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Triggering Glutamate Excretion in *Corynebacterium glutamicum* by Modulating the Membrane State with Local Anesthetics and Osmotic Gradients

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Corynebacterium glutamicum **can be triggered to excrete glutamate by the addition of local anesthetics, particularly tetracaine. Glutamate efflux is a carrier-mediated process and not due to unspecific membrane permeabilization. The concentration of local anesthetics triggering optimum excretion depended on the type of anesthetic and varied, ranging from 0.1 (chlorpromazine), 1.3 (tetracaine), and 2.6 mM (butacaine) to 15 mM (benzocaine), in close resemblance to the order of efficiency in anesthetic effect. The onset of glutamate excretion was not correlated to a change in the viscosity or fluidity of the membrane, as measured by electron spin resonance spectroscopy, nor was it related to an action of the anesthetic as an uncoupler. Tetracaine-triggered glutamate excretion was sensitive to changes in the transmembrane osmotic gradient, although an osmotic gradient alone could not trigger glutamate excretion. Tetracaine-triggered glutamate efflux was inhibited by an external rise in osmolality and stimulated by a corresponding decrease. The effects of osmotic gradients and the addition of local anesthetics on glutamate excretion were mutually exchangeable, indicating similar modes of action. We suggest that this common principle is a change in the membrane strain.** ***C. glutamicum*** **cells which excrete glutamate without manipulation of the membrane, e.g., biotin-limited cells or glutamate production mutants, were not stimulated by the addition of tetracaine.**

Corynebacterium glutamicum is widely used for its ability to produce large amounts of glutamate under particular conditions, e.g., biotin limitation. We and others have studied glutamate excretion (8–10, 22, 25, 26, 43), and different hypotheses have been put forward to explain this capacity (9, 12, 35, 36). We were able to attribute glutamate efflux under biotin limitation to the activity of a specific excretion carrier system (22, 25). We found that this activity is correlated to two different events, a metabolic overflow situation combined with growth arrest and a change in the physical state of the membrane (22, 26). The main focus of this paper is to elucidate the kind of alteration in the membrane which leads to increased activity of glutamate excretion in *C. glutamicum.*

Different explanations have been put forward for the changes responsible for triggering glutamate efflux, e.g., increased leakiness (8, 12), changed membrane fluidity (viscosity) and permeability, and other kinds of physical alterations (13). In general, glutamate excretion triggered by biotin starvation is explained by the inhibition of fatty acid synthesis. This leads to decreased availability of phospholipids and consequently to membrane alterations. Since there are many different ways to trigger glutamate excretion in coryneform bacteria (2, 35), the situation is obviously not that simple. Biotin limitation and inhibition of fatty acid synthesis, respectively, are complex ways to change membrane properties. Thus, we used a more straightforward approach to study the consequences of changes in membrane properties, i.e., addition of local anesthetics.

Local anesthetics are well-known for altering the physical state of a membrane. They have been applied in numerous studies in which mainly biophysical techniques were used, e.g., nuclear magnetic resonance, electron spin resonance (ESR), and fluorescence. There is general agreement that local anesthetics change the order of the lipid bilayer; it is, however, not clear whether the main effect is in the direction of disordering or ordering the membrane (7, 27, 32, 50). Most probably, the disordering (fluidizing) effect is due to the insertion of the uncharged form of a local anesthetic in the membrane, whereas the stabilizing effect (increasing the bilayer order) can be attributed to the charged form (4, 31).

In the present communication, we report that the addition of local anesthetics to *C. glutamicum* cells leads to effective glutamate excretion which is not due, however, to a change in the viscosity of the membrane. We present a general explanation as to why these unphysiological agents may induce a more or less physiological response in bacteria by relating the observed excretion to a situation more common for bacteria, namely, membrane strain by the application of osmotic stress.

MATERIALS AND METHODS

Organisms and culture conditions. *C. glutamicum* ATCC 13032 was grown aerobically on a rotary shaker (150 rpm) at 30°C. A minimal medium (MM1) with glucose (25 g/liter) or glutamate (100 mM) as the carbon source, with the following contents was used: $(NH_4)_2SO_4$, 5 g/liter; urea, 5 g/liter; KH_2PO_4, 2 g/liter; K_2HPO_4, 2 g/liter; $MgSO_4 \cdot 7H_2O$, 250 mg/liter; $FeSO_4 \cdot 7H_2O$, 10 mg/liter; $CaCl_2 \cdot 2H_2O$, 10 mg/liter; $MnSO_4 \cdot 1H_2O$, 10 mg/liter; biotin, 200 μg/liter; plus trace amounts of $ZnSO_4$, H_3BO_3, $CoCl_2$, $CuSO_4$, NiCl, and $NaMoO_4$ (pH 7.0) (14, 15). Alternatively, as indicated, cells were grown on MMYE medium containing $(NH_4)_2SO_4$ (10 g/liter), urea (3 g/liter), KH_2PO_4 (2 g/liter), K_2HPO_4 (2 g/liter), NaCl (0.05 g/liter), $MgSO_4 \cdot 7H_2O$ (0.4 g/liter), $FeSO_4 \cdot 7H_2O$ (2 mg/liter), $MnSO_4 \cdot 5H_2O$ (2 mg/liter), yeast extract (1 g/liter), and biotin (200 μg/liter) (pH 7.0). Biotin-limited wild-type cells were grown as described previously (22). A glutamate production mutant (strain 9087) was obtained from Kyowa-Hakko Kogyo Co., Tokyo, Japan. This strain was grown on a complex medium containing peptone (15.6 g/liter), yeast extract (2.8 g/liter), NaCl (5.6 g/liter), and biotin (200 μg/liter) (pH 7.0), with glucose (50 g/liter) as the carbon source. Cell mass was determined by measuring the optical density at 600 nm (OD_{600}); an OD_{600} of 1 corresponds to 0.32 mg (dry mass)/ml.

Measurement of the cytoplasmic volume, membrane potential, and pH gradient. All measurements were carried out as described previously (14, 15). Cells were separated from the medium by silicone oil centrifugation (34). For determinations of cytoplasmic volume, [^{14}C]taurine was used as the membrane-impermeable probe and ^3H_2O was used as the permeable probe. An average

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cytoplasmic volume of 1.6 μl · mg (dry mass)$^{-1}$ was found for *C. glutamicum* cells. Membrane potential was determined from the distribution of the [^{14}C]tetraphenylphosphonium cation. Values were corrected for the binding of the probe by using a [^{14}C]tetraphenylphosphonium bromide-^{86}Rb calibration curve (22). The pH gradient was determined from the distribution of the lipophilic probe [^{14}C]benzoic acid or [^{14}C]methylamine, depending on the external pH (38).

Determinations of external and cytosolic solute concentrations. For analyses of intra- and extracellular glutamate concentrations, cells from the incubation medium were centrifuged for 30 s in an Eppendorf centrifuge. The supernatant was carefully removed, and the pellet was vortexed with 500 μl of 0.1% (wt/wt) cetyltrimethylammonium bromide for 2 min. After addition centrifugation, the supernatant was removed and used to determine the concentration of internal glutamate. Both supernatants were then incubated for 15 min at 95°C to inactivate enzymes. Due to the high internal glutamate concentrations, a correction for the concentration of external glutamate (as well as separation by silicone oil centrifugation) was not necessary. Glutamate concentrations were determined enzymatically (6). The concentrations of other amino acids were determined by reversed-phase high-pressure liquid chromatography using *o*-phthalaldehyde precolumn derivatization (38). Nucleotides were extracted by injecting an aliquot of the bacterial suspension directly into dimethyl sulfoxide (13, 37). ATP concentrations were determined by firefly luciferase assay (39). Internal and external K^+ concentrations were determined by atomic absorption spectroscopy.

