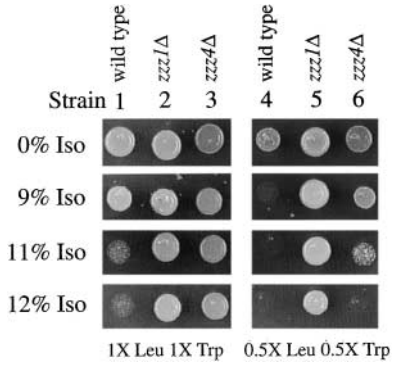

FIGURE 9.—Anesthetic MIC decreases on medium containing low levels of leucine and tryptophan. Approximately 10^4 cells from freshly saturated cultures of the indicated strains were spotted on medium containing our normal concentrations of leucine and tryptophan (1× Leu 1× Trp) or medium containing one-half the normal concentrations of these amino acids (0.5× Leu 0.5× Trp) and tested for response to isoflurane (Iso).

strain is similar in magnitude to that observed in the wild-type strain (Table 3A). However, since *zzz1*Δ cells import approximately twofold more leucine in the absence of isoflurane (Table 2), the 50% reduction induced by isoflurane leads to import levels similar to that for unexposed wild-type cells (Table 3B). Thus, the ability of *zzz1*Δ strains to grow in the presence of normally inhibitory concentrations of isoflurane may be due to their continued import of sufficient levels of amino acids.

Taken together, these results establish a relationship between ubiquitin metabolism and nutrient availability in anesthetic response of yeast. The data are also consistent with our previous finding that *zzz1*Δ mutants display a much higher level of resistance to isoflurane than do *zzz4*Δ mutants (WOLFE *et al.* 1999 and Figure 9).

Anesthetic MIC can be decreased: One difference in the behavior of anesthetic agents in yeast and humans is that an ~10-fold higher concentration of anesthetic is required to inhibit growth in yeast than is required to anesthetize a human (KEIL *et al.* 1996). It is possible that excess amino acids in our formulation of yeast growth medium may contribute to this high level of resistance. Thus, decreasing leucine and tryptophan levels in the medium may decrease the anesthetic MIC. To test this possibility, the isoflurane phenotypes of wild-type and *zzz* strains were compared on media containing 1× and 0.5× concentrations of leucine and tryptophan. Decreasing the amino acid levels in the medium reduced the MIC of the wild-type strain from the normal 12% to 9% (Figure 9, compare strains 1 and 4), indicating that it is possible to manipulate the anesthetic MIC simply by altering the leucine and tryptophan composition of the medium. Lowering the amino acid levels also increased the sensitivity of the *zzz4*Δ strain (Figure 9, compare strains 3 and 6). In contrast, the MIC of the strongly anesthetic-resistant *zzz1*Δ mutant was not affected on the 0.5× Leu 0.5× Trp media (Figure 9, compare strains 2 and 5) and may require an even greater reduction in the leucine and tryptophan concentrations to observe an effect.

DISCUSSION

Our results indicate that volatile anesthetics inhibit yeast cell division by affecting the availability of amino acids, in particular leucine and tryptophan for our strain, from the external environment. Numerous mutually supportive experimental findings are consistent with this statement. First, deletion or overexpression of amino acid permeases that transport leucine and/or tryptophan affects the anesthetic response of appropriately auxotrophic strains. Second, strains that are prototrophic for leucine and tryptophan are much more resistant to isoflurane than auxotrophic strains. Third, increased concentrations of leucine and tryptophan in the medium make auxotrophic strains resistant to these drugs, while decreased concentrations of these amino acids in the medium make them more sensitive. Fourth, uptake of radiolabeled leucine or tryptophan is inhibited during anesthetic exposure. These findings are consistent with models in which anesthetics affect amino acid availability by either directly or indirectly affecting amino acid permeases.

