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THE EFFECT OF FORMALDEHYDE EXPOSURE ON THE TRANSMEMBRANE DISTRIBUTION OF FREE AMINO ACIDS IN MUSCLES OF *MYTILUS EDULIS*

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Summary

Free amino acids in the posterior adductor muscle of mussels (*Mytilus edulis*) occur in a high-energy gradient group, with energy gradients of 15–18 kJ mol^{-1} (aspartate, beta-alanine, glycine, taurine and threonine), and a low-energy gradient group, with energy gradients around 12 kJ mol^{-1} (the rest of the amino acids). Two of the amino acids, glycine and taurine, are present at intracellular concentrations of 100–150 mmol kg^{-1}, while the other amino acids occur at concentrations below 50 mmol kg^{-1}.

Exposure of mussels to formaldehyde causes a marked influx of Na^+ into the muscle cells and an increase in cellular water content. The Na^+ gradient, which provides the energy for the cellular accumulation of free amino acids, is reduced. The drop in the Na^+ gradient is accompanied by a nearly proportional reduction in the energy gradients of all amino acids in the high-energy gradient group and a 150 mmol kg^{-1} reduction in the total intracellular concentration of free amino acids. Most of this reduction is made up by the Na^+-dependent amino acids aspartate, glycine and threonine, the concentrations of which are reduced by about 120 mmol kg^{-1}.

The transmembrane distribution of the low-energy gradient amino acids seems to be independent of the Na^+ gradient, and these amino acids display only moderate reductions in their intracellular concentrations when the Na^+ gradient is reduced.

The reduction in the concentrations of the free amino acids appears to be a volume-regulatory response, serving to bring the cell volume back to its optimal level after the formaldehyde-induced Na^+ influx has caused a cellular swelling. The basis of these differences in Na^+-dependence is discussed.

Taurine, which is the quantitatively dominating organic solute and an important volume-regulatory osmolyte in mussels, does not take part in the volume-regulatory response. This may be due the role of taurine in the protection against potentially toxic Ca^{2+}, which enters the cells in large quantities when mussels are exposed to formaldehyde.

Key words: *Mytilus edulis*, free amino acids, taurine, sodium gradient, formaldehyde.

Introduction

Free amino acids are important intracellular osmolytes in euryhaline osmoconforming organisms (Bricteux-Gregoire *et al.* 1962), and in blue mussels their intracellular concentrations are 100- to 1000-fold higher than the concentrations in the haemolymph (Aunaas and Zachariassen, 1994). The high intracellular concentrations allow the free amino acids to take part in cellular volume regulation. Osmotic swelling or shrinking of the cells gives rise to compensatory changes in the intracellular concentrations of free amino acids, allowing the cells to maintain an optimal volume when they are exposed to changes in extracellular osmolality or fluxes of osmolytes across the cell membrane. The changes in amino acid concentrations are due to movement of amino acids across the cell membranes, to the formation or destruction of proteins or to the activation of metabolic processes that have amino acids as substrate or product (Fugelli, 1980). The variations in intracellular levels of free amino acids cause osmotic movements of water across the cell membranes which bring the cell volume back to normal.

The high concentrations of intracellular free amino acids are the result of an energetically uphill transport of free amino acids from the extracellular to the intracellular fluid. The transport is a secondary active transport (symport), by which the influx of amino acids across the cell membrane is accompanied by influx of Na^+ (Schultz and Curran, 1970; Lerner, 1985). Na^+ is distributed across the cell membrane with a high electrochemical potential difference ('energy gradient'), which is maintained by the ATP-consuming Na^+/K^+ pump and which causes a passive net diffusion of Na^+ into the cells. Part of this passive influx of Na^+ appears to take place on membrane-bound transport molecules, which also possess binding sites for amino acids, and the passive influx of Na^+ is