Determination of amino acid excretion. *C. glutamicum* strains were grown to exponential phase (OD_{600}, 2 to 3), and cells were harvested by centrifugation, washed twice with 50 mM MES (morpholine ethanesulfonic acid)-Tris (pH 8.0)–1 mM KCl, and stored on ice at about 40 mg (dry weight) · ml^{-1}. Immediately prior to the experiment, cells were suspended at about 1 mg (dry weight) · ml^{-1} in 100 mM MES-Tris–5 mM KCl–10 mM NaCl–5 mM urea–10 mM ammonium sulfate–30 mM glucose (pH 8.0) at 30°C, if not otherwise indicated. Cell suspensions not exceeding an OD_{600} of 5.0 were vigorously stirred in small glass vessels at volumes not exceeding 5 ml to ensure sufficient aeration. At different times, samples were taken and analyzed as described above.

Determination of glutamate uptake. The uptake of glutamate was measured with a ^{14}C-labeled substrate. Termination of the reaction and the separation of cells after different time intervals were performed by rapid filtration on glass fiber filters (Whatman GF/F) or silicone oil centrifugation (38). It should be noted that the uptake of labeled glutamate can be measured during net glutamate excretion, which is determined enzymatically, by a measurement of the external glutamate concentration and corresponding correction of the actual specific activity. The uptake rates reported are initial values and were calculated from a linear kinetic range including at least four experimental points.

Preparation of membranes for ESR measurements. After growth in the indicated media to an OD_{600} of about 5, cells were harvested and washed twice by centrifugation in a sterile buffer containing 50 mM triethanolamine hydrochloride, 250 mM sucrose, and 1 mM EDTA (pH 7.5; NaOH). One gram of cells was suspended in 1 ml of the same buffer and passed six times through a French pressure cell at 16,000 lb/in^2. This resulted in vesicles with diameters of about 200 nm, as estimated by microscopy. Four milliliters of suspension was purified by centrifugation (16 h, 10°C, 25,000 rpm; Beckman SW28 rotor) on a continuous sucrose gradient (36 ml consisting of the buffer described above and a 70% [wt/vol] sucrose solution). The band containing the vesicles was diluted twofold in buffer containing 50 mM MOPS (morpholinepropanesulfonic acid), 60 mM KCl, 60 mM NaCl, and 5 mM $MgSO_4$ (pH 7.5). After centrifugation (10 min, 4°C, 12,000 × g), the sediment was suspended in 1 ml of MOPS buffer. To obtain highly purified vesicles, the suspension was centrifuged three times at 750 × *g* (10 min, 4°C) in MOPS buffer, with the sediment which contained whole cells always being discarded. Finally, vesicles were spun down at 12,000 × *g*. This sediment was resuspended in 100 μl of MOPS buffer, frozen at −30°C, and kept for further use.

ESR measurements. The fatty acid spin label was 2-(3-carboxypropyl)-2-dodecyl-4,4-dimethyl-3-oxazolidinyloxyl. A thin film of the label was prepared in a glass vessel by dissolving 25 μl of 1.1 mM label in CCl_4 and evaporating the solvent with nitrogen. Vesicles (50 to 100 μl) were added to limit the label to a maximum of 2% with respect to phospolipids. The suspension was shaken at room temperature for 10 min. When the influence of reagents on membrane fluidity was studied, they were present during incorporation of the label. ESR spectra were recorded by using an ESR spectrometer (ESC 106 Bruker) with a flat quartz cell for aqueous solutions. All measurements were carried out with the following settings: microwave power, 20 mW; modulation amplitude, 4 G; scan time, 168 s; time constant, 0.041 s (seven scans were accumulated). The temperature was kept constant with an accuracy of ± 0.1 K measured by a thermistor in the sample cell. The larger hyperfine splitting was derived from spectra with an enlarged *y* axis. The small portion of free label did not interfere with these measurements.

Chemicals. ^3H_2O, [U-^{14}C]taurine, and [U-^{14}C]isoleucine were purchased from Amersham International (Amersham, Buckinghamshire, United Kingdom). [U-^{14}C]tetraphenylphosphonium bromide was obtained from NEN/DuPont de Nemours (Brussels, Belgium). Biochemicals were supplied by Boehringer (Mannheim, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, Mo.).

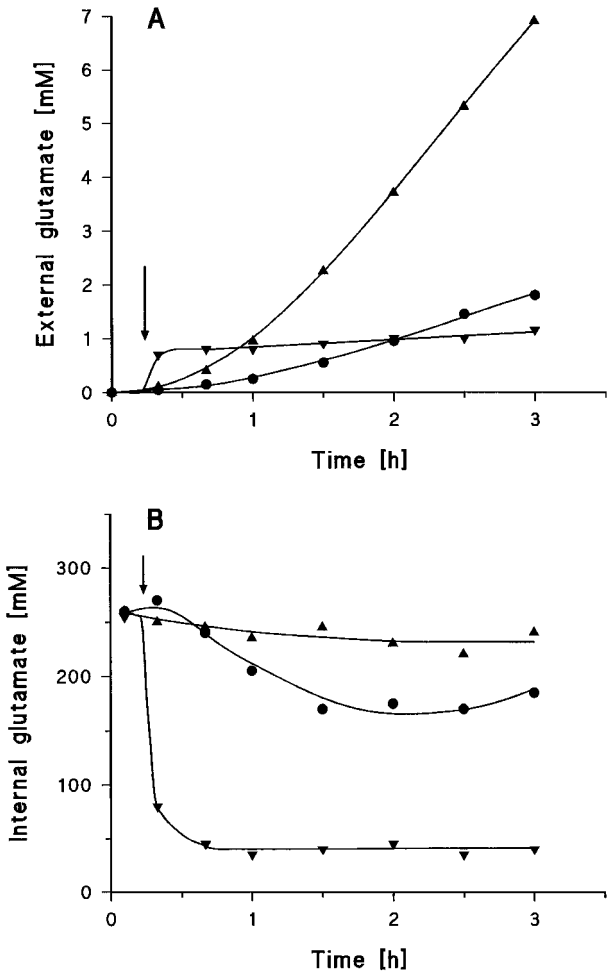

FIG. 1. Induction of glutamate excretion by tetracaine addition. Different concentrations of tetracaine were added to *C. glutamicum* cells grown on glutamate in MMYE medium at the beginning of short-term fermentation at pH 8.0 (see Methods) as indicated by an arrow in each panel. Time course of external (A) and internal (B) glutamate concentrations after the addition of 0.5 (●), 1.2 (▲), or 2.0 (▼) mM tetracaine.

RESULTS

Wild-type *C. glutamicum* cells grown on glutamate as the sole source of carbon and energy excrete glutamate when treated with local anesthetics. In the experiment shown in Fig. 1, 0.5, 1.2, or 2.0 mM tetracaine was added. Whereas the growth rate was only slightly decreased by 0.5 mM tetracaine, it was strongly reduced by 1.2 mM the local anesthetic and completely stopped when 2.0 mM was added (results not shown). The interpretation of these three concentrations as suboptimal (0.5 mM) and optimal (1.2 mM) with respect to glutamate excretion as well as lethal (2.0 mM) for the cell is reflected by the reactions of the external and internal glutamate concentrations. The addition of a suboptimal tetracaine concentration (0.5 mM) led to delayed glutamate excretion at low rates (2 to 4 nmol · min^{-1} · mg [dry weight]$^{-1}$), whereas the addition of an optimal concentration induced excretion at a high rate (up to 25 nmol · min^{-1} · mg [dry weight]$^{-1}$). The internal glutamate concentration was not changed (1.2 mM tetracaine) or was only slightly (0.5 mM tetracaine) changed. The addition of 2.0 mM tetracaine led to an immediate efflux of internal glutamate. During glutamate excretion under opti-