Role of amino acid permeases in anesthetic response: One potential role for amino acid permeases is that they may behave as drug pumps similar to the ATP-binding transporters that are involved in pleiotropic drug resistance in yeast (BALZI and GOFFEAU 1995). In this case, increased levels of permeases on the plasma membrane (*e.g.*, presence of YEp*TAT1*) would increase the export of volatile anesthetics that have entered the cell, thus decreasing the intracellular concentration of the anesthetic and rendering the cells resistant. Decreasing permease levels on the plasma membrane (*e.g.*, deletion of *TAT1*) would have the opposite effect, resulting in increased anesthetic sensitivity. Although this is an intriguing possibility, our data are not readily consistent with this model. First, while deletion of *TAT1* renders Leu^-Trp^-, Leu^+Trp^-, and Leu^-Trp^+ strains supersensitive to isoflurane, deletion in a Leu^+Trp^+ strain has no effect on anesthetic response (Figure 3, compare strain 4 *TAT1* to strain 4 *tat1*Δ). Second, overexpression of *TAT1* in the Leu^+Trp^+ strain background also has no effect (not shown). Because the level of Tat1p is irrelevant in a strain that is prototrophic for leucine and tryptophan, this suggests that the critical property of the permease is not a generalized ability to export anesthetics out of the cell, but rather is due to its ability to import amino acids from the external environment. The findings that overexpression of the leucine transporter, *BAP2*, or the tryptophan transporter, *TAT2*, confers anesthetic resistance only in strains with appropriate

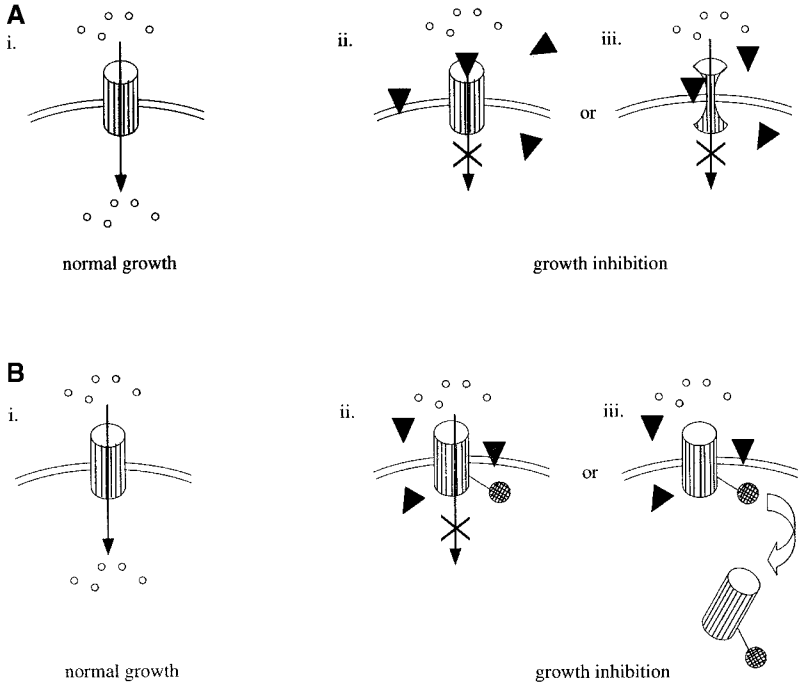

FIGURE 10.—Models for (A) direct and (B) indirect involvement of amino acid permeases in anesthetic response. See DISCUSSION for details of the models. Barrel, amino acid permease; open circles, amino acids; triangles, anesthetic; X, inhibition of amino acid transport; cross-hatched circle, post-translational modification of amino acid permease. The curved arrow indicates removal of the permease from the plasma membrane. Alternative models for the indirect involvement of amino acid permeases are possible.

prototrophies provide further evidence that the permeases are not functioning as drug export pumps. This is reminiscent of the findings of WOLFE *et al.* (1998) that the *p*leiotropic *d*rug *r*esponse genes *YAP1/PDR4* and *PDR5* do not affect anesthetic response.