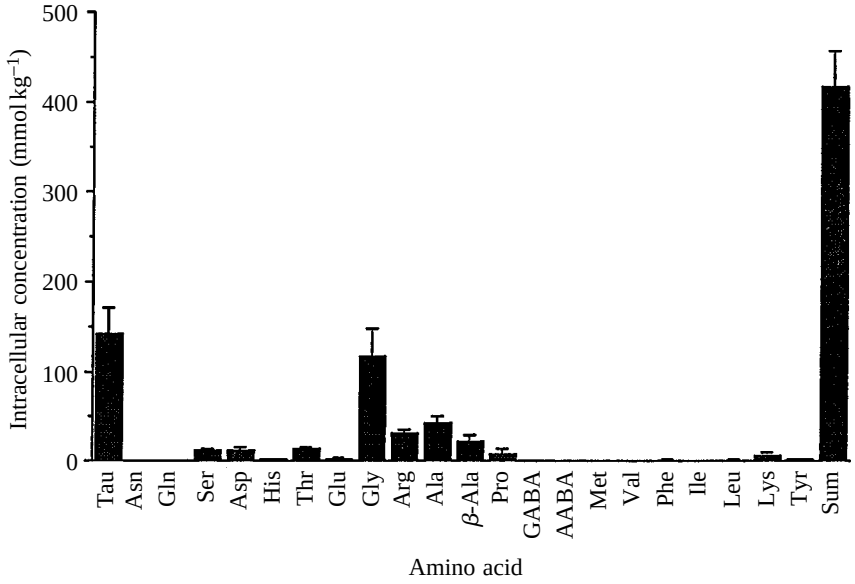

Fig. 1. Control values of intracellular concentration of free amino acids (mean + S.D.) in the posterior adductor muscle of blue mussels (*Mytilus edulis*), calculated as mmol kg^{-1} cell water. Each value is calculated from five parallel measurements.

thus associated with the influx of amino acids. Na^+ is pumped out by the Na^+/K^+ pump, while the amino acids remain inside the cells. The amino acids accumulate until their energy gradient creates a passive Na^+-coupled efflux that balances the Na^+-dependent influx. In this way, energy from the energy gradient for Na^+ is transformed into high-energy gradients for the various free amino acids, and the energy gradients of the amino acids are a function of the energy gradient for Na^+ and the stoichiometry of the cotransport (Schultz and Curran, 1970).

Most studies on the volume-regulatory functions of free amino acids have been carried out by exposing cells to changes in osmolality of the external medium. Exposure of mussels (*Mytilus edulis*) to formaldehyde and other metabolic inhibitors leads to an influx of Na^+ into the cells (Aunaas and Zachariassen, 1994) and to a depression of the transmembrane energy gradient for Na^+ (Børseth *et al.* 1995). The Na^+ influx is likely to cause osmotic cellular swelling and thus an activation of the volume-regulatory responses triggered under iso-osmotic conditions.

The purpose of the present study was to investigate how formaldehyde-induced iso-osmotic cellular swelling and a reduction of the transmembrane Na^+ gradient influence the transmembrane distribution of free amino acids in the posterior adductor muscle of the mussel *Mytilus edulis*.

Materials and methods

The experiments were carried out with blue mussels *Mytilus edulis* L. obtained from a commercial plant at the Trondheim Fjord, Norway. The mussels were kept in sea water (35 ‰) at 10 °C. The exposure experiments were carried out in a flow-through system described by Olsen and Nordtug (1991). The mussels were exposed to 60 p.p.m. formaldehyde, which causes a depression of the Na^+ gradient across the cell membrane of the posterior adductor muscle (Børseth *et al.* 1992, 1995). To ensure that the Na^+ gradient values were distributed over a wide range, samples from the formaldehyde-exposed group were taken after 24, 48, 72 and 96 h, whereas unexposed control animals were sampled at the beginning of the experiment and after 72 h.

Haemolymph and adductor muscle tissue were sampled as described by Olsen *et al.* (1991). The membrane potential, the intracellular concentration of Na^+ and the Na^+ gradient were determined from the haemolymph concentrations and whole-tissue contents of Na^+, K^+ and Cl^- according to a method described by Børseth *et al.* (1992). The Na^+ and K^+ concentrations were measured using a Radiometer FLM 3 flame photometer, whereas Cl^- concentrations were measured using a Radiometer CMT 10 chloride titrator. The free amino acids were prepared and measured using a Varian HPLC instrument according to the method described by Olsen *et al.* (1991). A correction factor, *b*, was used to correct for the stoichiometry of the Na^+/K^+ pumping and differences in membrane permeabilities of the two ions. In the present calculations, a *b*-value of 0.058 was used.

The electrochemical potential difference of Na^+ and free amino acids was calculated using the formula:

$$\Delta\mu_X = -L_X F E_m + \boldsymbol{R}T\ln([X]_e/[X]_i), \quad (1)$$

where $\Delta\mu_X$ is the electrochemical potential difference of the solute X (J mol^{-1}), L_X is the net electrical charge of X, F is the Faraday constant (96 500 C mol^{-1}), E_m is the membrane potential (V), $\boldsymbol{R}$ is the universal gas constant (J mol^{-1} K^{-1}), T is the absolute temperature (K) and $[X]_e$ and $[X]_i$ are the extracellular and intracellular concentrations of X, respectively (mmol kg^{-1} water). The membrane potential was calculated according to the Nernst equation.