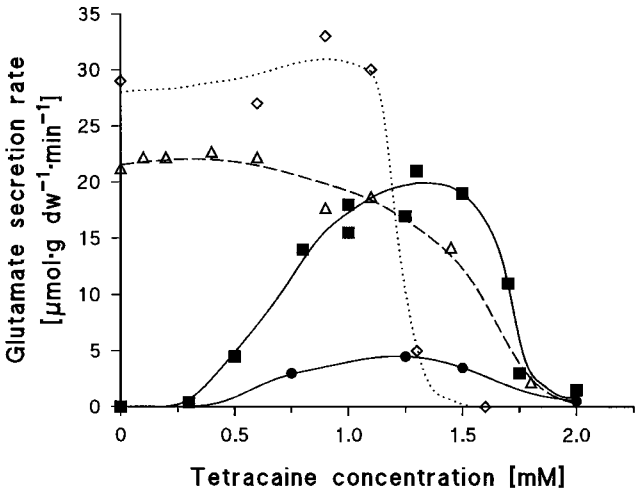

FIG. 2. Effects of tetracaine on glutamate excretion in different cells. Increasing concentrations of tetracaine were added to *C. glutamicum* cells under the conditions described in the legend to Fig. 1. Glutamate excretion rates were determined 1.5 to 2 h after the addition of the local anesthetic. The different cells used were wild-type *C. glutamicum* cells grown on MMYE medium under biotin supplementation with glutamate (■) or glucose as the carbon source (●) or under biotin limitation and with glucose (△). In addition, a glutamate production mutant of *C. glutamicum* (Kyowa-Hakko strain 9087) grown under biotin supplementation and in the presence of glucose (◇) was used. dw, dry weight.

mal conditions, the internal glutamate concentration was constant in spite of a massive efflux of glutamate for at least 2 h. At a concentration of 1.2 mM tetracaine, the effects both on growth and on glutamate excretion could be completely reversed by a washing step. After removal of the local anesthetic by washing, cells showed normal growth behavior and the addition of tetracaine was again needed to trigger glutamate excretion (experiments not shown).

Tetracaine-triggered glutamate excretion is a specific transport process. There are several mechanisms by which tetracaine may induce glutamate excretion in *C. glutamicum* cells. The most important one for it to be discriminated from is unspecific leakage caused by damage to the membrane. A large body of evidence argues against this possibility. (i) Although at a significantly reduced level, growth was still observed. (ii) The effects of local anesthetics could be completely reversed by the washing of cells. (iii) Glutamate excretion persisted for several hours at a constant rate, and no initial burst was observed (Fig. 1). (iv) The membrane potential retained values of 140 to 150 mV after the addition of tetracaine. (v) The internal glutamate concentration remained at a high level (Fig. 1). (vi) Immediate losses of only 3% of internal ATP (4.4 mM [total internal concentration]) and of less than 10% of K^+ (600 to 700 mM [internal concentration]) were observed after the addition of 1.2 mM tetracaine, presumably because of damage to a small portion of cells. Substantial leakage occurred only after the addition of tetracaine concentrations greater than 1.5 mM. Under these damaging conditions, the membrane potential was greatly decreased and the efflux of ATP as well as that of K^+ paralleled those observed for cytosolic glutamate (Fig. 2).

Besides these arguments which exclude the possibility of an unspecific efflux of glutamate caused by a damaged membrane, there is positive evidence that the excretion observed is a specific process. (i) During glutamate excretion, we never observed the efflux of another amino acid. This, however, is not a strong argument, since the internal concentration of glutamate is much higher than that of any other amino acid. (ii) Glutamate excretion was subject to regulation phenomena correlated to the energy state of the cell (Table 1). Excretion immediately stopped when either the carbon source or the energy source was lacking. The same behavior was observed after the inhibition of glucose uptake by bromophenacyl bromide. This result is not trivial since the internal glutamate concentration remained high under these conditions, providing a high driving force for glutamate excretion if it were a passive, unspecific process. (iii) As observed for glutamate excretion triggered by biotin starvation, the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not lead to a change in excretion activity (22, 25). This means that tetracaine-triggered glutamate excretion did not depend on the electrochemical potential across the membrane. (iv) Furthermore, glutamate excretion depended on the kind of carbon source on which the cells were grown.

Tetracaine-triggered glutamate excretion is different in cells of different metabolic states. The argument that the optimum concentration of tetracaine to be added (Table 1) is different in differently grown *C. glutamicum* cells may be raised. Figure 2 shows that this is not the case. Regardless of the growth substrate, the same concentration of tetracaine, namely, 1.0 to 1.4 mM, was needed for optimal glutamate excretion. The absolute rate of excretion, however, was significantly lower in the case of glucose-grown cells. The data in Table 1 show that this is not due to a lower internal glutamate concentration. The concentration dependence of tetracaine action is further elaborated in Fig. 2. For both glutamate- and glucose-grown wild-type *C. glutamicum* cells, a low concentration range at which

TABLE 1. Dependence of tetracaine-triggered glutamate excretion on cell type and metabolic state[a]

C source during growth	Presence in excretion assay[b]				Internal glutamate concn (mM)	Glutamate excretion rate (nmol · min^{-1} · mg [dw]$^{-1}$)
	Glucose	Oxygen	BPAB	CCCP		
Glutamate	+	+	−	−	220	14
Glutamate	+	−	−	−	185	<1
Glutamate	−	+	−	−	170	<1
Glutamate	+	+	+	−	290	—[c]
Glutamate	+	+	−	+	205	15.5
Glucose	+	+	−	−	190	1.5

[a] Tetracaine (1.2 mM) was added to all samples under the conditions described in the legend to Fig. 1. The internal glutamate concentration and glutamate excretion rate are given for 1.5 to 2 h after the addition of tetracaine.

[b] The conditions and additions were as follows (as indicated): 30 mM glucose as a source of carbon and energy; aeration by vigorous stirring (+ oxygen) or flushing with nitrogen (− oxygen); 50 μM SH reagent bromophenacyl bromide (BPAB), which blocks glucose uptake by the phosphotransferase system; 50 μM uncoupler CCCP. +, presence; −, absence.

[c] —, glutamate excretion is inhibited under these conditions because of the action of bromophenacyl bromide, whereas the glutamate uptake system still functions. Therefore, only glutamate uptake is observed.

tetracaine did not trigger glutamate efflux was observed. At above 1.5 mM, the optimum range was followed by a sharp decrease in efflux activity, indicating membrane damage induced by tetracaine.

The assay described in Fig. 2 offers the possibility of comparing tetracaine-triggered glutamate excretion with that brought about by other treatments, e.g., biotin limitation. The cells used for the experiments described so far were supplemented with biotin. Growth under limiting biotin concentrations leads to glutamate excretion, the properties of which have been studied previously (22, 25, 26). As shown in Fig. 2, glutamate excretion due to biotin limitation could not be enhanced by tetracaine. At concentrations above 1 mM, tetracaine led to inactivation of glutamate transport, as observed for biotin-supplemented cells. Interestingly, the maximum excretion rates were similar, regardless of whether glutamate efflux was triggered by biotin starvation or by the addition of tetracaine. Furthermore, a glutamate production strain (Kyowa-Hakko strain 9087) was tested. This strain excretes glutamate at elevated temperatures even in the presence of biotin. The results in Fig. 2 indicate that the production mutant closely resembled biotin-limited wild-type cells with respect to the action of tetracaine and clearly differed from the biotin-supplemented wild-type strain. The somewhat higher absolute excretion values and the membrane damage already observed at slightly lower tetracaine concentrations are due to the fact that the experiment with this mutant had to be carried out at 38°C in order to obtain optimum conditions.