The involvement of amino acid permeases in anesthetic response may be direct or indirect (Figure 10). Direct involvement would indicate that permeases are the primary targets of volatile anesthetics. In the absence of anesthetics, permeases would function normally, permitting the cell to grow (Figure 10A, i). During anesthetic exposure, the anesthetic could bind directly to the permease and inhibit amino acid transport (Figure 10A, ii), or the anesthetic could interact with the plasma membrane near the permease, inducing a perturbation that leads to altered permease function (Figure 10A, iii). In both cases, anesthetic interaction would lead to decreased permease activity and thus inhibition of amino acid transport.

An alternative explanation for decreased uptake of amino acids is that volatile anesthetics induce a post-translational modification of the permease that either directly decreases transport activity (Figure 10B, ii) or leads to altered cellular localization or degradation of the permease (Figure 10B, iii), thus affecting its activity. Our finding that mutation of a number of different ubiquitin metabolism genes affects anesthetic response (WOLFE *et al.* 1999) suggests that ubiquitination may be a potential modification. Further support for this possibility comes from the finding that a variety of plasma membrane proteins, including amino acid permeases, undergo ubiquitin-dependent endocytosis and degradation in response to environmental stimuli (HEIN *et al.* 1995; HICKE 1997; BECK *et al.* 1999). Of particular interest is the finding that ubiquitination and degradation of Tat2p are induced by nutrient limitation (BECK *et al.* 1999). Studies are currently in progress to determine which, if any, of these models accurately reflect the involvement of amino acid permeases in anesthetic response.

Specificity of amino acid permeases: We find that overexpression or deletion of *TAT1*, *BAP2*, and *TAT2* increases or decreases anesthetic resistance, respectively, in strains with appropriate amino acid auxotrophies. However, overexpression of *LYP1* has no effect on anesthetic response. These results indicate specificity in the activity of anesthetics in yeast. In addition, it suggests that these drugs are not affecting the proton gradient necessary to drive amino acid import, as this gradient is required for transport of all amino acids. While it is not clear why the levels of some amino acid permeases affect anesthetic response while others do not, it is interesting to note that Tat1p, Bap2p, and Tat2p are more closely related evolutionarily to each other than to Lyp1p (NELISSEN *et al.* 1997). In addition, transcription of *TAT1*, *BAP2*, and *TAT2* has been shown to be affected by Ssy1p (DIDION *et al.* 1996; IRAQUI *et al.* 1999), a component of a yeast plasma membrane sensor of extracellular amino acids (KLASSON *et al.* 1999; FORSBERG *et al.* 2001). Experiments are currently in progress to delineate which other amino acid permeases alter anesthetic response when their levels are genetically manipulated and to determine whether Ssy1p plays a role in anesthetic response. Results from these studies should provide insights into the nature of anesthetic specificity in yeast.

Anesthetic effects in yeast and mammals are similar:

In mammals, volatile anesthetics have been shown to dramatically affect metabolism in a variety of cells and tissues, including the brain. Of particular interest in regard to this study is the finding that anesthetics affect amino acid transport in mammalian systems. Since a number of neurotransmitters are amino acids (examples include glutamate, aspartate, and glycine) or amino acid derivatives (tryptophan is the precursor for serotonin, tyrosine is the precursor of catecholamines including dopamine, glutamate is the precursor for GABA, and histidine is the precursor of histamine), availability of amino acids and their derivatives is of critical importance for neuronal function. Shimada *et al.* (1995) showed that volatile anesthetics inhibit L-alanine transport in rat megakaryocytes (precursors of platelets). Volatile anesthetics also inhibit transport of dopamine (el-Maghrabi and Eckenhoff 1993) and serotonin (5-hydroxytryptamine; Martin *et al.* 1990) in rat brain synaptosomes. In addition, while branched-chain amino acids are neither neurotransmitters nor precursors, they (especially leucine) play a major role in regulating cellular pools of the neurotransmitter glutamate (Yudkoff *et al.* 1994; Yudkoff 1997). It is intriguing that a number of these amino acids are transported by permeases that are affected by volatile anesthetics in yeast: Tryptophan and tyrosine are transported by Tat1p and Tat2p (Schmidt *et al.* 1994); branched-chain amino acids are transported by Tat1p and Bap2p (Grauslund *et al.* 1995; Nelissen *et al.* 1997); and Tat1p has been shown to act as a low-affinity histidine transporter (Bajmoczi *et al.* 1998). Thus, effects of volatile anesthetics on permeases that transport any of these critical nutrients in neuronal cells as well as in other cells and tissues may play an essential role in the cellular activities of these drugs in mammals.

Biologically relevant targets: The search for cellular targets of anesthetics that are critical for inducing anesthesia has been a predominant theme in research on these drugs for years. One standard applied in this search has been whether effects on potential targets are observed when a clinically relevant concentration of anesthetic is used. This standard has sparked a lively exchange (Eckenhoff and Johansson 1999, 2001; Eger *et al.* 2001). Finding that activities of a wide range of cellular proteins are affected by clinical concentrations of anesthetics *in vitro* (for a review see Eckenhoff and Johansson 1997) makes it difficult to argue that this is an informative guidepost to distinguish between targets that are biologically significant for anesthetic action and targets that are inconsequential. In addition, Eckenhoff and Johansson (1999) caution against extrapolating effects determined with clinically relevant anesthetic concentrations *in vitro* to *in vivo* systems, citing a lack of understanding of how various *in vitro* systems contribute to integrated responses in an intact organism. A clear advantage of using a genetic approach to investigate anesthetic action *in vivo* is that genes identified as altering cellular responses to these drugs must reflect molecular effects of anesthetics that are biologically relevant, although it does not demonstrate if this involvement is direct or indirect. In several model organisms, including yeast, concentrations higher than those used clinically have been employed to identify mutants with altered anesthetic response (Overton 1901; Morgan *et al.* 1988; Keil *et al.* 1996). One reason suggested for the necessity of high doses is the need of these organisms to protect themselves from potentially harmful compounds in an environment on which they exert little control (Sedensky *et al.* 1994). While this may be true, the findings reported here show that for yeast the relatively high biologically relevant anesthetic dose is due, at least in part, to: (1) the ability of yeast to efficiently import critical nutrients, (2) the culture conditions used to grow yeast in our lab, and (3) the particular auxotrophic markers present in our wild-type strain. Specifically, anesthetic MIC in yeast can be dramatically influenced by (1) the cellular concentration of a putative protein target [*e.g.*, compare MIC for *TAT1* (12%), YEp*TAT1* (>14%), and *tat1*Δ (9%) strains (Figures 2 and 3)], (2) the concentration of essential metabolites in the environment [*e.g.*, compare MIC for the wild-type strain grown on 3× Leu 3× Trp (>14%) *vs.* 1× Leu 1× Trp (12%) *vs.* 0.5× Leu 0.5× Trp (9%; Figures 7 and 9)], and (3) the auxotrophic requirements of the cell [*e.g.*, compare MIC for the Leu^-Trp^- strain (12%) to that of the Leu^+Trp^+ strain (>14%; Figure 7A)]. Experiments are in progress to determine if strains auxotrophic for other nutrients have an even lower anesthetic MIC than the yeast strain currently used. The ability to manipulate MIC in yeast genetically or environmentally suggests that determination of effective concentrations in other *in vivo* or *in vitro* systems may be subject to similar constraints. Thus, finding that the effective concentration in a particular experimental system approximates a clinical concentration may be a fortuitous mix of experimental conditions and seems likely to provide limited insight regarding the biological importance of a given target.

