The Journal of Experimental Biology **198**, 311–324 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

THE OSMOLALITY-SENSITIVE TAURINE CHANNEL IN FLOUNDER ERYTHROCYTES IS STRONGLY STIMULATED BY NORADRENALINE UNDER HYPO-OSMOTIC CONDITIONS

STEINAR M. THOROED[1], MARGRETHE SOERGAARD[1], EDWARD J. CRAGOE, JR[2] AND KJELL FUGELLI[1]

[1]*The Division of General Physiology, University of Oslo, PO Box 1051 Blindern, N-0316 Oslo, Norway* and [2]*PO Box 1548, Nacogdoches, TX 75963-1548, USA*

Summary

Stimulation of flounder erythrocytes by noradrenaline under isosmotic conditions (330 mosmol kg^{-1}) and physiological Na^+ concentration (113 mmol l^{-1}) caused swelling of the cells. The EC_{50} of this cell swelling was 0.65 μmol l^{-1} noradrenaline. The effect of the noradrenaline-induced cell swelling on the taurine channel under isosmotic conditions was negligible. However, when the cells were stimulated by noradrenaline (1.0 μmol l^{-1}) before, simultaneously with or after reduction of osmolality (255 mosmol kg^{-1}), the volume regulatory efflux of taurine mediated by the taurine channel was transiently accelerated. The rate coefficient for taurine efflux was more than four times higher than in osmolality-stimulated cells not exposed to noradrenaline. The present paper deals with the accelerating effect of noradrenaline on the taurine channel under hypo-osmotic conditions and the lack of effect of noradrenaline-induced cell swelling on the channel under iso-osmotic conditions.

Noradrenaline initiated the cell swelling by interacting with β-receptors which appeared to be more related to the mammalian β_1-receptors than to the β_2-receptors. The receptor interaction activated the adenylate cyclase system and, in the presence of 1.0 μmol l^{-1} noradrenaline, the cellular cyclic AMP concentration increased about 23 times. Noradrenaline also stimulated the Na^+/H^+ and Cl^-/HCO_3^- antiporters and this affected the extracellular pH as well as the cell volume. Depending on the extracellular Na^+ concentration, the incubation medium was acidified (113 mmol l^{-1} Na^+) or alkalized (2.7 mmol l^{-1} Na^+). Under these two conditions, the accelerating effects of noradrenaline on the taurine efflux were of similar magnitude. Similar effects on the cell volume, the extracellular pH and the volume regulatory taurine efflux were obtained in the presence of the cyclic AMP analogue 8-bromo-cyclic AMP. Under hypo-osmotic conditions in the absence of noradrenaline, the cellular level of cyclic AMP was not elevated. There was no significant positive correlation between the water content of the cells (cell volume) under different conditions in the presence or absence of noradrenaline and the state of activation of the osmolality-sensitive taurine channel.

We conclude that the mechanism(s) which activate(s) the osmolality-sensitive taurine channel in flounder erythrocytes is transiently and strongly accelerated by noradrenaline, but not triggered by the noradrenaline-induced events. The acceleration does not appear to be due to increased activity of the antiporters, but to increased cellular levels of cyclic AMP.

Key words: cell volume regulation, cyclic AMP, erythrocytes, fish, flounder, noradrenaline, osmolality-sensitive taurine channel, *Platichthys flesus.*

Introduction

Animal cells are able to maintain a constant volume despite alterations in the osmolality of the extracellular fluid. Under hypo-osmotic conditions, the recovery of the cell volume following the initial cell swelling is achieved by a reduction in the content of certain cellular solutes, the osmo-effectors, and an osmotically obligated efflux of water (for reviews, see Chamberlin and Strange, 1989; Hoffmann *et al.* 1993). Flounder erythrocytes re-adjust their volume by efflux of taurine, γ-aminobutyric acid (GABA), K^+ and Cl^- (Fugelli and Zachariassen, 1976; Fugelli and Rohrs, 1980; Fugelli and Thoroed, 1986; Thoroed and Fugelli, 1994*a*). The volume regulatory efflux of taurine and GABA is mediated by the osmolality-sensitive taurine channel, which is strongly and transiently stimulated under hypo-osmotic conditions (Fugelli and Thoroed, 1986; Thoroed and Fugelli, 1994*a*,*b*).

The taurine channel, like other transport systems that mediate transport of osmo-effectors in other types of cells, is regulated by intracellular signal systems (Thoroed and Fugelli, 1994*b*,*c*). However, the signal system(s) involved have been investigated thoroughly in only a few cell types. In most types of cells, activation of the volume regulatory processes seems to be dependent on the intracellular concentration of free Ca^{2+} (see McCarty and O'Neill, 1992; Hoffmann *et al.* 1993). The regulatory roles of other signal systems, such as leukotrienes,

hepoxilins, calmodulin and inositol trisphosphate, seem to differ in different cells (Leite and Goldstein, 1987; Hoffmann *et al.* 1988; Dahl *et al.* 1991; Margalit *et al.* 1993; Lambert, 1994; Thoroed and Fugelli, 1994*b*,*c*; Thoroed *et al.* 1994).

Little is known about the role of cyclic AMP in the control of osmolality-sensitive pathways. Flounder erythrocytes possess catecholamine receptors (Fugelli and Reiersen, 1978; Fugelli and Thoroed, 1986). Interaction of noradrenaline ($1.0\,\mu mol\,l^{-1}$) with these receptors in an iso-osmotic medium at physiological Na^+ concentration causes swelling of the cells. During 4 h of incubation, the erythrocytes gradually swell to the same extent as when the osmolality of the incubation medium is reduced from $330\,mosmol\,kg^{-1}$ to $255\,mosmol\,kg^{-1}$ (Fugelli and Thoroed, 1986). Unlike cells exposed to hypo-osmotic medium, the noradrenaline-stimulated cells do not shrink towards their original volume, and the stimulatory effect of noradrenaline on taurine efflux is negligible (Fugelli and Thoroed, 1986). This suggests that something in the noradrenaline signal-transduction cascade mediates an inhibitory action on the processes activating the volume regulatory taurine transport or that the volume regulatory mechanism does not respond to changes in the cell volume *per se*. In this study, we have therefore investigated the mechanisms behind the cell swelling induced by noradrenaline and the effect of this noradrenaline signal-transduction cascade on the osmolality-sensitive taurine channel.

Materials and methods

Animals

Flounders (*Platichthys flesus* L.) (28–45 cm) were caught by gill nets or Danish seines at different localities in the Oslofjord. They were kept at 7–9 °C in seawater aquaria (33 ‰) for at least 7 days before the experiments. They were fed on shrimps during captivity.

Reagents

Adrenaline (L-epinephrine bitartrate), amiloride hydrochloride, ascorbic acid, atenolol, 8-bromoadenosine 3′,5′-cyclic monophosphate) (8Br-cAMP), choline chloride, clonidine hydrochloride, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid), dimethyl sulphoxide, forskolin, GABA (γ-amino-*n*-butyric acid), isoprenaline (L-isoproterenol-D-bitartrate), noradrenaline (L-arterenol bitartrate), L-phenylephrine hydrochloride, prazosin hydrochloride, DL-propranolol hydrochloride, salbutamol, taurine, theophylline, timolol maleate and yohimbine hydrochloride were purchased from Sigma Chemical Co. (St Louis, USA). The ^3H-labelled cyclic-AMP assay kit and [^{14}C]taurine were purchased from Amersham International (Amersham, Great Britain) and EIPA [5-(*N*-ethyl-*N*-isopropyl) amiloride], HMA [5-(*N*,*N*-hexamethylene) amiloride], MGCMA {5-[*N*-methyl-*N*-(guanidinocarbonylmethyl)] amiloride} and MIBA [5-(*N*-methyl-*N*-isobutyl) amiloride] from Cragoe, Jr (Texas, USA). ICI 118 551 was a gift from ICI Pharma (Cheshire, Great Britain) and DMA [5-(*N*,*N*-dimethyl) amiloride] was a gift from Merck Sharp and Dohme Research Laboratories (New Jersey, USA). All other reagents were of analytical grade.