These results strongly depended on the temperatures at which the experiments were performed. We routinely take measurements at 30°C, which is the optimum temperature for the metabolism and growth of *C. glutamicum*. Glutamate production, however, is usually carried out at higher temperatures (35 to 37°C). When these experiments (Fig. 2) were performed at higher temperatures, several aspects were changed (results not shown). (i) Glutamate excretion became faster, and the stability of cells decreased, i.e., constant excretion rates were observed only for shorter periods. (ii) The differences between cells became less prominent. At temperatures above 35°C, even wild-type cells without biotin limitation or tetracaine addition started to excrete glutamate. This was also true to a significant extent for glucose-grown cells. By investigating the temperature range from 20°C (no excretion activity) to 43°C (complete growth inhibition), 30°C proved to be the optimum temperature for both the handling of cells and studying the effects described in Fig. 1 and 2.

Tetracaine-triggered glutamate excretion and membrane fluidity. The data presented above suggest that tetracaine-triggered glutamate excretion is very similar to excretion induced by biotin limitation in terms of kinetics and energetics (22, 26). The question of the common parameter(s) which may be changed by these completely different methods to trigger glutamate excretion then arose. In other words, are the two different methods in fact not so different with respect to their influence on the membrane?

As for most local anesthetics, tetracaine can be uncharged or positively charged, depending on the pH. It was thus of primary interest to determine which species was the active form. The pK of tetracaine in aqueous solution is 8.5; however, when tetracaine is inserted into phospholipid bilayers, this value shifts to 7.5 (47). In Fig. 3 are shown the results of a series of experiments similar to that depicted in Fig. 2; they were carried out at various external pH values. In every experiment, the concentration of tetracaine leading to optimum glutamate excretion was determined. Obviously, this value depended strongly on the external pH. On the basis of a pK of 7.5, if only the concentration of the uncharged species was considered, the optimum concentration became virtually independent of pH. Just the opposite was true for the charged species. This result is a strong argument for the uncharged species of tetracaine being the form responsible for altering the activity of the glutamate excretion system. This is elaborated by the data listed in Table 2. The results of experiments on the effects of tetracaine at different external pH values are combined both with respect to the optimum concentration for triggering glutamate excretion and that for damaging effect. Despite a significant dependence on pH, the pattern with respect to the difference in the

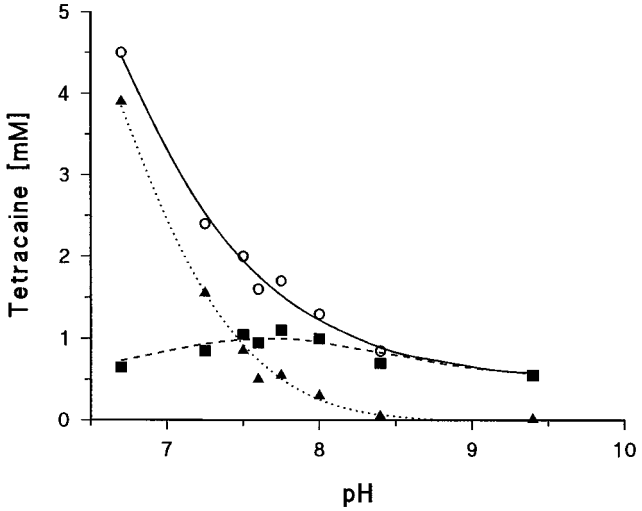

FIG. 3. Tetracaine concentrations necessary to trigger optimum glutamate excretion at different external pH values. Experiments were performed as described in the legend to Fig. 2 except that different pH values were used and controlled throughout the experiment. For example, the optimum tetracaine concentration under the conditions used in Fig. 2 is 1.3 mM. The dependence of the optimum tetracaine concentration on the external pH value is related to the total concentration (○) and the concentrations of the uncharged (■) and charged species (▲). The pK of tetracaine inserted into membranes is 7.5 (49).

TABLE 2. Effects of various local anesthetics and other membrane-active agents on glutamate excretion as a function of pH[a]

Reagent	External pH	Maximum excretion rate (nmol · min^{-1} · mg [dw]$^{-1}$)	Reagent concn (mM) to induce:	
			Maximum excretion[b]	Half-maximum inactivation[c]
Tetracaine	6.7	7.5	4.5	5.8
	7.25	14.5	2.4	ND[d]
	7.55	18.8	2.0	2.5
	7.75	16.5	1.7	ND
	8.0	17.9	1.3	1.7
	8.4	16.0	0.85	1.1
	9.4	13.9	0.55	1.0
Butacaine	8.0	21.0	2.6	3.3
Benzocaine	8.0	13.4	15	>30
Chlorpromazine	8.0	8.5	0.1	0.1
CCCP	8.0	<1.0	—[e]	ND

[a] Data are based on a series of experiments similar to that shown in Fig. 2.
[b] Concentration necessary to achieve maximum glutamate excretion activity (cf. Fig. 2).
[c] Concentration necessary to reduce the internal level of glutamate to 50% of the original value.
[d] ND, not determined.
[e] —, not detectable since the reagent does not induce the corresponding effect.

effect inducing excretion and that causing cell damage was more or less constant.

In the experiments whose results are reported in Table 2, furthermore, different local anesthetics were compared. This provided another argument in favor of the uncharged species being the active form, since benzocaine, which is not charged, was able to trigger glutamate excretion, although at relatively high concentrations. In comparison to tetracaine, butacaine was somewhat less effective, whereas chlorpromazine exceeded tetracaine in its potency. Nevertheless, chlorpromazine was not used, since the safety distance between the concentration which induces glutamate excretion and that which damages the cell was unfavorably small. This is indicated by the fact that the concentration leading to optimum induction of excretion caused a 50% loss of internal glutamate.

In order to test the specificities of the actions of local anesthetics, other membrane-active reagents were tested. Neither ethanol nor benzyl alcohol, which are known to increase the fluidity of lipid bilayers (28) and to affect the activities of membrane proteins (19), was able to trigger glutamate excretion when applied in concentrations not leading to substantial cell damage, as measured by the leakage of ATP (data not shown). Furthermore, the uncoupler CCCP was used as a control since uncoupling (and decoupling) by local anesthetics has previously been reported (18, 53). It has already been shown above (Table 1) that CCCP did not interfere with tetracaine-triggered glutamate excretion. As expected, CCCP led to a breakdown of membrane potential, but the addition of this uncoupler did not stimulate excretion activity, even at very high concentrations.

In view of the hypotheses which have been put forward to explain the actions of local anesthetics, the question of what is the common parameter influenced by these agents that leads to the observed effects on membrane function arises. The data in Table 2 prove that the order of efficiency of different local anesthetics in triggering glutamate excretion is exactly the same as that observed for anesthetic effect (27). Since the actions of these drugs are generally discussed as being mediated by changing the fluidity (or viscosity) of a lipid bilayer and since their effects were positively related to the temperature applied, we investigated the influence of anesthetics on the viscosity of the *C. glutamicum* plasma membrane by using spin-labeled fatty acids. They were inserted into isolated plasma membranes from *C. glutamicum* cells grown or treated under conditions found to be relevant for the activity of glutamate excretion. It is customary to derive an order parameter from the axial hyperfine splittings, $A\| - A\perp$, in the ESR spectrum of the membrane-bound fatty acid spin label (21). For the qualitative comparison intended here, it suffices to use the larger hyperfine splitting, $A\|$, which can be easily obtained from the spectrum. This parameter alone is sensitive to the angular amplitude of the segmental motion. Therefore, it is related to the viscosity of the membrane, i.e., increased larger hyperfine splitting means increased viscosity or decreased fluidity. In Fig. 4A, an original spectrum of isolated *C. glutamicum* membranes at 30°C, illustrating the derivation of the larger hyperfine splitting used in the present study, is shown. Figure 4B demonstrates that the addition of local anesthetics in concentrations which led to effective glutamate excretion did not change the viscosity of the phospholipid bilayer to any significant extent. This became even clearer when the dependence of viscosity on temperature was studied (Fig. 5). A temperature change of a few degrees had a much stronger influence on excretion than did the addition of local anesthetics (Fig. 4). The data in Fig. 5 are interesting in another aspect. The two types of cells found to exhibit higher membrane fluidity (wild-type cells grown with glucose in different media) are those cells which are not able to excrete glutamate effectively. On the other hand, the cell types showing reduced fluidity are those that excrete glutamate well after tetracaine addition (biotin-supplemented wild-type cells grown on glutamate) or even without tetracaine addition (production mutant and biotin-limited wild-type cells). Obviously, a correlation between membrane fluidity and the ability to excrete can be found. However, cells excreting glutamate show decreased membrane fluidity, and the difference is relatively small compared with the influence of a moderate change in temperature.