In agreement with data provided by West *et al.* (1966), the calculations have been made on the assumption that alanine, beta-alanine, glycine, proline, serine and threonine have no net electrical charge, whereas arginine is considered to be basic and aspartate acidic. Taurine is also assumed to be electroneutral (Dawson *et al.* 1959). Exposure of mussels to

formaldehyde activates anaerobic pathways (Børseth *et al.* 1995) and leads to an acidification of the body fluids. Because amino acids are ampholytic, a reduction in pH might affect the net charge of amino acids and thus their transmembrane electrochemical potential difference. However, determination of intracellular pH using nuclear magnetic resonance reveals that formaldehyde exposure causes the intracellular pH to drop from approximately 7.6 to approximately 7.0, which is far from the isoelectric point of the amino acids. Thus, the changes in intracellular pH are not likely to have any significant effect on the electrochemical potential difference of the amino acids.

The statistical tests for significance levels of slopes of regression lines were carried out according to the method of Zar (1984).

Results

Fig. 1 shows the intracellular concentrations of the various free amino acids in the posterior adductor muscle of the unexposed control group of *Mytilus edulis* mussels. The results show that the quantitatively dominating amino acids are taurine, glycine, alanine, arginine and beta-alanine. Other free amino acids present at relatively high concentrations are threonine, aspartate, serine and proline. In the following, attention is focused on the effects on these amino acids.

Fig. 2 shows the variations in intracellular Na^+ concentration, relative water content and membrane potential across the adductor muscle membrane as formaldehyde exposure caused the energy gradient for Na^+ to drop. The results show that there was a marked increase in the intracellular Na^+ concentration and a marked reduction in the membrane potential. The relative water content showed a substantial, but less obvious, increase. The slope of the linear regression line of the water content data is significantly different from horizontal at $P<0.002$.

Fig. 3 shows that in the control animals the intracellular concentration of taurine was in the range 100–180 mmol kg^{-1} and that of glycine was 70–150 mmol kg^{-1}, both substantially higher than the concentrations of other amino acids. Alanine was present at relatively high concentrations, ranging from 30 to 60 mmol kg^{-1}. The figure also reveals that the reduction in the Na^+ gradient was accompanied by a marked reduction in the intracellular concentrations of arginine, aspartate, glycine, serine and threonine. There was probably also a reduction in proline concentration. For proline the slope of the linear regression line is significantly different from horizontal at $P<0.05$, whereas for the other five amino acids the deviation from horizontal was significant at $P<0.001$. The reduction in total amino acid concentration was about 150 mmol kg^{-1}. Most of this reduction was made up by aspartate, glycine and threonine, in that the total concentration of these three amino acids dropped by about 120 mmol kg^{-1}. For some quantitatively important amino acids, such as alanine and taurine, there was no reduction. The concentration of beta-alanine was very variable and was not correlated with the Na^+ energy gradient.