Equilibration media

The normal osmolality medium ($330\,mosmol\,kg^{-1}$) contained ($mmol\,l^{-1}$): NaCl, 113; KCl, 3.20; choline chloride, 43.85; $CaCl_2$, 3.75; $MgSO_4$, 1.25; taurine, 0.30; GABA, 0.30 and Tris, 12.2. HCl was added to give a pH of 7.40 at 10.0 °C. The low osmolality medium ($255\,mosmol\,kg^{-1}$) was obtained by reducing the concentration of choline chloride to $3.60\,mmol\,l^{-1}$. Sodium-free, low osmolality medium was obtained by replacing NaCl iso-osmotically by choline chloride. Urea medium with normal osmolality was obtained by iso-osmotic replacement of some of the choline chloride with $75\,mmol\,l^{-1}$ urea. The osmolality was measured using an osmometer (Knauer, Berlin, Germany). The media were aerated for 30 min before the experiments.

Amiloride, the amiloride analogues, 8Br-cAMP, DIDS, forskolin, noradrenaline and the various catecholamine agonists and antagonists were added to the incubation media immediately before use. Amiloride, the amiloride analogues, atenolol, DIDS and prazosin were dissolved in dimethyl sulphoxide and forskolin was dissolved in ethanol. The maximum concentrations of these solvents in the incubation media were 0.5 % and 0.1 % (v/v), respectively. Ascorbic acid was added (0.1 mol per mol of substance tested) to prevent oxidation of noradrenaline and the various catecholamine agonists and antagonists. These concentrations of dimethyl sulphoxide (see Table 1), ethanol (data not shown) and ascorbic acid *per se* (Fugelli and Thoroed, 1986) had no significant effect on the cellular water content.

Experimental procedure

The fish was stunned with a blow in the head, and blood was obtained by puncturing the bulbus arteriosus with a heparinized syringe. The blood was transferred to an incubation syringe, and the cells were sedimented by centrifugation for 25 s at 3940 ***g*** and 8–10 °C (RC2B from Sorvall, Connecticut, USA). The plasma and buffy coat were removed by aspiration. To reduce the number of non-erythrocytic cells in the cell pellet, the cells were washed four times in normal osmolality medium by resuspension and sedimentation by centrifugation. The duration of the centrifugation period was reduced in steps from 16 to 13 s. The efficiency of this washing procedure was verified by flow cytometry. In experiments where erythrocytes from more than one fish were used, the cells were pooled after the first wash.

Following the fourth wash, the cells were resuspended in normal osmolality medium (15 % haematocrit) and pre-incubated for 120 min in a temperature-controlled water bath at 10.0±0.1 °C, according to Fugelli and Rohrs (1980). The incubation medium was renewed after 30 min and again after 60 min. During the pre-incubation period, the cells shrink to a stable level. This level is reached after 120 min and is maintained for an additional 240 min (Thoroed and Fugelli, 1994*a*).

Cyclic AMP content and cell volume

Following the pre-incubation period, the cells were incubated in various test media (10 % haematocrit). Samples were taken at fixed time intervals to determine the concentration of cells, the haematocrit, the content of cyclic AMP in the cell suspension and in the incubation medium, and/or the cellular water content. The concentration of cells was determined by a Coulter Counter (Coulter Electronics Ltd, Dunstable, Great Britain) after the sample had been diluted in normal osmolality medium. The haematocrit was measured using standard microhaematocrit tubes. The samples (500 μl) for determination of the cyclic AMP content were transferred to test tubes containing 1.5 ml of 1.0 mmol l^{-1} theophylline. The tubes were immediately put in a bath of boiling water for 3 min and subsequently cooled to 0 °C. After centrifugation, the cyclic-AMP-containing supernatant was isolated and immediately frozen at −18 °C. The amount of cyclic AMP was measured in 50 μl of the clear supernatant by radioimmunoassay. The cellular cyclic AMP content was calculated from the cyclic AMP contents of the cell suspension and the incubation medium and the haematocrit. The extracellular cyclic AMP represented less than 1 % of the content of cyclic AMP in the cell suspension. The water content of the cells (g intracellular water g^{-1} dry mass) is used as an equivalent for the cell volume. The sample was transferred to a centrifuge tube and the erythrocytes were sedimented by centrifugation for 10 min as described. The water content was calculated as described by Fugelli and Thoroed (1986).

Na^+ concentration

Samples of the incubation media were diluted in CsCl solution [final Cs^+ concentration: 0.1 % (w/v)]. The Na^+ concentration (mmol l^{-1}) was determined by atomic absorption spectrophotometry (SpectrAA-10, Varian, Mulgrave, Australia).

Extracellular pH

Following the pre-incubation period, the erythrocytes were washed three times in the incubation medium. Finally, the cells were resuspended in 18 ml of normal osmolality medium and transferred to a 50 ml test tube. The tube, fitted with a stirrer and pH electrode, was placed in the temperature-controlled water bath, and the extracellular pH was monitored continously (M 72 Mk2 digital acid–base analyzer, Radiometer, Copenhagen, Denmark). After the extracellular pH had remained stable for at least 10 min, the test substance(s) dissolved in 2 ml of incubation medium were added (final haematocrit 8 %).

Taurine efflux

Following the fourth wash, the erythrocytes were loaded for 240 min with [^{14}C]taurine in normal osmolality medium. The measurements were carried out as described by Fugelli and Thoroed (1986). The rate coefficient for efflux of taurine (k_e, min^{-1}) was calculated for each of the 20 min periods according to the formula (Caldwell and Keynes, 1969):

$$k_e = \frac{M_n}{t(C_n + 0.5M_n)}, \quad (1)$$

where M_n is the content of [^{14}C]taurine in the medium at the end of the nth sampling period (cts min^{-1}), C_n is the total cellular [^{14}C]taurine content at the end of the last sampling period (cts min^{-1}) and t is the duration of the sampling period (min).

Taurine influx

Following the pre-incubation period, the erythrocytes were incubated for 220 min in various test media. During the incubation period, the media were renewed three times: after 40 min, 80 min and 120 min. Before measuring the taurine influx, the erythrocytes were washed three times by centrifugation and resuspension in their respective tracer-free test medium. The measurements were carried out as described by Thoroed and Fugelli (1993). The influx of taurine (nmol g^{-1} dry mass min^{-1}) was calculated from the specific activity of [^{14}C]taurine in the influx medium (cts min^{-1} $nmol^{-1}$), the cellular [^{14}C]taurine content (cts min^{-1} g^{-1} wet mass), the cellular dry mass (g dry mass g^{-1} wet mass) and the duration of the incubation period (min). The taurine influx mediated by the taurine channel was calculated from the total influx of taurine in the cells and the carrier-mediated, osmolality-insensitive influx of taurine (Thoroed and Fugelli, 1993, 1994*b*).

Statistics

Data were analysed by Student's *t*-test for paired observations. Differences with a *P*-value of less than 0.05 were considered significant.

Results

The effect of noradrenaline on the volume regulatory taurine efflux under hypo-osmotic conditions

Flounder erythrocytes stimulated by 1.0 μmol l^{-1} noradrenaline under iso-osmotic conditions gradually swell. After 4 h of exposure, the degree of cell swelling corresponds to the cell swelling induced by a reduction of the osmolality from 330 mosmol kg^{-1} to 255 mosmol kg^{-1} (Fugelli and Thoroed, 1986). The stimulatory effect of noradrenaline on the taurine efflux is very small (Fugelli and Thoroed, 1986), which suggests that noradrenaline could impair the activation of the osmolality-sensitive taurine channel. To examine this hypothesis, we incubated flounder erythrocytes in iso-osmotic medium containing 1.0 μmol l^{-1} noradrenaline for 20 min before reducing the osmolality from 330 mosmol kg^{-1} to 255 mosmol kg^{-1}, and measured the effect on the rate coefficient for taurine efflux.

