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Local Anesthetics Block Induction of the *Pseudomonas alk* Regulon

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The local anesthetics procaine and piperocaine blocked induction of the plasmid-determined enzymatic activities involved in the metabolism of *n*-alkanes in *Pseudomonas putida*. Procaine reversibly inhibited existing alkane hydroxylase activity. Induction of a soluble aliphatic amidase activity was not affected. These results support the hypothesis that induction of the plasmid-determined alkane metabolic system in *P. putida* involves a membrane component(s).

Pseudomonas putida strains are able to grow on *n*-alkanes (C_6-C_{10}) by virtue of specific *alk* genes which are found only on certain Inc-P2 *Pseudomonas* plasmids (1, 6, 7). These genes determine biochemical and regulatory functions required for growth on alkanes (1, 3, 6). Recent work has shown that the plasmid-determined alkane hydroxylase and alcohol dehydrogenase steps involve cytoplasmic membrane components (1, 3; S. Benson, J. Shapiro, and M. Oppici, manuscript in preparation). The important question of whether or not the inducer recognition takes place within the membrane structure has not been clearly addressed. Several considerations suggest that inducer recognition involves the membrane structure: (i) alkanes have a high affinity for the hydrophobic environment of the membrane and a low affinity for the hydrophilic environment of the cytoplasm; (ii) unoxidized alkanes are efficient inducers (2); and (iii) long-chain alcohols induce by virtue of their unoxidized hydrophobic tails (6). We have not been able to identify an *alk*-specific membrane peptide with a regulatory role so far. To test the hypothesis that induction involves a membrane component(s), the effects of local anesthetics on induction of the *alk* system were examined.

Local anesthetics affect a number of membrane processes in both mammalian (10) and bacterial cells (11, 12). Although the exact mechanism(s) of action of this class of drugs remains unknown, it has been demonstrated that they cause structural alterations in biological membranes (10, 11) and in artificially constructed bilayers (9, 13). It is generally accepted that the membrane disruption property accounts at least in part for their ability to block a number of seemingly unrelated membrane processes.

To test the effects of the local anesthetic procaine on induction, *alk*$^+$ *P. putida* cells were induced by dicyclopropylmethanone and heptane (6) in glycerol-salts medium containing various concentrations of procaine. The results in Fig. 1A show that cells grown in the presence of 0.4% (wt/vol) procaine were blocked more than 90% for induction of alkane hydroxylase activity. A concentration of 0.2% blocked induction by approximately 50%. Concentrations as high as 0.6% did not block induction of the soluble aliphatic amidase system (Fig. 1A). Since alkane hydroxylase activity involves both a particulate (AlkB) and a soluble (AlkA) activity, each of which can be assayed by in vitro complementation (1), it was important to show that the lack of induced activity reflects a lack of induction and not a secondary effect procaine might be having on the particulate AlkB activity. To show further that the lack of hydroxylase activity in Fig. 1A is due to an effect on induction, I measured induction of the soluble AlkA activity in a strain carrying the *alkR524* allele (5). This allele confers a low level of constitutivity and retains the ability to be induced to a normal wild-type level (5). Figure 1B shows that procaine blocked induction of the AlkA activity at a concentration (0.5%) that did not affect induction of the amidase system. The observation that AlkA activity did not drop to zero indicated that preexisting AlkA activity is not affected by the anesthetic. The degree of induction blockage seen in Fig. 1A (hydroxylase activity) was greater than that seen in Fig. 1B (AlkA activity). This was unexpected, since the genes determining each of these activities are contained within a single regulon (5). The apparent difference results from two factors: (i) the sensitivity of the AlkA assay system is less than that of the hydroxylase, since in vitro complementation is required in the former case; and (ii) the AlkA activity was determined using a strain that contains a low-level constitutive

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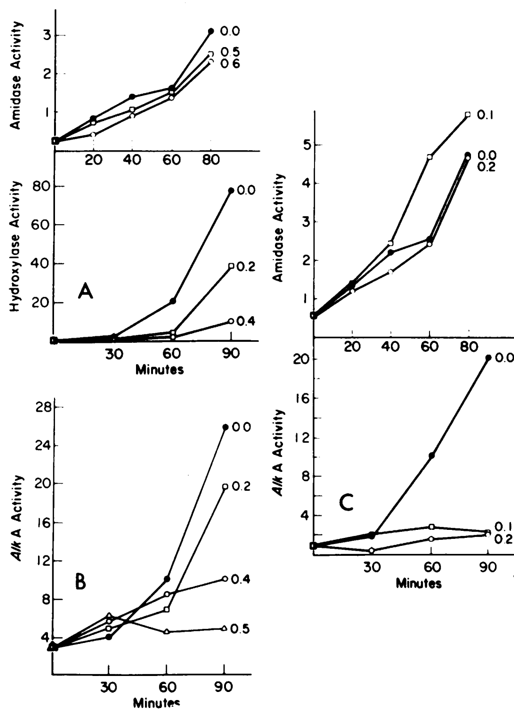