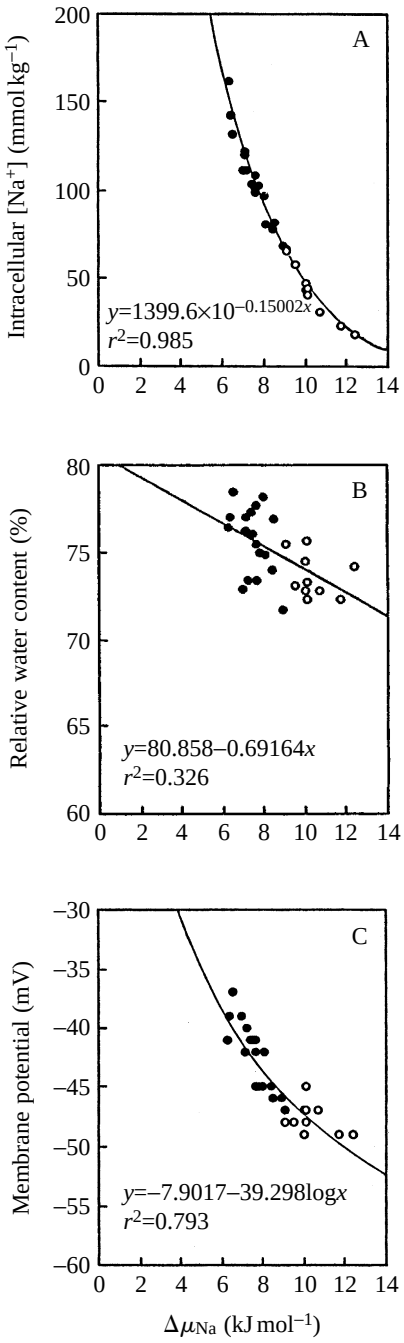

Fig. 2. Intracellular Na^+ concentration (A), relative water content of muscle tissue (B) and membrane potential (C) in the posterior adductor muscle of mussels (*Mytilus edulis*) before (open circles) and during (filled circles) exposure to 60 p.p.m. formaldehyde. All results are plotted as a function of the transmembrane electrochemical potential difference of Na^+ ($\Delta\mu_{Na}$). Further details are given in the text.

Fig. 4 shows the transmembrane electrochemical potential difference of the nine quantitatively dominating free amino acids in the adductor muscle plotted as a function of the electrochemical potential difference for Na^+. The results show that in control mussels the free amino acids are distributed