Noradrenaline did not inhibit the volume regulatory taurine efflux. On the contrary, the mean rate coefficient for taurine efflux during the first 20 min period after reduction of osmolality was more than four times higher in the cells stimulated by noradrenaline than in the non-stimulated cells (Fig. 1). The transport activity remained at this high level during the next

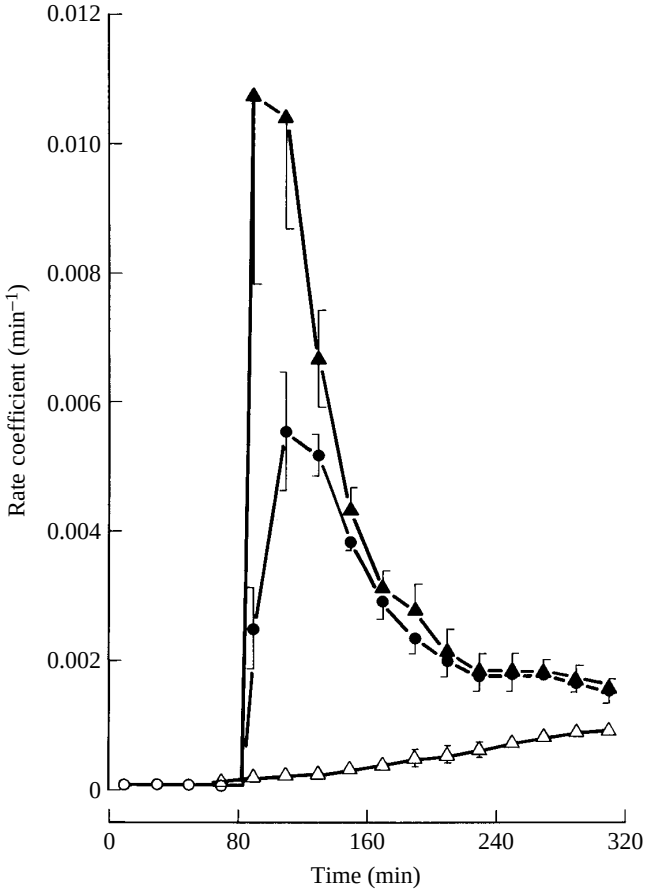

Fig. 1. The effect of noradrenaline and reduction of osmolality on the rate coefficient (min^{-1}) for taurine efflux from flounder erythrocytes. ○, 330 mosmol kg^{-1}; ●, 255 mosmol kg^{-1}; △, 330 mosmol kg^{-1}, 1.0 μmol l^{-1} noradrenaline; ▲, 255 mosmol kg^{-1}, 1.0 μmol l^{-1} noradrenaline. Each point represents the mean value for each 20 min period. The media were renewed at the beginning of each period. Mean values ± S.D. are shown except where error bars are masked by the symbol. The number of experiments (*N*)=4.

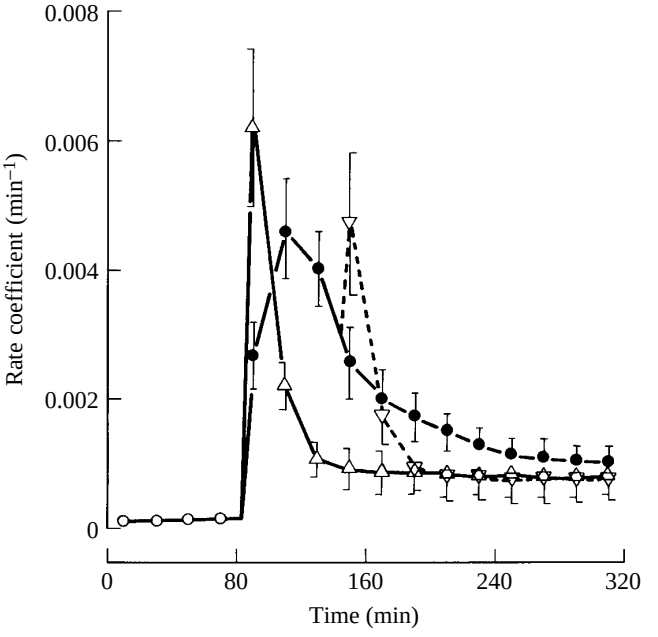

Fig. 2. The effect of noradrenaline on the osmolality-induced increase in the rate coefficient (min^{-1}) for taurine efflux from flounder erythrocytes. ○, 330 mosmol kg^{-1}; ●, 255 mosmol kg^{-1}; △, 255 mosmol kg^{-1}, 1.0 μmol l^{-1} noradrenaline added at the beginning of the fifth period; ▽, 255 mosmol kg^{-1}, 1.0 μmol l^{-1} noradrenaline added at the beginning of the eighth period. Each point represents the mean value for each 20 min period. The media were renewed at the beginning of each period. Mean values ± S.D. (*N*=5) are shown except where error bars are masked by the symbol.

period before declining to the activity level seen in the non-stimulated cells. Similar stimulation of the taurine efflux was also seen when the cells were exposed to noradrenaline simultaneously with reduction of osmolality (Fig. 2) and 20 min (data not shown), 40 min (data not shown) and 60 min after the reduction (Fig. 2). However, under these four conditions, the increased transport activity lasted for only one 20 min period and was quickly reduced to a level that was significantly lower than in the cells not stimulated by noradrenaline.

Thus, our hypothesis was not correct: noradrenaline accelerates the cellular mechanism(s) responsible for the activation of the osmolality-sensitive taurine channel in flounder erythrocytes. In order to reveal the mechanism(s) behind this acceleration, we studied several steps of the noradrenaline signal-transduction cascade in the erythrocytes in more detail.

The noradrenergic response of flounder erythrocytes

About 83 % of the swelling obtained after 240 min incubation in an iso-osmotic medium containing 1.0 μmol l^{-1} noradrenaline occurs during the first 120 min (Fugelli and Thoroed, 1986). To obtain the dose–response curve of this noradrenaline-induced uptake of water, the erythrocytes were exposed for 120 min to media containing 0.2–10.0 μmol l^{-1} noradrenaline.

The water content of the non-stimulated cells was 1.94±0.13 g intracellular water g^{-1} dry mass. Each of the tested noradrenaline concentrations resulted in a significant swelling of the cells (Fig. 3). 50 % of the observed maximum swelling was obtained at 0.65 μmol l^{-1} noradrenaline. Concentrations above 5.0 μmol l^{-1} had no additional significant effect on the water content.

Figs 1–3 indicate that the flounder erythrocytes possess catecholamine receptors. In order to characterize these receptors, we used different agonists and antagonists for the mammalian adrenergic receptor subtypes (Table 1). Many cell types contain receptors from more than one of these subgroups (Minneman *et al.* 1979). Therefore, when testing an agonist for a particular receptor subtype, an antagonist for the most closely related subtype(s) was also added to the incubation medium. The concentration of the various drugs was 1.0 μmol l^{-1}.

The water content in the non-stimulated erythrocytes was 1.87±0.07 g intracellular water g^{-1} dry mass (*N*=25). Stimulation by 1.0 μmol l^{-1} noradrenaline for 120 min caused

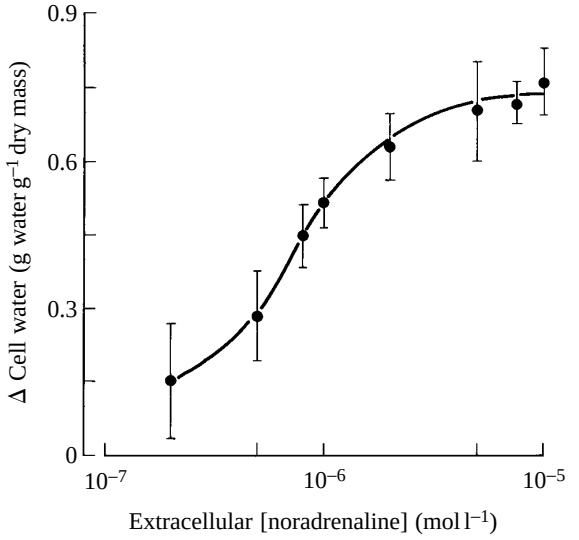

Fig. 3. The dose–response curve of the noradrenaline-induced swelling (g intracellular water g^{-1} dry mass) of flounder erythrocytes. Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 120 min in a normal osmolality medium with the addition of noradrenaline. Mean values ± S.D. (N=5) are shown.