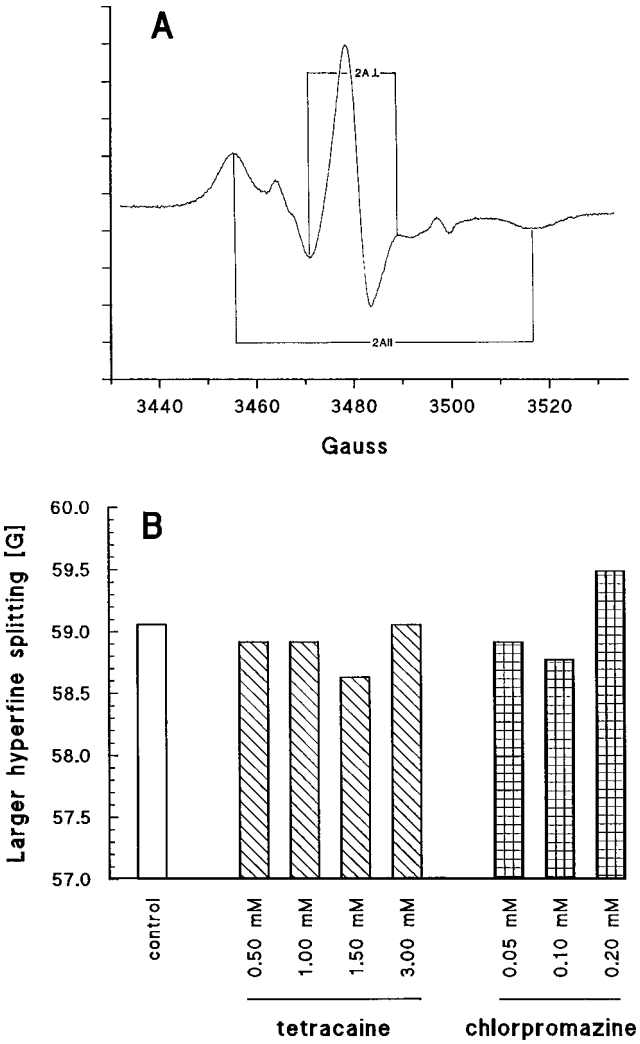

FIG. 4. Effects of local anesthetics on the viscosity of *C. glutamicum* plasma membranes as measured by larger hyperfine splitting in ESR spectroscopy with spin-labeled fatty acids. (A) Representative ESR spectrum of the fatty acid spin label incorporated in isolated *C. glutamicum* membranes at 30°C. The derivation of the larger hyperfine splitting, $A\|$, is shown. (B) Influence of the addition of tetracaine and chlorpromazine in different concentrations on isolated plasma membranes (see Methods). These experiments were carried out at 30°C.

Correlation of glutamate excretion to membrane strain. Other authors investigating the actions of local anesthetics on membranes have interpreted the effect in terms of imposing a mechanical stress on the membrane, which may also be called membrane strain or tension (28, 41, 49). In order to use a different way of changing the membrane strain, we tested a shift in the osmotic gradient across the plasma membrane (Fig.

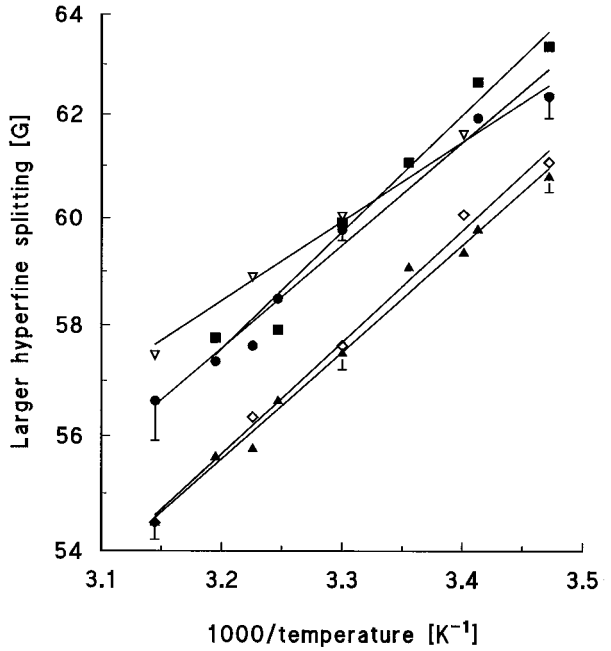

FIG. 5. Effects of temperature on the viscosity of *C. glutamicum* plasma membranes as measured by larger hyperfine splitting in ESR spectroscopy with spin-labeled fatty acids. Isolated plasma membranes of the following cells were used in these experiments: wild-type cells grown on MM1-glucose under biotin supplementation (▲) or biotin limitation (■) or grown on MMYE medium under biotin supplementation with glucose (◇) or glutamate (●) as the carbon source or a production mutant grown on MM1-glucose with biotin supplementation (△). Data are the averages of at least three experiments; in some cases, standard deviations are indicated.

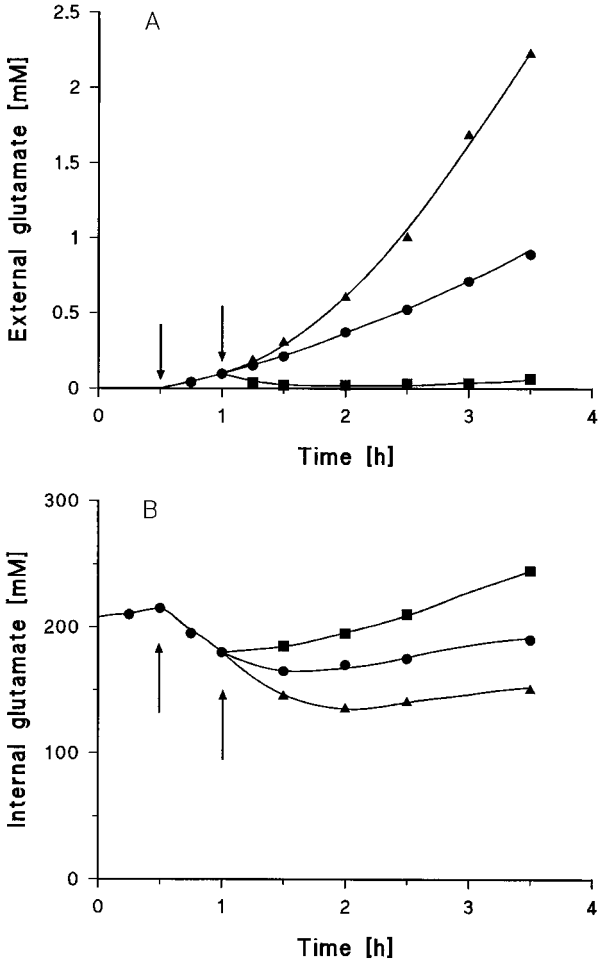

FIG. 6. Influence of a shift in external osmolality on glutamate excretion activity (A) and internal glutamate concentration (B). *C. glutamicum* cells were incubated in short-term fermentation buffer at 170 mosmol/kg (pH 8.0). After 30 min (first arrow), 0.7 mM tetracaine was added. After 1 h of incubation (second arrow), an aliquot of cells was diluted in water (▲), incubation buffer (●), or incubation buffer with NaCl (■), thereby reaching external osmolalities of 85, 170, and 340 mosmol/kg, respectively. External glutamate concentrations were measured, and the first two values (at 45 min and 1 h) were corrected for the higher cell density before the dilution after 1 h of incubation.