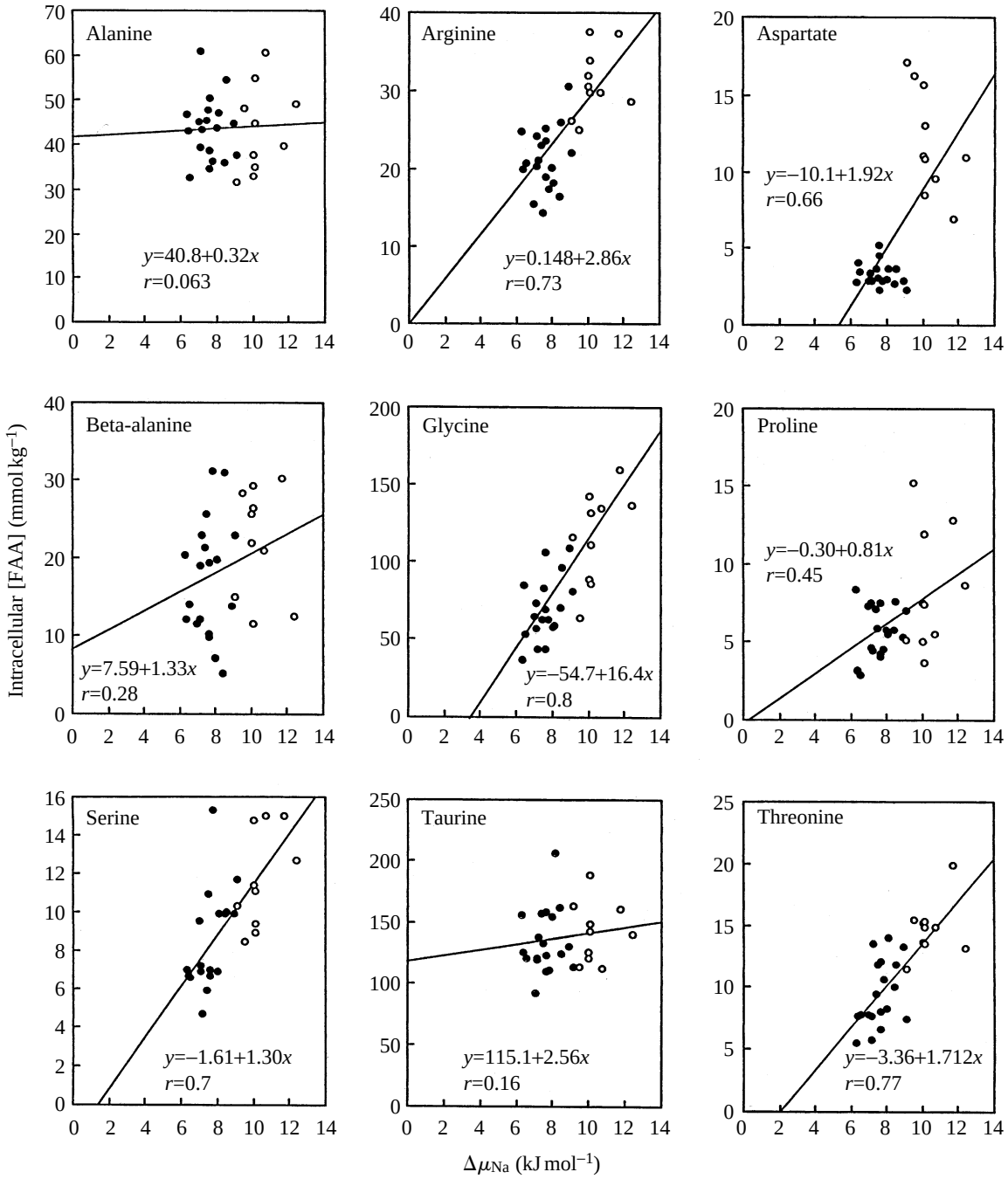

Fig. 3. Intracellular concentration of free amino acids (FAA) in the posterior adductor muscle of mussels (*Mytilus edulis*) before (open circles) and during (filled circles) exposure to 60 p.p.m. formaldehyde. The concentrations are calculated as mmol kg^{-1} cell water. All results are plotted as a function of the transmembrane electrochemical potential difference of Na^+ ($\Delta\mu_{Na}$). The lines are linear regression lines, calculated according to the method of Zar (1984).

across the cell membrane with different energy gradients. Aspartate, beta-alanine, glycine and taurine have energy gradients of approximately 18 kJ mol^{-1}, whereas alanine, arginine, proline and serine have gradients of approximately 12 kJ mol^{-1}. The energy gradient of threonine seems to be between the values of these two groups, with a value about 15 kJ mol^{-1}.

As the Na^+ gradient drops from the high values of the control animals to the lower values of the formaldehyde-exposed animals, there is also a marked reduction in the energy gradients of aspartate, beta-alanine, glycine, taurine and threonine. For these five amino acids, the slope of the linear regression line differs significantly from the horizontal ($P<0.001$) but not significantly from hypothetical lines representing a proportional reduction between the energy gradients for Na^+ and the amino acids ($P>0.1$). In the