Table 1. *The effect of noradrenaline and various catecholamine receptor agonists on the water content (g intracellular water g^{-1} dry mass) in flounder erythrocytes in the presence of various catecholamine receptor antagonists*

	Receptors		Increase in cellular water content
Addition	Stimulated	Inhibited	(%)
None			0
NA			100
DMSO			–0.3±3.3
NA + DMSO			100.0±3.3
Phenylephrine	α_1		
+timolol		β	0.2±9.6
Clonidine	α_2		
+prazosin + DMSO		α_1	8.9±15.7
Isoprenaline	β		
+ICI 118 551		β_2	107.4±15.6
Salbutamol	β_2		
+atenolol + DMSO		β_1	–1.1±6.5
NA + prazosin + DMSO		α_1	100.1±12.3
NA + yohimbine		α_2	97.9±14.3
NA + propranolol		β	21.4±7.8*
NA + atenolol + DMSO		β_1	76.6±10.3*
NA + ICI 118 551		β_2	107.5±11.8

Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 120 min in normal osmolality medium with addition of noradrenaline and/or various catecholamine receptor agonists and antagonists to a final concentration of 1.0 μmol l^{-1}. The concentration of dimethyl sulphoxide was 0.5 % (v/v).

Mean values ± S.D. (N=5) are shown.

*Significant inhibition (P<0.05) of the noradrenaline-induced cell swelling.

NA, noradrenaline; DMSO, dimethyl sulphoxide.

the uptake of 0.37±0.08 g water g^{-1} dry mass. The β-receptor agonist isoprenaline was the only compound which mimicked this swelling effect of noradrenaline (Table 1). Phenylephrine (α_1-receptor agonist), clonidine (α_2-receptor agonist) and salbutamol (β_2-receptor agonist) did not affect the water content significantly. When the erythrocytes were incubated in media containing noradrenaline (1.0 μmol l^{-1}) and one of the various antagonists (1.0 μmol l^{-1}), only the β-receptor blocker propranolol and the β_1-receptor blocker atenolol inhibited the swelling effect of noradrenaline, the latter much less than the former (Table 1). Prazosin (α_1-receptor blocker), yohimbine (α_2-receptor blocker) and ICI 118 551 (β_2-receptor blocker) had no inhibitory effect. Atenolol and prazozin were dissolved in dimethyl sulphoxide. The final concentration of the solvent was 0.5 % (v/v). At this concentration, dimethyl sulphoxide had no significant effect on the cellular water content, either in the absence or in the presence of noradrenaline (Table 1).

The results shown in Table 1 indicate that the receptors involved in the cellular swelling induced by noradrenaline are closely related to mammalian β_1-receptors. This conclusion was confirmed by experiments testing noradrenaline, isoprenaline and adrenaline (1.0 μmol l^{-1}) for 120 min. The effect of isoprenaline on cell volume was not significantly different from that of noradrenaline. The water content in the cells was 2.33±0.16 g intracellular water g^{-1} dry mass (N=5) in the presence of isoprenaline and 2.30±0.16 g intracellular water g^{-1} dry mass in the presence of noradrenaline. In the absence of agonists, the water content was 1.87±0.02 g intracellular water g^{-1} dry mass. The effect of adrenaline on the cellular water content, however, was 42 % smaller than that of noradrenaline.

The β-receptor-induced cellular swelling at iso-osmotic conditions indicates activation of transport systems through the adenylate cyclase/cyclic AMP system, which mediates the accumulation of osmolytes. Therefore, we investigated the effect of 1.0 μmol l^{-1} noradrenaline on the cyclic AMP level in flounder erythrocytes incubated in normal osmolality medium.

In unstimulated cells, the cyclic AMP concentration was about 0.4 nmol g^{-1} intracellular water. 5 min after the addition of noradrenaline, the cyclic AMP concentration was 23 times higher than in the non-stimulated cells (Fig. 4). During the following 235 min, the concentration declined gradually to about 4 nmol g^{-1} intracellular water. This reduction was due to net degradation of the cyclic AMP (Fig. 4) and to a diluting effect of the accumulated water (Fig. 3; Fugelli and Thoroed, 1986).

To examine further the participation of cyclic AMP and cyclic-AMP-dependent processes in the noradrenaline-induced cell swelling, we tested the effect of forskolin and of 8Br-cAMP on the cellular water content. Forskolin stimulates the adenylate cyclase system directly, without interacting with the

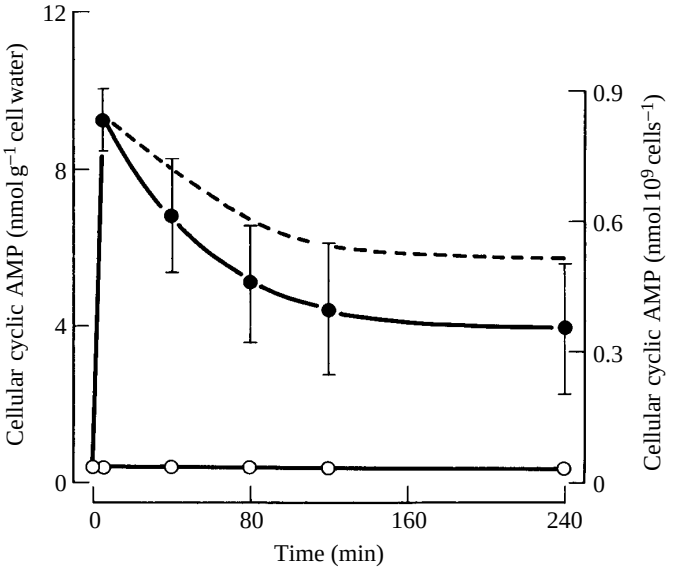

Fig. 4. The effect of noradrenaline on the cyclic AMP concentration (nmol g^{-1} intracellular water) in flounder erythrocytes. Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 240 min in normal osmolality medium (○) or normal osmolality medium with addition of noradrenaline to the final concentration of 1.0 μmol l^{-1} (●). Mean values ± S.D. (N=6) are shown except where error bars are masked by the symbol. The effect of noradrenaline on the intracellular cyclic AMP content (nmol 10^9 cells^{-1}) is indicated by the broken line.

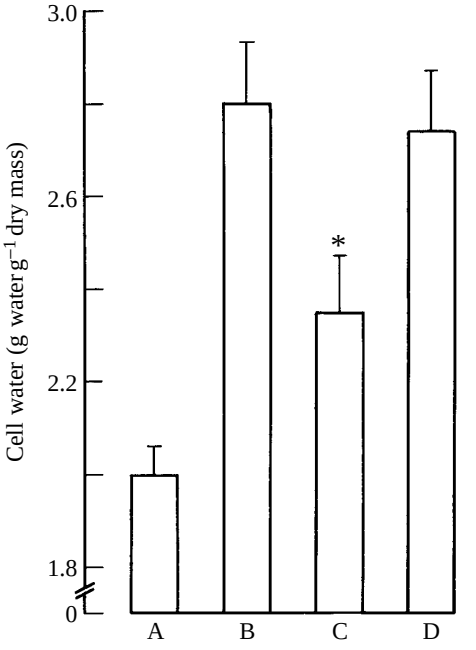

Fig. 5. The effect of forskolin and of 8-bromo-cyclic AMP on the water content (g intracellular water g^{-1} dry mass) in flounder erythrocytes. Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 120 min in normal osmolality medium (A), normal osmolality medium with addition of 1.0 μmol l^{-1} noradrenaline (B), 1.0 μmol l^{-1} forskolin (C) or 1.0 mmol l^{-1} 8-bromo-cyclic AMP and 1.0 mmol l^{-1} theophylline (D). Mean values + S.D. (N=5) are shown. *Cell swelling significantly less (P<0.05) than in the presence of noradrenaline.

catecholamine receptors. However, forskolin is generally less potent than noradrenaline (Seamon and Daly, 1981).

Fig. 5 shows that exposure of erythrocytes to 1.0 μmol l^{-1} forskolin for 120 min caused swelling of the cells. However, this cell swelling was only about 44% of that caused by 1.0 μmol l^{-1} noradrenaline. The effect of 1.0 mmol l^{-1} 8Br-cAMP on the cell volume was not significantly different from that of noradrenaline.