6A). A hyperosmotic shift immediately stopped tetracaine-triggered glutamate excretion, whereas a hypo-osmotic shift had a stimulating effect. These changes in the excretion rate cannot be attributed to changes in the glutamate gradient across the membrane. On the contrary, the inhibition of excretion by increased external osmolality led to an increase in the internal glutamate pool and vice versa (Fig. 6B). The inhibition of amino acid excretion could be overcome by the addition of glycine betaine (data not shown). This osmoprotective substance is effectively taken up by *C. glutamicum* and is accumulated in the cytosol as a compatible solute (17). Furthermore, the observed inhibition of glutamate excretion could not be attributed to changes in the ion concentration, since hyperosmotic shifts with mannitol led to very similar effects.

If the hypothesis that a change in membrane strain is the basic trigger of glutamate excretion is true, the two procedures, i.e., the addition of tetracaine and a change in the osmotic gradient, should be mutually compensative. The data in Table 3 show that this is in fact the case. Under conditions of constant external osmolality, 1.1 mM tetracaine is the optimum concentration to trigger glutamate excretion at pH 8.0. A concentration of 0.5 mM tetracaine, which is suboptimal under these conditions, was found to trigger excretion optimally when a moderate hypo-osmotic shift was applied. After a hyperosmotic shift, however, 1.5 mM tetracaine was necessary to obtain optimum excretion. The same held true for the toxic effects of local anesthetics. The amount of tetracaine (1.1 mM) which after a hyperosmotic shift did not induce glutamate excretion at all was optimal under iso-osmotic conditions and led to cell damage when a hypo-osmotic gradient was applied.

DISCUSSION

Many different models to explain the ability of *C. glutamicum* and related species to excrete glutamic acid under particular metabolic conditions still exist (35, 36). In recent studies we have provided an explanation for the excretion of amino acids, such as lysine, isoleucine, and threonine, in physiological terms (35, 36). The metabolic significance of glutamic acid excretion, however, remains obscure. We showed that at least two factors are needed to induce persisting glutamate excretion in *C. glutamicum* (22, 25, 26). First, a situation characterized as metabolic overflow is essential (22). This is characterized by growth inhibition due to the lack of an essential compound in the presence of unlimited availability of carbon and energy (52). Second, unspecific alterations of the membrane have to occur, e.g., by a block of phospholipid synthesis (biotin limitation) or by the addition of membrane-active reagents (13) or antibiotics (2). The only hypothesis so far which relates these effects to glutamate excretion assumes that the membrane becomes unspecifically leaky (12), which is not the

TABLE 3. Effects of different osmotic gradients and tetracaine concentrations on glutamate excretion[a]

External osmolality after shift (mosmol/kg)	Tetracaine concn (mM)	Growth rate (h^{-1})	Internal glutamate concn (mM)	Glutamate excretion rate (nmol · min^{-1} · mg [dw]$^{-1}$)
85	0.7	0.05	140	21
	1.1	0	40	10[b]
	1.5	0	<10[c]	0
170	0.7	0.12	175	4.5
	1.1	0.06	140	20.5
	1.5	0	25	3.0
340	0.7	0.15	210	<0.5
	1.1	0.15	205	<0.5
	1.5	0	140	11.0

[a] *C. glutamicum* cells were incubated in the buffer for amino acid excretion described in Materials and Methods, and the temperature was kept constant at 30°C during the experiment. At 30 min, tetracaine in the indicated concentration was added. After a further 30 min, the osmotic shift was introduced by dilution with either water, medium, or medium containing additional NaCl. Prior to the osmotic shift, the osmolality of the medium was 170 ± 5 mosmol/kg, as determined in an osmometer. The internal glutamate concentration as well as the glutamate excretion rate was determined at 1.5 h after the osmotic shift. The growth rate was measured by the change in OD_{600} between 0.5 and 2.5 h after the osmotic shift.

[b] Under these conditions, cells become leaky to a significant extent.

[c] Cells are completely leaky, and the internal glutamate concentration is below the detection limit.

case according to our data (22, 25). In order to avoid a metabolically complex mixture of events triggering glutamate excretion, in this work we used a more direct method to change the membrane state, i.e., the addition of local anesthetics.

We found that the addition of local anesthetics to glutamate-grown *C. glutamicum* cells triggers effective glutamate excretion. Excretion was shown to be a specific, carrier-mediated process since (i) no other metabolites were excreted in significant amounts; (ii) the gradients across the plasma membrane, e.g., $\Delta\Psi$ or the K^+ gradient, remained more or less unchanged; (iii) in spite of fast glutamate efflux, the internal glutamate concentration remained constant; (iv) excretion could be stopped by the removal of tetracaine; and (v) cells were able to regulate glutamate excretion. In summary, tetracaine-triggered glutamate excretion closely resembles glutamate efflux caused by biotin limitation (22).

In numerous reports, local anesthetics were shown to influence the membrane in the direction of either higher or lower fluidity, depending on the state of protonation (see Results). We investigated the fluidity of *C. glutamicum* membranes by ESR spectroscopy with spin-labeled fatty acid probes. We in fact found a difference in membrane viscosity between cells which are able to excrete glutamate and those which are not. However, the difference was in the wrong direction, i.e., the membranes of excreting cells had a higher viscosity. It is generally accepted that the permeability of a membrane increases as the bilayer becomes more fluid (40). Furthermore, the differences were small in comparison to the changes in viscosity brought about by a temperature change in the physiological range. The positive effect of temperature on excretion also contradicts the interpretation that the difference in fluidity is the reason for the difference in excretion. Most importantly, tetracaine did not significantly change the viscosity of isolated membranes from *C. glutamicum* cells. Thus, we conclude that a change in viscosity is not a major reason for the triggering of glutamate excretion by local anesthetics. The same holds true for excretion triggered by biotin limitation. Although membrane changes do occur, it is not the change in membrane fluidity which causes glutamate excretion. Recently, Neubeck et al. came to a similar conclusion with respect to biotin-limited cells (43). In contrast to our measurements, they did not find a difference in membrane fluidity between biotin-supplemented cells and biotin-limited cells of a *Brevibacterium* sp. In those studies, however, a fluorescence probe was used; because of the problem of the location of this probe in whole cells, it is not as accurate as ESR with purified membranes.

Another effect of certain local anesthetics is the ability to act as uncouplers (33, 44, 53), which was correlated to the charged form of anesthetic. We showed that the effectivity in triggering glutamate excretion was related to the uncharged form of tetracaine. Furthermore, benzocaine, which does not occur in a charged form, was also able to trigger glutamate excretion. The effects of local anesthetics have also been explained by direct action on membrane proteins (1, 3, 28, 51). Although direct interaction with the glutamate excretion system cannot be ruled out, this possibility seems to be unlikely since agents with different structures (chlorpromazine, tetracaine, and benzocaine) exert the same influence and since exactly the same result can be obtained by treatments such as biotin limitation, which cannot be explained by a direct effect on a protein.