We thank Drs. Anita K. Hopper and Roderic G. Eckenhoff for their critical comments regarding this manuscript, members of the Keil laboratory for their helpful discussions, and M. Hall and M. C. Kielland-Brandt for providing plasmids used in this work. This work was supported in part by grant GM57822 from the National Institutes of Health to R.L.K.

LITERATURE CITED

Bajmoczi, M., M. Sneve, D. J. Eide and L. R. Drewes, 1998 *TAT1* encodes a low-affinity histidine transporter in *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. **243:** 205–209.

Balzi, E., and A. Goffeau, 1995 Yeast multidrug resistance: the PDR network. J. Bioenerg. Biomembr. **27:** 71–76.

Batai, I., M. Kerenyi and M. Tekeres, 1999 The impact of drugs used in anaesthesia on bacteria. Eur. J. Anesthesiol. **16:** 425–440.

Beck, T., A. Schmidt and M. N. Hall, 1999 Starvation induces

vacuolar targeting and degradation of the tryptophan permease in yeast. J. Cell Biol. **146:** 1227–1238.

Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer *et al.*, 1979 Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:** 17–24.

Carlson, M., and D. Botstein, 1982 Two differentially regulated mRNAs with different 5′ ends encode secreted with intracellular forms of yeast invertase. Cell **28:** 145–154.

Didion, T., M. Grausland, C. Kielland-Brandt and H. A. Andersen, 1996 Amino acids induce expression of *BAP2*, a branched-chain amino acid permease gene in *Saccharomyces cerevisiae*. J. Bacteriol. **178:** 2025–2029.

Eckenhoff, R. G., and J. S. Johansson, 1997 Molecular interactions between inhaled anesthetics and proteins. Pharmacol. Rev. **49:** 343–367.

Eckenhoff, R. G., and J. S. Johansson, 1999 On the relevance of "clinically relevant concentrations" of inhaled anesthetics in *in vitro* experiments. Anesthesiology **91:** 856–860.

Eckenhoff, R. G., and J. S. Johansson, 2001 What are "relevant" concentrations? Anesthesiology **95:** 1537–1539.

Eger, II, E. I., D. M. Fisher, J. P. Dilger, J. M. Sonner, A. Evers *et al.*, 2001 Relevant concentrations of inhaled anesthetics for in vitro studies of anesthetic mechanisms. Anesthesiology **94:** 915–921.

el-Maghrabi, E. A., and R. G. Eckenhoff, 1993 Inhibition of dopamine transport in rat brain synaptosomes by volatile anesthetics. Anesthesiology **78:** 750–756.

Forsberg, H., M. Hammar, C. Andreasson, A. Moliner and P. O. Ljungdahl, 2001 Suppressors of *ssy1* and *ptr3* null mutations define novel amino acid sensor-independent genes in *Saccharomyces cerevisiae*. Genetics **158:** 973–988.

Ghislain, M., R. J. Dohmen, F. Levy and A. Varshavsky, 1996 Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. **15:** 4884–4899.

Gietz, R. D., and A. Sugino, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

Grauslund, M., T. Didion, M. C. Kielland-Brandt and H. A. Andersen, 1995 *BAP2*, a gene encoding a permease for branched-chain amino acids in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1269:** 275–280.

Guldener, U., S. Heck, T. Fielder, J. Beinhauer and J. H. Hegemann, 1996 A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:** 2519–2524.

Hein, C., J. Y. Springael, C. Volland, R. Haguenauer-Tsapis and B. Andre, 1995 NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. **18:** 77–87.

Heitman, J., A. Koller, J. Kunz, R. Henriquez, A. Schmidt *et al.*, 1993 The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 5010–5019.

Helliwell, S. B., S. Losko and C. A. Kaiser, 2001 Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. J. Cell Biol. **153:** 649–662.