Various transport systems (i.e. $Na^+/K^+/2Cl^-$ cotransporter and Na^+/H^+ and HCO_3^- antiporters) are activated when vertebrate erythrocytes are stimulated by catecholamines (Kregenow, 1973; Borgese *et al.* 1986; Haas, 1989). To identify the type of transport systems activated in flounder erythrocytes, we examined the effect of DIDS, amiloride and amiloride analogues on the noradrenaline-induced cell swelling. DIDS inhibits the Cl^-/HCO_3^- antiporter (Jennings, 1989), whereas amiloride and the amiloride analogues inhibit the Na^+/H^+ antiporter (Simchowitz and Cragoe, 1986).

DIDS, at a concentration of 100 μmol l^{-1}, completely inhibited the swelling produced by 240 min of exposure to 1.0 μmol l^{-1} noradrenaline (Table 2). Amiloride also had a strong inhibitory effect, causing 93% inhibition. The inhibitory effects of MGCMA and DMA were intermediate, whereas the effect of MIBA was small but significant. The other amiloride analogues that were tested had no significant inhibitory effect.

To demonstrate further the presence of catecholamine-activated Na^+/H^+ and anion antiporters, we recorded the change in extracellular pH during 20 min of exposure of erythrocytes to either 1.0 μmol l^{-1} noradrenaline or 1.0 mmol l^{-1} 8Br-cAMP, incubated in normal osmolality medium containing 12.2 mmol l^{-1} Tris buffer. The pH of the incubation medium (113 mmol l^{-1} Na^+) started to fall about 1 min after the addition of noradrenaline. During the period 2–4 min after the stimulation, the extracellular pH fell by about 0.026 units min^{-1} (Fig. 6). The rate of acidification then declined and, 8–10 min after the addition of noradrenaline, the pH reached a minimum level 0.13 units lower than the initial level of 7.40. Throughout the last 10 min of the incubation period, the pH slowly increased. The acidifying effect of 8Br-cAMP was less than that of noradrenaline, and it was not seen until 5 min after the addition. When the concentration of Na^+ in the normal osmolality medium was reduced from 113 mmol l^{-1} to 2.7 mmol l^{-1} Na^+, stimulation by noradrenaline caused weak extracellular alkalization (Fig. 6). During the first 8 min, the pH increased from 7.40 to 7.42. This pH level was maintained during the rest of the incubation period. When erythrocytes incubated at 113 mmol l^{-1} Na^+ were stimulated by noradrenaline in the presence of 100 μmol l^{-1} DIDS, the medium became more acidified than it did in the absence of DIDS (Fig. 6). During the period 2–10 min after the stimulation, the pH fell by about

Table 2. *The effect of DIDS, amiloride and amiloride analogues on the noradrenaline-induced swelling of flounder erythrocytes*

Addition	Water content (g intracellular water g^{-1} dry mass)
None	1.91±0.04
NA	2.61±0.12
NA + DIDS	1.92±0.01*
NA + amiloride	1.96±0.05*
NA + DMA	2.42±0.14*
NA + EIPA	2.58±0.16
NA + HMA	2.68±0.17
NA + MGCMA	2.29±0.13*
NA + MIBA	2.56±0.12*

Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 240 min in normal osmolality medium with different additions. The concentration of noradrenaline was 1.0 μmol l^{-1}, while the concentration of DIDS, amiloride and the amiloride analogues was 100 μmol l^{-1}.

Mean values ± S.D. (N=6) are shown.

*Significant inhibition (P<0.05) of the noradrenaline-induced cell swelling.

NA, noradrenaline.

0.062 units min^{-1}. The acidification then continued at a declining rate for the rest of the incubation period. At the end of the experiment, the extracellular pH was 0.67 units lower than the initial level.

From the results presented in the latter paragraphs, we suggest that the noradrenergic swelling of the erythrocytes is caused by accumulation of Na^+ and Cl^- due to increased transport activity of the Na^+/H^+ and anion antiporters. The increase in the transport activity is caused by the increased intracellular level of cyclic AMP, which in turn is due to interaction of noradrenaline with the β_1-related receptors of the cells.

The part(s) of the noradrenaline signal-transduction cascade that accelerate(s) the activation of the osmolality-sensitive taurine channel

To distinguish between effects from the activation of the Na^+/H^+ and anion antiporters and other effects from increased cyclic AMP concentration, we wished to inhibit the antiporters without affecting the cyclic AMP production induced by noradrenaline. Moreover, this inhibition should not affect the volume regulatory efflux of taurine in the absence of noradrenaline.

Table 2 shows that, at 100 μmol l^{-1}, the amiloride analogues HMA and EIPA, unlike amiloride, did not inhibit the cellular swelling induced by noradrenaline. The inhibitory effect of these two substances on the Na^+/H^+ antiporters in mammalian tissues is much stronger than the effect of amiloride. The IC_{50} values are 0.16 μmol l^{-1} for HMA, 0.38 μmol l^{-1} for EIPA and 83.8 μmol l^{-1} for amiloride (Simchowitz and Cragoe, 1986). This difference suggests that the Na^+/H^+ antiporter in flounder erythrocytes is structurally

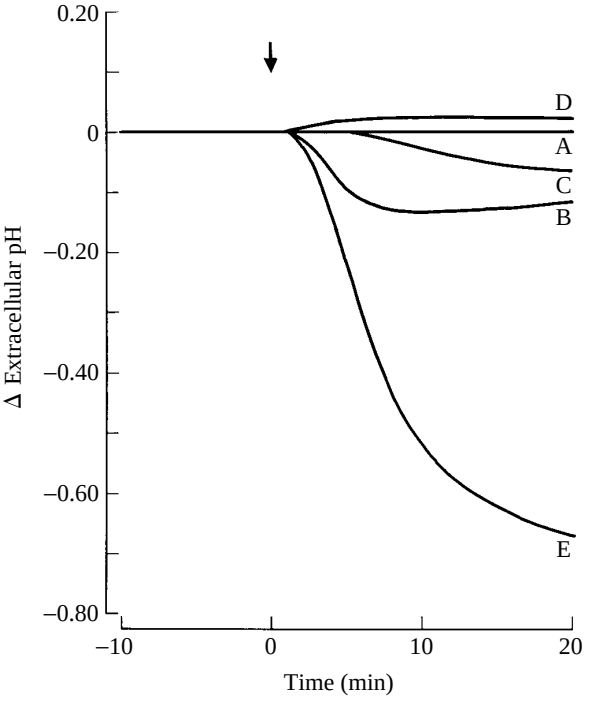

Fig. 6. The effect of noradrenaline and of 8-bromo-cyclic AMP on the extracellular pH in a suspension of flounder erythrocytes (8 % haematocrit; total volume 21.7 ml). The incubation media contained 12.2 mmol l^{-1} Tris buffer. The cells were pre-incubated in normal osmolality medium (330 mosmol kg^{-1}). Prior to the incubation period, the cells were washed three times in their respective incubation medium (330 mosmol kg^{-1}) containing 113 mmol l^{-1} or 2.7 mmol l^{-1} Na^+. The test substances were added to the cell suspension when the extracellular pH had been stable for at least 10 min. The results from one representative experiment out of four are shown. (A) 113 mmol l^{-1} or 2.7 mmol l^{-1} Na^+; (B) 113 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline; (C) 113 mmol l^{-1} Na^+, 1.0 mmol l^{-1} 8-bromo-cyclic AMP, 1.0 mmol l^{-1} theophylline; (D) 2.7 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline; (E) 113 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline, 100 μmol l^{-1} DIDS.

different from the antiporters in mammalian cells. It also suggests that the effect of amiloride, DMA, MGCMA and MIBA shown in Table 2 might be due to inhibition of steps other than the Na^+/H^+ antiporter in the noradrenaline signal-transduction cascade. Mahé *et al.* (1985) have shown in trout erythrocytes that amiloride inhibits both adenylate cyclase and the Na^+/H^+ antiporter, with the strongest effect being on the adenylate cyclase. We therefore measured the content of cyclic AMP production in cells stimulated by noradrenaline in the presence of amiloride or the amiloride analogues that inhibited the noradrenaline-induced swelling of the cells.

Amiloride, DMA, MGCMA and MIBA all inhibited cyclic AMP production (Table 3). The most potent inhibitor was amiloride. 8 min after addition of noradrenaline, the cyclic AMP content of these cells was 90 % lower than in the cells not treated with amiloride. Amiloride and the amiloride analogues were thus not suitable for further experiments. Nor was DIDS, since it strongly inhibited the volume regulatory efflux of taurine in the absence of noradrenaline (Fig. 7).