The actions of local anesthetics have also been attributed to influences on the membrane curvature, thus inducing membrane strain (28, 41, 49). A concept of dynamic lateral tension of a bilayer membrane was first developed by Helfrich (24). The correlation between lipid bilayer elasticity and bilayer shape was extended to the bending energy concept (45). These properties are difficult to measure, particularly in intact cells. However, membrane strain can be influenced by a change in the transmembrane osmotic gradient, which is also considered in the concept of bilayer elasticity (30, 45). Recently, membrane strain in artificial phospholipid bilayers was directly correlated to the osmotic gradient (16, 23). We tested this possibility by applying different osmotic gradients. Combined with the addition of tetracaine, osmotic stress influenced glutamate excretion. A rise in the external osmolality inhibited efflux, whereas a hypo-osmotic shift enhanced the action of tetracaine. The effects of local anesthetics and changes in the osmotic gradient were mutually exchangeable. The efficiency of tetracaine was enhanced by hypo-osmotic conditions and vice versa. This held true not only for the specific effect in triggering glutamate excretion but also for the unspecific (toxic) effect of tetracaine in permeabilizing the cell. Interestingly enough, the toxic effects of high concentrations of tetracaine could be avoided by increasing the external osmolality. It should be noted that in contrast to *Escherichia coli* (46), hypo-osmotic shock alone did not lead to fast efflux of glutamate in *C. glutamicum* (unpublished observations). Glutamate efflux in *C. glutamicum* is not, as in *E. coli*, an immediate and direct answer to an osmotic downshift. A well-studied example of carrier activity which is in fact modulated by membrane stress is the proline betaine carrier ProP in *E. coli* (42). Obviously, this carrier senses the osmotic gradient and consequently changes its activity (11, 42). In principle, this type of regulation also occurs in *C. glutamicum*, since we found that the activity of the glycine betaine transport is even more effectively modulated in *C. glutamicum* by variations in the osmotic gradient than it is in *E. coli* (17). Although tetracaine-triggered glutamate efflux seems to be a fairly simple model for glutamate excretion in *C. glutamicum*, a general model correlating metabolic overflow and the membrane trigger is still lacking. However, it is important, to realize that cells which do not need an additional change in membrane state to excrete glutamate, i.e., biotin-limited cells or glutamate production mutants, cannot be stimulated by tetracaine. In such cases, tetracaine did not cause a

specific effect (excretion) and the unspecific effect (permeabilization) was unchanged. Obviously, these cells have already accomplished the membrane state which is appropriate for glutamate excretion.

The results obtained may also shed new light on the general actions of local anesthetics. The finding that the order of efficiency of different local anesthetics in triggering glutamate excretion was the same as that found for anesthetic action (48) may have two explanations. There may in fact be a common functional principle, or the reason may simply be different tendencies to insert into the membrane, leading to identically effective concentrations. Regardless of this question, the arguments discussed above indicate that the action of a local anesthetic is not a direct interaction with a certain protein but a general action on the membrane, which triggers the carrier's activity. In contrast to the common model in which a change in membrane fluidity is responsible for the action of a local anesthetic, the effect on the plasma membrane of *C. glutamicum* is not a change in its viscosity. Taking into account that the concepts of the viscosity of a membrane with high-level protein contents and of membrane strain are still relatively ill-defined phenomena, we tentatively interpret our results as showing that a change in membrane strain may be correlated to a change in the activity of the glutamate excretion system.

Besides the fact that osmotic gradients can change the activities of carrier proteins (29), this explanation is in line with the observation that local anesthetics can mimic the effects of a change in the osmotic gradient, as has been shown for the EnvZ-OmpR system. Sublethal concentrations of local anesthetics influence the transcription of certain outer membrane channel proteins (OmpF and OmpC) in *E. coli* (20). This is triggered by the two-components EnvZ-OmpR system. In a manner similar to that of modulation by osmotic stress, the membrane sensor EnvZ can be triggered by the addition of local anesthetics (54). In our case, by mimicking a change in membrane strain, tetracaine would not trigger a signal cascade but would change the activity of a membrane protein, i.e., the glutamate excretion carrier. Effects of this kind, i.e., changing the activity of a transport protein by a change in the osmotic gradient or membrane stress, are also well-known for the mechanosensitive ion channels of *E. coli*, which are responsible for the fast solute efflux after osmotic downshock (5). These channels have been shown not only to respond to osmotic effects but also to be triggered by the addition of local anesthetics (41).

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REFERENCES

1. **Adale, A. B., K. L. O'Brien, and G. Vanderkooi.** 1987. Temperature dependence and mechanism of local anesthetic effects on mitochondrial adenosine triphosphatase. Biochemistry **26:**7297–7303.
2. **Aida, K., I. Chibata, I. Nakayama, K. Takinami, and H. Yamada.** 1986. Biotechnology of amino acid production. Kodansha, Tokyo.
3. **Arias, H. R., M. B. Sankaram, D. Marsh, and F. J. Barrantes.** 1990. Effect of local anesthetics on steroid-nicotinic acetylcholine interactions in native membranes of *Torpedo marmorata* electric organ. Biochim. Biophys. Acta **1027:**287–294.
4. **Auger, M., H. C. Jarell, I. C. P. Smith, D. J. Siminovitch, H. H. Mantsch, and P. T. T. Wong.** 1988. Effects of the local anesthetic tetracaine on the structural and dynamic properties of lipids in model membranes. A high-pressure Fourier transform infrared study. Biochemistry **27:**6086–6093.
5. **Berrier, G., A. Coulombe, I. Szabo, M. Zoratti, and A. Ghazi.** 1992. Gadolinium ion inhibits loss of metabolites induced by osmotic shock and large stretch-activated channels in bacteria. Eur. J. Biochem. **206:**559–565.
6. **Beutler, H. O., and G. Michal.** 1974. L-Glutamatbestimmung mit Glutamat-Dehydrogenase, Diaphorase und Tetrazoliumsalzen, p. 1753–1759. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen analyse, vol. II. Verlag Chemie, Weinheim, Germany.
7. **Bottner, M., and R. Winter.** 1993. Influence of the local anesthetic tetracaine on the phase behavior and the thermodynamic properties of phospholipid bilayers. Biophys. J. **65:**2041–2046.
8. **Bunch, A. W., and R. E. Harris.** 1986. The manipulation of micro-organisms for the production of secondary metabolites. Biotechnol. Genet. Eng. Rev. **4:**117–144.
9. **Clement, Y., C. Escoffier, M. C. Trombe, and G. Laneelle.** 1984. Is glutamate excreted by its uptake system in *Corynebacterium glutamicum*? A working hypothesis. J. Gen. Microbiol. **130:**2589–2594.
10. **Clement, Y., and G. Laneelle.** 1986. Glutamate excretion mechanism in *Corynebacterium glutamicum*: triggering by biotin starvation or by surfactant addition. J. Gen. Microbiol. **132:**925–929.
11. **Culham, D. E., B. Lasby, G. Maragoni, J. L. Milner, B. A. Steer, R. W. van Nues, and J. M. Wood.** 1993. Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter ProP. J. Mol. Biol. **229:**268–276.
12. **Demain, A. L., and J. Birnbaum.** 1968. Alteration of permeability for the release of metabolites from microbial cells. Curr. Top. Microbiol. Immunol. **46:**1–25.
13. **Duperray, F., D. Jezequel, A. Ghazi, L. Letellier, and E. Shechter.** 1992. Excretion of glutamate from *Corynebacterium glutamicum* triggered by amine surfactants. Biochim. Biophys. Acta **1103:**250–258.
14. **Ebbighausen, H., B. Weil, and R. Krämer.** 1989. Transport of branched-chain amino acids in *Corynebacterium glutamicum*. Arch. Microbiol. **151:**238–244.
15. **Ebbighausen, H., B. Weil, and R. Krämer.** 1989. Isoleucine excretion in *Corynebacterium glutamicum*: evidence for a specific efflux carrier system. Appl. Microbiol. Biotechnol. **31:**184–190.
16. **Ertel, A., A. G. Marangoni, J. Marsh, F. R. Hallett, and J. M. Wood.** 1993. Mechanical properties of vesicles. I. Coordinated analyses of osmotic swelling and lysis. Biophys. J. **64:**426–434.
17. **Farwick, M., R. Siewe, and R. Krämer.** 1995. Glycine betaine uptake after hyperosmotic shift in *Corynebacterium glutamicum*. J. Bacteriol. **177:**4690–4695.
18. **Garlid, K. D., and R. A. Nakashima.** 1983. Studies on the mechanism of uncoupling by amine local anesthetics. J. Biol. Chem. **258:**7974–7980.
19. **Gordon, L. M., R. D. Sauerheber, J. A. Esgate, I. Dipple, R. J. Marchmont, and M. D. Houslay.** 1980. The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. J. Biol. Chem. **255:**4519–4527.
20. **Granett, S., and M. Villarejo.** 1982. Regulation of gene expression in *Escherichia coli* by the local anesthetic procaine. J. Mol. Biol. **160:**363–367.
21. **Griffith, D. H., and P. Jost.** 1976. Lipid spin labels in biological membranes, p. 454–523. *In* L. J. Berliner (ed.), Spin labeling, theory and application. Academic Press, New York.
22. **Gutmann, M., C. Hoischen, and R. Krämer.** 1992. Carrier mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim. Biophys. Acta **1112:**115–123.
23. **Hallett, F. R., J. Marsh, B. G. Nickel, and J. M. Wood.** 1993. Mechanical properties of vesicles. II. A model for osmotic swelling and lysis. Biophys. J. **64:**435–442.
24. **Helfrich, W.** 1978. Steric interactions of fluid membranes in multilayer systems. Z. Naturforsch. **33a:**305–315.
25. **Hoischen, C., and R. Krämer.** 1989. Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. Arch. Microbiol. **151:**342–347.
26. **Hoischen, C., and R. Krämer.** 1990. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J. Bacteriol. **172:**3409–3416.
27. **Hornby, A. P., and P. R. Cullis.** 1981. Influence of local and neutral anesthetics on the polymorphic phase preferences of egg yolk phosphatidylethanolamine. Biochim. Biophys. Acta **647:**285–292.
28. **Houslay, M. D., and L. M. Gordon.** 1983. The activity of adenylate cyclase is regulated by the nature of its lipid environment. Curr. Top. Membr. Transp. **18:**179–231.
29. **Houssin, C., N. Eynard, E. Shechter, and A. Ghazi.** 1991. Effect of osmotic pressure on membrane energy-linked functions in *Escherichia coli*. Biochim. Biophys. Acta **1056:**76–84.
30. **Käs, J., and E. Sackmann.** 1991. Shape transitions and shape stability of giant phospholipid vesicles in pure water induced by area-to-volume changes. Biophys. J. **60:**825–844.
31. **Kelusky, E. C., Y. Boulanger, S. Schreier, and I. C. P. Smith.** 1986. A ^{2}H-NMR study on the interaction of the local anesthetic tetracaine with membranes containing phosphatidylserine. Biochim. Biophys. Acta **856:**85–90.
32. **Kelusky, E. C., and I. C. P. Smith.** 1983. Charactrization of the binding of the