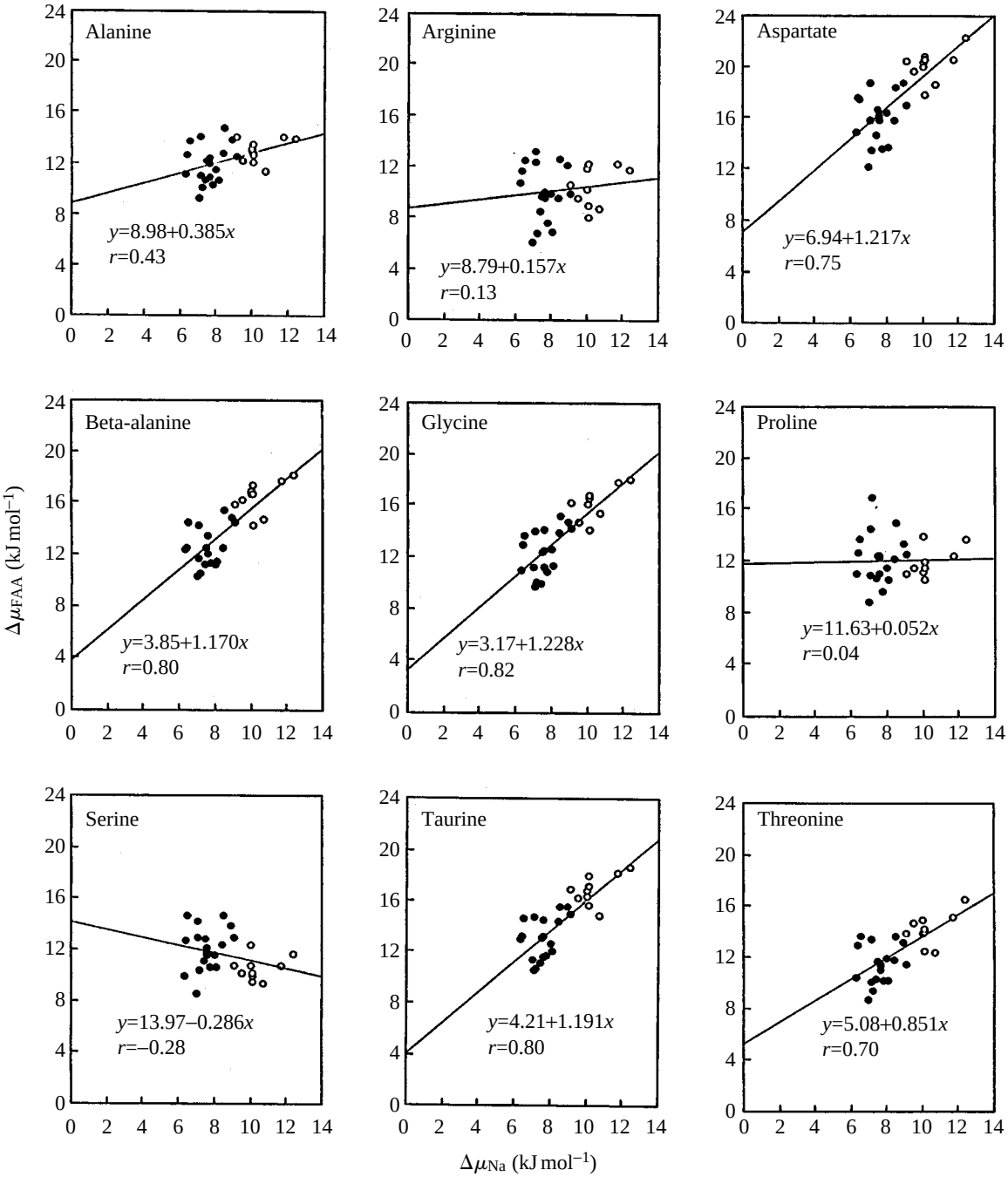

Fig. 4. Transmembrane chemical potential difference of free amino acids ($\Delta\mu_{FAA}$) in the posterior adductor muscle of blue mussels (*Mytilus edulis*) before (open circles) and during (filled circles) exposure to 60 p.p.m. formaldehyde. All results are plotted as a function of the electrochemical potential difference of Na^+ ($\Delta\mu_{Na}$). The lines are linear regression lines, calculated according to the method of Zar (1984).

following, these five amino acids are called Na^+-dependent. For the other amino acids, there was no significant change in the energy gradient when the Na^+ gradient was depressed.

Discussion

The results in Fig. 2A,B confirm that, as the transmembrane electrochemical potential difference of Na^+ drops following formaldehyde exposure, there is a marked increase in the intracellular Na^+ concentration and the relative water content. This implies that the exposure has caused substantial osmotic swelling and probably an activation of volume-regulatory mechanisms.

This is confirmed by the results in Fig. 3, which reveal that the cells respond to the cellular swelling and the reduced Na^+ gradient by marked reductions in the intracellular concentrations of a number of amino acids.

The intracellular concentration of amino acids displays a total reduction of about 150 mmol kg^{-1}. However, the amino acids do not respond uniformly, in that the concentration reduction is restricted to arginine, aspartate, glycine, proline, serine and threonine, while the intracellular concentrations of alanine, beta-alanine and taurine seem to be unaffected. Thus, the formaldehyde exposure seems to give rise to volume-regulatory responses that involve most, but not all, intracellular amino acids.

For the amino acids that display reduced concentrations, the reductions amount to about 70 % of the control values. The relative water content concomitantly increased from 73 to 77 %, which corresponds to an increase in cellular water content of about 17 % of the control value. Thus, the increase in cellular water content is too small to have given rise to the observed reduction in amino acid concentration by a dilution effect, i.e. the cellular contents of these amino acids must have been reduced. This reduction in intracellular concentrations of free amino acids is sufficient to have caused a substantial reduction in cell volume, i.e. the cell volume has been regulated back towards its normal size. However, the observation that the cellular water content is still higher than control values indicates that the cells have not performed a complete volume regulation.

For an osmolyte to be able to reduce cell volume, it must occur in intracellular concentrations sufficiently high to create a substantial reduction in the number of intracellular osmolytes by leaving the cells, i.e. the amino acids that display the highest intracellular concentrations are those that are most fitted to act as volume-reducing osmotic effectors. Since increased cellular accumulation of an amino acid from the extracellular medium will be associated with an increased transmembrane energy gradient for that same amino acid, those amino acids that display the highest energy gradients are also likely to be the most important effectors of cellular osmoregulation. This conforms well with the present results, in that by far the greatest part of the decrease in the concentration of cellular amino acids is made up by amino acids of the high-energy gradient group (glycine, aspartate and threonine). These Na^+-dependent amino acids make up as much as 120 mmol kg^{-1} of the total reduction of 150 mmol kg^{-1} in intracellular amino acid concentration. Arginine, proline and serine, which have energy gradients that are independent of the Na^+ gradient, take part in cell volume regulation, but their contribution is only about 25 mmol kg^{-1}. Hence, as proposed above, it is the Na^+-dependent amino acids that are responsible for by far the greatest part of the volume-regulatory response of the cells.