Table 3. *The effect of amiloride and amiloride analogues on the noradrenaline-induced increase of the cyclic AMP content in flounder erythrocytes*

Addition	Cyclic AMP content (nmol 10^9 cells^{-1})
None	0.036±0.010
NA	0.780±0.099
NA + amiloride	0.063±0.017*
NA + DMA	0.094±0.008*
NA + MGCMA	0.130±0.034*
NA + MIBA	0.179±0.072*

Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}), the cells were incubated for 8 min in normal osmolality medium with different additions. The noradrenaline concentration was 1.0 μmol l^{-1}, while the concentration of amiloride and the amiloride analogues was 100 μmol l^{-1}.

Mean values ± S.D. (N=4) are shown.

*The cyclic AMP content is significantly lower (P<0.05) than in the cells only exposed to noradrenaline.

NA, noradrenaline.

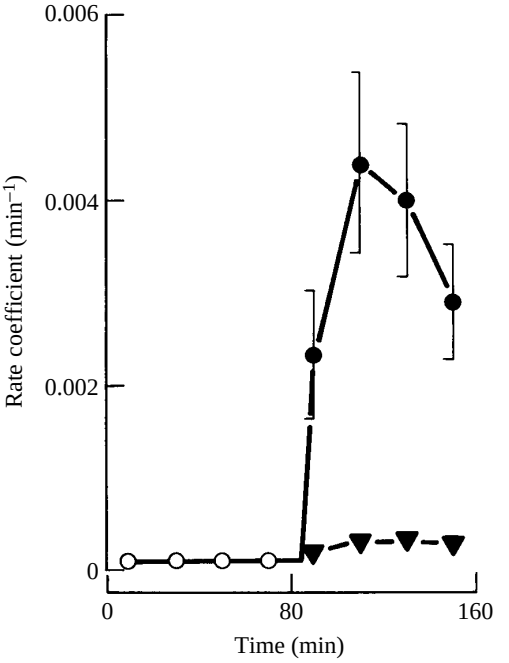

Fig. 7. The effect of DIDS on the osmolality-induced increase in the rate coefficient (min^{-1}) for taurine efflux from flounder erythrocytes. ○, 330 mosmol kg^{-1}; ●, 255 mosmol kg^{-1}; ▼, 255 mosmol kg^{-1}, 100 μmol l^{-1} DIDS. Each point represents the mean value for each 20 min period. The media were renewed at the beginning of each period. Mean values ± S.D. (N=5) are shown except where error bars are masked by the symbol.

The effect of noradrenaline-stimulated Na^+/H^+ antiporter activity was clearly dependent on the extracellular Na^+ concentration (Fig. 6). The effect of the antiporter activity might thus be isolated from other noradrenaline effects by reducing the extracellular Na^+ concentration. We therefore investigated whether the volume regulatory efflux of taurine in the first 20 min period after the reduction of osmolality in the absence of noradrenaline was dependent on the extracellular Na^+ concentration. This was examined by replacing the normal osmolality medium containing 113 mmol l^{-1} Na^+ by low osmolality medium containing 113 mmol l^{-1} or 0 mmol l^{-1} Na^+. Since it was impossible to remove the Na^+-containing medium completely, the final Na^+ concentration in the latter situation was 2.7±0.5 mmol l^{-1} (N=6). The reduction in the extracellular Na^+ concentration had no significant effect on the activity of the taurine efflux system. The rate coefficient for efflux of taurine was 0.0036±0.0011 min^{-1} (N=5) at 113 mmol l^{-1} Na^+ and 0.0036±0.0005 min^{-1} at 2.7 mmol l^{-1} Na^+.

We then examined whether the noradrenaline-induced increase in the intracellular cyclic AMP level was affected by reduction of the extracellular Na^+ concentration from 113 mmol l^{-1} to 2.7 mmol l^{-1}. 8 min after the addition of noradrenaline and the reduction in osmolality, the cyclic AMP concentration in cells incubated at 113 mmol l^{-1} Na^+ was 5.9±2.9 nmol g^{-1} intracellular water (N=6). This level was not significantly different from the cyclic AMP level in cells stimulated by noradenaline at normal osmolality and 113 mmol l^{-1} Na^+ (5.1±2.3 nmol g^{-1} intracellular water). At low osmolality and 2.7 mmol l^{-1} Na^+, however, the cyclic AMP concentration in the cells stimulated by noradrenaline was significantly lower (3.0±1.2 nmol g^{-1} intracellular water).

To investigate the role of cyclic AMP in the noradrenaline-induced acceleration of the volume regulatory efflux of taurine, we examined the effect of 1.0 μmol l^{-1} noradrenaline on the rate coefficient for this efflux in low osmolality media containing 2.7 mmol l^{-1} or 113 mmol l^{-1} Na^+ and the effect of low osmolality media containing 1.0 mmol l^{-1} 8Br-cAMP and 113 mmol l^{-1} Na^+.

In the presence of 8Br-cAMP, the rate coefficient for the volume regulatory efflux of taurine was 24% higher than in non-stimulated erythrocytes (Fig. 8). The stimulatory effect of noradrenaline on this taurine efflux was not significantly affected by the reduction in the extracellular Na^+ concentration. Thus, whether noradrenaline stimulation of the erythrocytes elevated the intracellular concentration of cyclic AMP to either approximately 3 or approximately 6 nmol g^{-1} intracellular water in the middle of the first 20 min period after the reduction of osmolality, the accelerating effect of noradrenaline on the volume regulatory efflux of taurine efflux was not affected.

The stimulatory effect of noradrenaline on intracellular cyclic AMP production (Fig. 4) and the negligible effect of noradrenaline on the volume regulatory efflux of taurine under iso-osmotic conditions (Fig. 1) suggest that cyclic AMP is not part of the signal-transduction mechanisms responsible for the activation of the taurine channel under hypo-osmotic conditions. To investigate this hypothesis, we measured the content of cyclic AMP in cells incubated in low osmolality media containing 113 mmol l^{-1} or 0 mmol l^{-1} Na^+ and in cells incubated in normal osmolality medium containing 75 mmol l^{-1} urea and 113 mmol l^{-1} Na^+. Under each of these

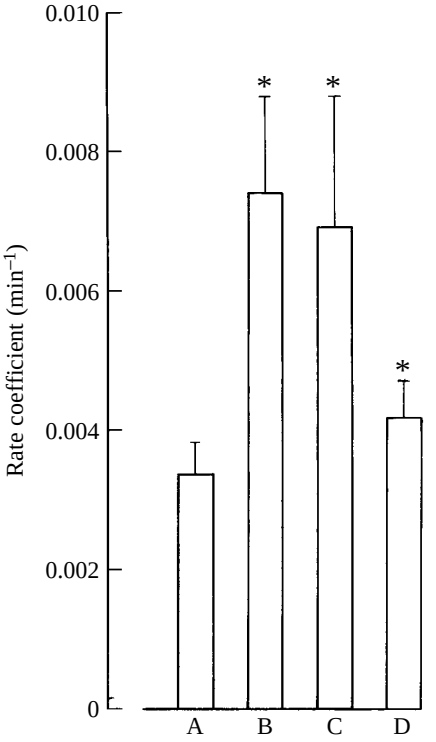

Fig. 8. The effect of noradrenaline at various extracellular Na^+ concentration and of 8-bromo-cyclic AMP on the mean rate coefficient (min^{-1}) for taurine efflux from flounder erythrocytes during the first 20 min after reduction of osmolality. Following incubation in normal osmolality medium (330 mosmol kg^{-1}) for 80 min (i.e. periods 1–4 in Figs 1 and 2), the incubation medium was replaced by low osmolality media (255 mosmol kg^{-1}). (A) 113 mmol l^{-1} Na^+; (B) 113 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline; (C) 2.7 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline; (D) 113 mmol l^{-1} Na^+, 1.0 mmol l^{-1} 8-bromo-cyclic AMP. Mean values + S.D. (N=6) are shown. *The rate coefficient for the efflux of taurine is significantly greater (P<0.05) than in A.