local anesthetics procaine and tetracaine to model membranes of phosphatidylethanolamine: a deuterium nuclear magnetic resonance study. Biochemistry **22:**6011–6017.
33. **Klein, R. A., M. J. Moore, and M. W. Smith.** 1971. Selective diffusion of neutral amino acids across lipid bilayers. Biochim. Biophys. Acta **233:**420–433.
34. **Klingenberg, M., and E. Pfaff.** 1967. Means of terminating reactions. Methods Enzymol. **10:**680–684.
35. **Krämer, R.** 1994. Secretion of amino acids by bacteria—physiology and mechanism. FEMS Microbiol. Rev. **13:**75–93.
36. **Krämer, R.** 1994. Systems and mechanisms of amino acid uptake and secretion in prokaryotes. Arch. Microbiol. **162:**1–13.
37. **Krämer, R., and C. Lambert.** 1990. Uptake of glutamate in *Corynebacterium glutamicum*. II. Evidence for a primary active transport system. Eur. J. Biochem. **194:**937–944.
38. **Krämer, R., C. Lambert, H. Ebbighausen, and C. Hoischen.** 1990. Uptake of glutamate in *Corynebacterium glutamicum*. I. Kinetic properties and regulation by internal pH and potassium. Eur. J. Biochem. **194:**929–935.
39. **Lundin, A., A. Richardson, and A. Thore.** 1976. Continuous monitoring of ATP converting reactions by firefly luciferase. Anal. Biochem. **75:**611–620.
40. **Magin, R. L., M. R. Niesman, and G. Bacic.** 1990. Influence of fluidity on membrane permeability—correspondence between studies of membrane models and simple biological systems. Adv. Membr. Fluid. **4:**221–239.
41. **Martinac, B., J. Adler, and C. Kung.** 1990. Mechanosensitive ion channels of *E. coli* activated by amphipaths. Nature (London) **348:**261–263.
42. **Milner, J. L., S. Grothe, and J. M. Wood.** 1988. Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J. Biol. Chem. **263:**14900–14905.
43. **Neubeck, M., E. Prenner, P. Horvat, R. Bona, A. Hermetter, and A. Moser.** 1993. Membrane fluidity in glutamic acid-producing bacteria Brevibcterium sp. ATCC-13869. Arch. Microbiol. **160:**101–107.
44. **Porter, R. K., J. M. Scott, and M. D. Brand.** 1992. Choline transport into rat liver mitochondria—characterization and kinetics of a specific transporter. J. Biol. Chem. **267:**14637–14646.
45. **Sackmann, E.** 1994. Membrane bending energy concept of vesicle- and cell-shapes and shape transitions. FEBS Lett. **346:**3–16.
46. **Schleyer, M., R. Schmid, and E. P. Bakker.** 1993. Transient, specific and extremely rapid release of osmolytes from growing cells of *Escherichia coli* K-12 exposed to hypoosmotic shock. Arch. Microbiol. **160:**424–431.
47. **Schreier, S., W. A. Frezzatti, P. S. Araujo, H. Chaimovich, and I. M. Cuccovia.** 1984. Effect of lipid membranes on the apparent pK of the local anesthetic tetracaine. Spin label and titration studies. Biochim. Biophys. Acta **769:**231–237.
48. **Seeman, P.** 1972. Membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. **24:**583–655.
49. **Sheetz, M. P., and S. J. Singer.** 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc. Natl. Acad. Sci. USA **71:**4457–4461.
50. **Shimooka, T., A. Shibata, and H. Terada.** 1992. The local anesthetic tetracaine destabilizes membrane structure by interaction with polar headgroups of phospholipids. Biochim. Biophys. Acta **1104:**261–268.
51. **Shoshan-Barmatz, V., and S. Zchut.** 1993. The interaction of local anesthetics with the ryanodine receptor of the sarcoplasmic reticulum. J. Membr. Biol. **133:**171–181.
52. **Tempest, D. W., and O. M. Neijssel.** 1992. Physiological and energetic aspects of bacterial metabolite overproduction. FEMS Microbiol. Lett. **100:**169–176.
53. **Terada, H., O. Shima, K. Yoshida, and Y. Shinohara.** 1990. Effects of the local anesthetic bupivacaine on oxidative phosphorylation in mitochondria. J. Biol. Chem. **265:**7837–7842.
54. **Villarejo, M., and C. C. Case.** 1984. *envZ* mediates transcriptional control by local anesthetics but is not required for osmoregulation in *Escherichia coli*. J. Bacteriol. **159:**883–887.