The results (Figs 3, 4) also reveal that, although taurine has a high intracellular concentration and a high energy gradient across the cell membrane, this amino acid is not involved in the volume-regulatory responses activated under the present experimental conditions. This result differs from the osmoregulatory response of taurine observed by other investigators. Lange (1963) demonstrated that acclimation of *Mytilus edulis* to low salinities was accompanied by an almost complete removal of taurine from the muscle cells. A marked reduction in taurine levels has also been observed in various cells and tissues of flounder undergoing freshwater acclimation (Fugelli, 1967; Fugelli and Vislie, 1975; Fugelli and Zachariassen, 1976; Vislie, 1983). However, the experiments in which taurine has been shown to respond as a volume-regulatory osmolyte have all been carried out using cells that have undergone an initial swelling in a hypo-osmotic medium involving reduced concentrations of all intracellular solutes. In the present experiments, the cells underwent an iso-osmotic swelling due to Na^+ influx. Thus, it appears that taurine efflux is activated by factors that are associated with swelling in a hypo-osmotic medium, which is likely to cause reduced intracellular concentrations of Na^+ and other osmolytes. The failure to mobilize the taurine-efflux response may be the reason why the formaldehyde-exposed cells still display incomplete volume regulation.

The lack of participation of taurine in cell volume regulation in the present experiments may be associated with the role of taurine in cellular Ca^{2+} regulation. As pointed out by Huxtable (1992), cellular taurine has important roles in the protection of cells which are threatened by elevated levels of potentially toxic Ca^{2+}. Børseth *et al.* (1995) found that formaldehyde-exposure of *Mytilus edulis* leads to a marked increase in the content of Ca^{2+} in the adductor muscle cells. The Ca^{2+} has to be dealt with by protective mechanisms, and an osmoregulatory efflux of taurine may reduce the capacity of these protective mechanisms. The absence of a volume-regulatory reduction in the concentration of taurine suggests that the volume-regulatory role of taurine under the present experimental conditions has been overruled by its Ca^{2+} protective functions.

Since cells accumulate free amino acids across cell membranes in cotransport (symport) with Na^+, and since the energy for cellular accumulation of amino acids thus comes from the energy gradient for Na^+, the transmembrane energy gradient of the amino acids should be expected to be influenced by variations in the Na^+ gradient (Schultz and Curran, 1970). Furthermore, if the system is energetically balanced, a reduction in the Na^+ gradient should be accompanied by a proportional reduction in the energy gradients for the free amino acids (Fugelli and Zachariassen, 1976; Eddy, 1985).

The slopes of the linear regression lines of the data points of the five Na^+-dependent amino acids shown in Fig. 4 do not deviate significantly from lines passing through the origin, suggesting that the energy gradients of these amino acids are depressed approximately in proportion with the Na^+ gradient. This agrees with the pattern expected if these five amino acids were cotransported with Na^+ across the cell membrane. The observation that these five amino acids have about the same energy gradient value in the unexposed control mussels, together with the observation that the linear regression lines of the data points of these amino acids have about the same slopes, further suggests that these amino acids are transported on the same transport system. A reservation has to be made for aspartate, which is negatively charged and which is therefore probably transported *via* a separate system for negative amino acids.

The amino acids that display a reduction in the energy gradient when the Na^+ gradient is reduced all have high energy gradients (15–18 $kJ\,mol^{-1}$) in the unexposed control mussels.

Assuming that the energy gradients of the cotransported solutes remain balanced throughout the exposure period, the slopes of the regression lines in Fig. 4 can be used to calculate the stoichiometry of the cotransport (Eddy, 1985). The slopes of the regression lines of four of these amino acids, aspartate, beta-alanine, glycine and taurine, are all around 1.2. A slope of 1.0 would reflect a 1:1 stoichiometry of the cotransport between Na^+ and amino acids, whereas a slope of 1.5 would reflect a stoichiometry of 3:2. The slope observed in the present study does not fit precisely to either of these slopes, indicating either that there is a more complex stoichiometric relationship or that some kind of methodological error has influenced the data used to estimate the slopes.

The greatest methodological error influencing the calculated energy gradients in the present study is probably attached to the determination of the intracellular solute concentrations by means of the total cellular contents of solutes and water. Binding of a solute to intracellular components will cause the concentration of the free solute to be lower than the values determined by this method, while binding of water as hydration water will reduce the amount of solvent water and thus cause the concentrations of the free solutes to be higher than those measured. For the adductor muscle of *Mytilus edulis*, Børseth *et al.* (1992) found that the Na^+ concentrations determined on the basis of chemical analyses were higher than those obtained by using intracellular Na^+-sensitive microelectrodes, and this was interpreted as reflecting that the intracellular activity of Na^+ is more influenced by water binding than by Na^+ binding.