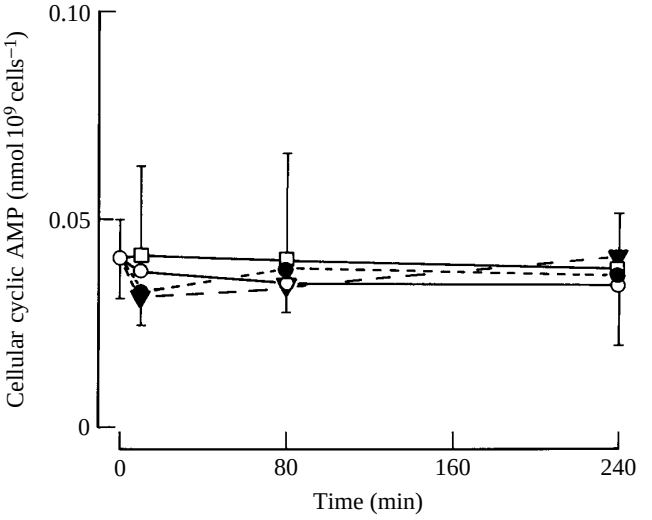

Fig. 9. The cyclic AMP content (nmol 10^9 $cells^{-1}$) of flounder erythrocytes incubated under different conditions that cause swelling of the cells and activation of the taurine channel. Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}, 113 mmol l^{-1} Na^+), the cells were incubated for 240 min in the following media: ○, 330 mosmol kg^{-1}, 113 mmol l^{-1} Na^+; □, 330 mosmol kg^{-1}, 75 mmol l^{-1} urea, 113 mmol l^{-1} Na^+; ●, 255 mosmol kg^{-1}, 113 mmol l^{-1} Na^+; ▼, 255 mosmol kg^{-1}, 0 mmol l^{-1} Na^+. Mean values (N=5) and the S.D. of the lowest and highest mean value at each time point are shown.

three conditions, the osmolality-sensitive taurine channel is strongly stimulated (Fugelli and Thoroed, 1986).

Incubation in the urea-containing medium and in the low osmolality media had no significant effect on the cellular cyclic AMP content (Fig. 9). Because of the swelling of the erythrocytes (Fugelli and Thoroed, 1986), the cyclic AMP concentration in these cells was reduced during the incubation period. Thus, in the absence of noradrenaline, activation of the osmolality-sensitive taurine channel is not associated with increased cellular levels of cyclic AMP.

Lack of correlation between the cell volume and the level of activation of the taurine channel in flounder erythrocytes

The water content of the cells at the end of the experiments shown in Fig. 1 and Fig. 2 was greater in cells exposed to noradrenaline and reduced osmolality [Fig. 1 (▲): 2.86±0.23 g intracellular water g^{-1} dry mass; Fig. 2 (△): 3.27±0.24 and (▽): 3.12±0.27 g intracellular water g^{-1} dry mass) than in cells only exposed to redused osmolality (Fig. 1 (●): 2.16±0.08 g intracellular water g^{-1} dry mass; Fig. 2 (●): 2.40±0.08 g intracellular water g^{-1} dry mass]. Thus, there was no positive correlation between the cell volume and the level of activation of the taurine channel. The relationship between these two variables was further investigated by measuring influx of taurine in erythrocytes incubated for 220 min in six different incubation media: normal osmolality medium containing 113 mmol l^{-1} Na^+, low osmolality media containing 113 mmol l^{-1} Na^+ or 0 mmol l^{-1} Na^+, normal osmolality media containing 75 mmol l^{-1} urea and 113 mmol l^{-1} Na^+ or 0 mmol l^{-1} Na^+, and normal osmolality medium containing 113 mmol l^{-1} Na^+ and 1.0 μmol l^{-1} noradrenaline. Under four of these conditions (i.e. urea-containing media and low osmolality media), efflux and influx of taurine mediated by the taurine channel are strongly stimulated (Fugelli and Thoroed, 1986).

There was a significant positive correlation (P<0.05) between the water content in the cells incubated in normal osmolality medium, urea-containing media or low osmolality media and the influx of taurine mediated by the taurine channel (Fig. 10). However, when the cells stimulated by noradrenaline were included in the regression analysis, a significant correlation was no longer obtained. Noradrenaline does not inhibit the activation of the taurine channel (Figs 1, 2). Thus, this lack of correlation between the cell volume and the state of activation of the taurine channel strongly suggests that the volume regulatory mechanisms are not activated by changes in the cell volume *per se*.

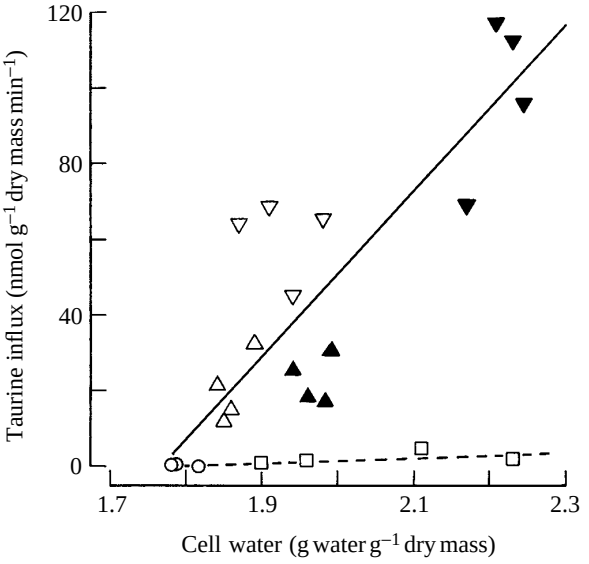

Fig. 10. The relationship between the water content (g intracellular water g^{-1} dry mass) and the influx of taurine mediated by the taurine channel (nmol g^{-1} dry mass min^{-1}) in flounder erythrocytes incubated under different conditions. Following pre-incubation in normal osmolality medium (330 mosmol kg^{-1}; 113 mmol l^{-1} Na^+), the cells were incubated for 220 min in various media. During the incubation period the media were renewed after 40 min, 80 min and 120 min. ○, 330 mosmol kg^{-1}, 113 mmol l^{-1} Na^+; △, 330 mosmol kg^{-1}, 113 mmol l^{-1} Na^+, 75 mmol l^{-1} urea; ▽, 330 mosmol kg^{-1}, 0 mmol l^{-1} Na^+, 75 mmol l^{-1} urea; ▲, 255 mosmol kg^{-1}, 113 mmol l^{-1} Na^+; ▼, 255 mosmol kg^{-1}, 0 mmol l^{-1} Na^+; □, 330 mosmol kg^{-1}, 113 mmol l^{-1} Na^+, 1.0 μmol l^{-1} noradrenaline. Each medium contained 0.30 mmol l^{-1} taurine. The influx mediated by the taurine channel was calculated from the total influx of taurine and the influx of taurine mediated by the osmolality-insensitive Na^+-accelerated transport system. The regression line for the mean values obtained in each of the various noradrenaline-free media (y=222.1x−393.2) (P<0.05) is shown and a broken line connects the values obtained in the normal osmolality medium and in noradrenaline-containing normal osmolality medium.

Discussion

Steps in the noradrenaline signal-transduction cascade of flounder erythrocytes

Noradrenaline induced swelling of flounder erythrocytes incubated in iso-osmotic medium (Figs 3, 5; Tables 1, 2). The EC_{50} of this response was 0.65 μmol l^{-1} noradrenaline (Fig. 3). This confirms earlier studies from our laboratory (Fugelli and Reiersen, 1978; Fugelli and Thoroed, 1986). A similar catecholamine response has been demonstrated in erythrocytes from rainbow trout and striped bass both *in vivo* and *in vitro* (Nikinmaa, 1982; Baroin *et al.* 1984*a*; Nikinmaa and Huestis, 1984). Neither the various α-agonists (clonidine and phenylephrine) nor the α-antagonists (prazosin and yohimbine) that were tested had any effect on the cellular water content or the noradrenaline (NA) response (Table 1). This suggests that the catecholamine receptors on the flounder erythrocytes are exclusively of the β type.