Hicke, L., 1997 Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J. **11:** 1215–1226.

Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles *et al.*, 1999 Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:** 989–1001.

Keil, R. L., D. Wolfe, T. Reiner, C. J. Peterson and J. L. Riley, 1996 Molecular genetic analysis of volatile-anesthetic action. Mol. Cell. Biol. **16:** 3446–3453.

Klasson, H., G. R. Fink and P. O. Ljungdahl, 1999 Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol. Cell. Biol. **19:** 5405–5416.

Koblin, D. D., 1994 Inhaled anesthetics: mechanisms of action, pp. 67–99 in *Anesthesia*, edited by R. Miller. Churchill-Livingstone, New York.

Lin, Y. H., and R. L. Keil, 1991 Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast. Genetics **127:** 31–38.

Martin, D. C., R. J. Adams and R. S. Aronstam, 1990 The influence of isoflurane on the synaptic activity of 5-hydroxytryptamine. Neurochem. Res. **15:** 969–973.

Morgan, P. G., M. M. Sedensky, P. M. Meneely and H. F. Cascorbi, 1988 The effect of two genes on anesthetic response in the nematode *Caenorhabditis elegans*. Anesthesiology **69:** 246–251.

Nelissen, B., R. De Wachter and A. Goffeau, 1997 Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **21:** 113–134.

Omura, F., Y. Kodama and T. Ashikari, 2001 The N-terminal domain of the yeast permease Bap2p plays a role in its degradation. Biochem. Biophys. Res. Commun. **287:** 1045–1050.

Overton, C. E., 1901 *Studies of Narcosis*. Gustav Fischer, Jena, Germany.

Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt and S. Holmberg, 1999 Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. Curr. Genet. **36:** 317–328.

Rose, M. D., F. Winston and P. Hieter, 1990 *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schiestl, R. H., and R. D. Gietz, 1989 High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:** 339–346.

Schmidt, A., M. N. Hall and A. Koller, 1994 Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, *TAT1* and *TAT2*, encode amino acid permeases mediating tyrosine and tryptophan uptake. Mol. Cell. Biol. **14:** 6597–6606.

Sedensky, M. M., H. F. Cascorbi, J. Meinwald, P. Radford and P. G. Morgan, 1994 Genetic differences affecting the potency of stereoisomers of halothane. Proc. Natl. Acad. Sci. USA **91:** 10054–10058.

Shimada, H., Y. Tomita, G. Inooka and Y. Maruyama, 1995 Sodium-coupled neutral amino acid cotransporter inhibited by the volatile anesthetic, halothane, in megakaryocytes. Jpn. J. Physiol. **45:** 165–176.

Sychrova, H., and M. R. Chevallier, 1993 Cloning and sequencing of the *Saccharomyces cerevisiae* gene *LYP1* coding for a lysine-specific permease. Yeast **9:** 771–782.

Wolfe, D., P. Hester and R. L. Keil, 1998 Volatile anesthetic additivity and specificity in *Saccharomyces cerevisiae*: implications for yeast as a model system to study mechanisms of anesthetic action. Anesthesiology **89:** 174–181.

Wolfe, D., T. Reiner, J. L. Keeley, M. Pizzini and R. L. Keil, 1999 Ubiquitin metabolism affects cellular response to volatile anesthetics in yeast. Mol. Cell. Biol. **19:** 8254–8262.

Yashiroda, H., T. Oguchi, Y. Yasuda, E. A. Toh and Y. Kikuchi, 1996 Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 3255–3263.

Yudkoff, M., 1997 Brain metabolism of branched-chain amino acids. Glia **21:** 92–98.

Yudkoff, M., Y. Daikhin, Z. P. Lin, I. Nissim, J. Stern *et al.*, 1994 Interrelationships of leucine and glutamate metabolism in cultured astrocytes. J. Neurochem. **62:** 1192–1202.

Communicating editor: M. Lichten