Water binding is likely to have the same effect on the activities of Na^+ and free amino acids, whereas solute binding is likely to be of importance only for Na^+. Thus, in the present study, Na^+ binding is probably the most important factor that has influenced the relationship between the energy gradients for Na^+ and amino acids, i.e. the intracellular Na^+ concentrations are overestimated in relation to the intracellular concentrations of free amino acids. This implies that the energy gradients for Na^+ are likely to be underestimated compared with the corresponding gradients for free amino acids and, consequently, the correct slopes of the regression lines are likely to be more close to 1.0. Accordingly, the stoichiometry of the cotransport of amino acids and Na^+ is likely to be 1:1. This is in agreement with the stoichiometry previously reported by Eddy (1985) for cotransport of Na^+ and free amino acids.

The intracellular concentrations of beta-alanine and taurine remain high, although their energy gradients drop in proportion with the Na^+ gradient. For these amino acids, the reduction of the energy gradient is associated with an increase in the extracellular concentration. Owing to the large difference between the amino acid concentrations in the intracellular and extracellular compartments, a moderate efflux of amino acids from the cells may give rise to a marked increase in extracellular amino acid concentration and a reduction in the energy gradient without any noticeable reduction in intracellular level. The amino acids in the low-energy gradient group (alanine, arginine, proline and serine) do not display any noticeable response to the formaldehyde-induced reduction in the Na^+ gradient, suggesting that these amino acids do not depend on the Na^+ gradient in the same way as the amino acids mentioned above. However, even these amino acids have substantial energy gradients across the cell membranes (about 12 $kJ\,mol^{-1}$), implying that a high energy input is required to maintain the high intracellular concentrations of these free amino acids.

Ion-coupled transport, most commonly with Na^+, is the energy source for cellular accumulation of free amino acids (Eddy, 1985), and it is not clear why the energy gradients of some amino acids fail to respond to the change in the Na^+ gradient. The majority of the amino acids are neutral at physiological pH, and their energy gradient is consequently not affected by the depolarization of the cell membrane observed during formaldehyde exposure (Fig. 2C, Børseth *et al.* 1992). Since amino acids are ampholytic, it may be that a reduced pH can cause an undetected change in the net charge of the amino acids and thus a change in their energy gradient. However, nuclear magnetic resonance measurements have revealed that during formaldehyde exposure the intracellular pH dropped from about 7.6 to about 7.0 (T. Aunaas, unpublished results). Since the amino acids have pK values ranging from 2 to 3, this change in pH is not likely to be of any importance.

The observations that alanine, arginine, proline and serine have energy gradients of only about 12 $kJ\,mol^{-1}$ in control mussels and that their energy gradients do not respond to the formaldehyde-induced depression of the Na^+ gradient may be related to regulation of cellular volume. The continuous Na^+-driven influx of free amino acids into the cells is likely to represent a potential stress to cell volume regulation. The Na^+ influx is compensated for by the Na^+ pump, but the intracellular amino acid level would increase until the Na^+-coupled amino acid efflux matches the Na^+-dependent influx, i.e. until the energy gradients are in balance. If the balanced energy gradients were 18 $kJ\,mol^{-1}$, and the extracellular concentrations were as observed, the total intracellular concentration of alanine, arginine, proline and serine would have to be more than 800 $mmol\,kg^{-1}$ higher than the observed values. This would have caused osmotic influx of water across the cell membrane and a cell volume far greater than optimal. To prevent amino acid influx from causing lethal cellular swelling, the cells must ensure that some amino acids leak out to reduce the levels of intracellular osmolytes. As a result, the energy gradients of these amino acids drop below the value representing energetic balance with the Na^+ gradient. This might be the reason why amino acids such as alanine, arginine, proline and serine have relatively low energy gradients in the unexposed control animals.

Cells must be able to respond adequately to volume stress from a variety of causes and of a variety of magnitudes. They must not only reverse small and large volume changes caused by acute changes in the osmolality of the extracellular fluid, but also continuously correct for osmolyte fluxes which

threaten to displace cell volumes from their optimal value. Their response must also be adjusted to fit the total physiological status of the cells, i.e. amino acids with more than one physiological function, such as taurine, must respond according to the total physiological needs of the cells. This type of flexible response is likely to require a graduated series of volume-regulatory mechanisms triggered by different signals. The presence of free amino acids with a wide range of intracellular concentrations and energy gradients may enable cells to make such a graduated and adequate volume regulatory response.

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