FIG. 1. *Effects of local anesthetics on induction of alkane hydroxylase activity and aliphatic amidase activity in P. putida. P. putida PpS 124 CAM-OCT (alk⁺) (6) (A and C) and PpS 524 trp-338 alcA81 CAM- OCT (alkR184) (5) (B) were grown overnight in minimal salts medium supplemented with 0.5% glycerol (PA-glycerol) as previously described (1). Cells were then diluted into fresh medium containing 0.5 mM dicyclopropylmethanone, 0.1% (vol/vol) heptane, and various concentrations of procaine (A and B) or piperocaine (C). Cells were incubated at 32°C with constant shaking; at the times indicated on the abscissa, samples were removed, and the cells were washed with an equal volume of PA-salts and frozen (−20°C) for 1 to 3 days. Cell-free sonic extracts were prepared from thawed cell pellets and assayed for hydroxylase activity (A) or the soluble component of the alkane hydroxylase activity (AlkA activity) (B and C) as previously described. Amidase activity was induced by growing cells as described above with substitution of the amidase inducer acetamide (10 mM) for the hydroxylase inducers dicyclopropylmethanone plus heptane. Amidase activity was determined by the method of Brammar et al. (4). In those cases where AlkA activity was measured, excess cell-free extract from an alkA B⁺ strain was provided as previously described (1). The designation alcA defines the chromosomal gene(s) which determine(s) growth on hexanol through dodecanol (3). Anesthetics were purchased from Sigma Chemical Co. (procaine) and Lilly Pharmaceuticals Co. (piperocaine). (A) Top panel: (●) no anesthetic, (□) 0.5% procaine, (○) 0.6% procaine; bottom panel: (●) no anesthetic, (□) 0.2% procaine, (○) 0.4% procaine. (B) (●) No anesthetic, (□) 0.2% procaine, (○) 0.4% procaine, (△) 0.5% procaine. (C) (●) No anesthetic, (□) 0.1% piperocaine, (○) 0.2% piperocaine.*

superinducible phenotype (5). Thus, it is difficult to compare the degree of blockage directly in the two experiments. In both cases the absolute level of induced activity was greatly reduced by the presence of procaine. This reduction in activity cannot be due to procaine being carried into the assay mixtures, since control experiments where induced cells were treated with 0.5% procaine showed no reduction in alkane hydroxylase or in AlkA activity. The direct addition of procaine to 0.5% into the alkane hydroxylase mixture reduced activity by approximately 50%. This is much less than the blockage seen in Fig. 1A at 0.4% or Fig. 1B at 0.5% procaine.

I reasoned that if procaine blocked induction due to its general membrane disruption property, then other local anesthetics should behave similarly. To test this I used a second anesthetic, piperocaine. Piperocaine is a more potent anesthetic which has a higher octanol-water partition coefficient due to the increased hydrophobicity of its molecular structure (8). Figure 1C shows that a concentration of 0.1% was sufficient to block induction of AlkA activity by greater than 90%, whereas induction of the amidase system was not affected at concentrations as high as 0.2%.

These results show that induction of the plasmid-encoded *alk* system is sensitive to the effects of the local anesthetics procaine and piperocaine. The exact mechanism by which these compounds block induction remains to be determined. The fact that two structurally different anesthetics, neither of which resembles known inducers of the *alk* system, block induction suggests that the blockage is not due to a competitive anti-inducer mechanism. The possibility that the induction defect is caused by a blockage of inducer permeability seems unlikely in light of the fact that two different inducers were used: dicyclopropylmethanone, a gratitious inducer of low water solubility, and heptane, which is not soluble in water. The most attractive explanation is that these compounds prevent induction by altering the membrane structure in such a way that inducer recognition cannot take place. This is supported by the observation that piperocaine, with its greater affinity for a hydrophobic environment, is more effective at blocking induction than procaine. However, other less likely explanations cannot be ruled out at this time.

Thus the hypothesis that induction of the *alk* system involves a membrane component(s) is supported by the fact that membrane disruption agents (i.e., procaine and piperocaine) block induction. Direct proof that the inducer recognition protein is a membrane protein will require the identification of an *alk*-specific membrane protein encoded by a regulatory gene.

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