Adrenaline (A) and isoprenaline (ISOP), in contrast, caused swelling of the cells. The mammalian β_1- and β_2-receptors are characterized by the potency orders ISOP>NA>A and ISOP>A≫NA, respectively (Lands *et al.* 1967; Stene-Larsen, 1981). The effect on the flounder erythrocytes was ISOP=NA≫A. This result indicates that the flounder receptors are closely related to the mammalian β_1-receptors. This conclusion was supported by the lack of effect of the β_2-specific antagonist (ICI 118 551) and agonist (salbutamol) and by the observation that the antagonist with a general β-receptor effect (propranolol) was more potent than the β_1-specific antagonist (atenolol) (Table 1). The erythrocyte catecholamine receptors have been characterized in different vertebrates. Like flounder erythrocytes, rainbow trout erythrocytes (Tetens *et al.* 1988) and turkey red cells (André *et al.* 1981) have β_1-related receptors. Frog red cell receptors (Mukherjee *et al.* 1975), however, appear to be more related to the β_2-receptors on human (Sager, 1983) and rat erythrocytes (Kaiser *et al.* 1978).

The noradrenaline-induced swelling of flounder erythrocytes is partly due to accumulation of Na^+ and K^+. During 4 h of exposure to 1.0 μmol l^{-1} noradrenaline, the cellular content of these cations increases by 133 μmol g^{-1} dry mass for Na^+ and 36 μmol g^{-1} dry mass for K^+ (A. Tufte and K. Fugelli, unpublished results). It is well documented that noradrenaline-induced accumulation of Na^+ in vertebrate erythrocytes is mediated by Na^+/H^+ antiporters (Borgese *et al.* 1986) and $Na^+/K^+/2Cl^-$ cotransporters (Kregenow, 1973; Haas, 1989). In flounder erythrocytes, the Na^+ accumulation was mediated by Na^+/H^+ antiporters and not by cotransporters. This conclusion is supported by the following observations. (1) The accumulation of Na^+ was accompanied by extracellular acidification (Fig. 6). (2) Transport mediated by Na^+/H^+ antiporters is driven by the coupled energy gradients of Na^+ and H^+ (Aronson, 1985). If the accumulation of Na^+ in flounder red cells and the extracellular acidification were due to increased Na^+/H^+ antiport activity, reduced acidification caused by reduction of the extracellular Na^+ concentration was to be expected. Na^+ is in electrochemical equilibrium across the membrane of flounder red cells at an extracellular concentration of about 17 mmol l^{-1} (Fugelli and Rohrs, 1980). Fig. 6 shows that, at a concentration lower than this (2.7 mmol l^{-1}), the noradrenaline-induced extracellular acidification was blocked. The pH of the incubation medium was slightly increased. This suggests that the Na^+/H^+ antiporter worked in the opposite direction, exchanging intracellular Na^+ for extracellular H^+. (3) 50 % of the amount of Na^+ accumulated during 4 h of exposure to noradrenaline (1.0 μmol l^{-1}) is accumulated after about 20 min. During this period, there is no significant change in the cellular content of K^+ (A. Tufte and K. Fugelli, unpublished results). (4) The noradrenaline-induced accumulation of K^+ is completely inhibited by the addition of ouabain (A. Tufte and K. Fugelli, unpublished results), which strongly suggests that the Na^+/K^+-ATPase exchanges accumulated Na^+ for extracellular K^+.

Additional extracellular acidification was obtained in the presence of DIDS (Fig. 6). This strongly indicates that the noradrenaline-induced swelling was also due to accumulation of Cl^- mediated by the Cl^-/HCO_3^- antiporter. The anion antiporter is stimulated by the intracellular alkalization and the increased cellular levels of HCO_3^- caused by the extrusion of H^+ by the Na^+/H^+ antiporter.

Similar effects of catecholamine stimulation on the extracellular pH, and the effects of DIDS and the extracellular Na^+ concentration on this response, have been demonstrated in erythrocytes from fish species living in fresh water (Baroin *et al.* 1984*b*; Nikinmaa and Huestis, 1984).

Binding of noradrenaline to the β_1-related receptors was the first step in the noradrenaline signal-transduction cascade of flounder erythrocytes. The last step was activation of the antiporters. Fig. 4 shows that one of the intermediate steps was production of cyclic AMP. The swelling of the cells induced by forskolin and by 8Br-cAMP (Fig. 5) and the effect of 8Br-cAMP on the extracellular pH (Fig. 6) support this conclusion. Other authors have shown similar effects on the cellular cyclic AMP level in erythrocytes from rainbow trout (Mahé *et al.* 1985) and carp (Salama and Nikinmaa, 1990). The mechanism underlying the propagation of the signal from cyclic AMP to the Na^+/H^+ antiporter in flounder erythrocytes is not yet known. It seems reasonable to suggest that the Na^+/H^+ antiporter in the flounder cells, like the antiporter in erythrocytes from rainbow trout, is probably activated by a phosphorylation at two sites on the cytoplasmic part of the antiporter. The phosphorylation is mediated by cyclic-AMP-dependent protein kinase A (Borgese *et al.* 1992; Guizouarn *et al.* 1993).

The part(s) of the noradrenaline signal-transduction cascade that accelerate(s) the osmolality-sensitive taurine channel

Noradrenaline induced a very small, but significant, increase in the rate coefficient for efflux of taurine from flounder erythrocytes under iso-osmotic condition (Fig. 1). This result confirms earlier investigations from our laboratory (Fugelli and Thoroed, 1986). Under hypo-osmotic conditions, however, the volume regulatory efflux of taurine was temporarily strongly accelerated (Figs 1, 2). This synergistic effect of noradrenaline and reduction of osmolality was more than four times greater than the effect of osmolality reduction alone. The noradrenaline-induced swelling of flounder erythrocytes was due to increased Na^+/H^+ and anion antiporter activity. However, the reduced activity of these antiporters (Fig. 6) did not affect the synergistic effect of noradrenaline on the osmolality-sensitive taurine channels (Fig. 8). This leaves a key role to earlier steps in the signal-transduction cascade, probably cyclic AMP. The accelerating effect of 8Br-cAMP on the volume regulatory efflux of taurine supports this conclusion (Fig. 8). One might argue that the small effect of 8Br-cAMP on the taurine channel, compared with the corresponding effect of noradrenaline (Fig. 8), does not fit this hypothesis. However, noradrenaline induced an abrupt increase in the intracellular concentration of cyclic AMP (Fig. 4). Although the extracellular concentration of 8Br-cAMP was about 100 times higher than the peak concentration of cyclic AMP, the intracellular concentration of 8Br-cAMP increased considerably more slowly towards this level, if it was reached at all. This was partly due to the low permeability of the cell membrane (see Materials and methods). The difference between the effects of noradrenaline and of 8Br-cAMP on the extracellular pH (Fig. 6) supports this assumption.

The effect of noradrenergic stimulation on efflux of taurine appears to have been studied most thoroughly in mammalian glial cells and pineal organs. Efflux of taurine from these cells and tissues is also strongly stimulated by catecholamines and cyclic AMP (Wheler and Klein, 1979, 1980; Madelian *et al.* 1985). However, unlike the efflux of taurine from flounder erythrocytes, the taurine efflux from these cells is stimulated under iso-osmotic conditions. In glial cells, as in flounder erythrocytes, release of taurine contributes to the cell shrinkage following the cell swelling induced by reduction of the osmolality of the incubation medium (Pasantes-Morales and Schousboe, 1988). The osmolality-stimulated efflux of taurine from the astroglial cells studied by Martin *et al.* (1990) appears to be mediated by the same transport system as the β-receptor-induced taurine efflux. These different effects of β-receptor stimulation on the taurine efflux at normal osmolality suggest that the efflux of taurine from cells is controlled by several different mechanisms.

The results presented in this investigation are apparently not in accordance with the results obtained using erythrocytes from starry flounder incubated under depolarizing conditions ($150\,mmol\,l^{-1}$ K^+) by Fincham *et al.* (1987). 8Br-cAMP induced swelling of erythrocytes from both flatfish species. But, by assuming that the volume regulatory efflux of taurine is positively correlated to the cell volume, Fincham and co-workers concluded that the volume regulatory transport of taurine in erythrocytes from starry flounder is inhibited by 8Br-cAMP.

Little is known about the mechanisms participating in the activation of the osmolality-sensitive taurine channel in flounder erythrocytes. Ca^{2+}/calmodulin appear to be involved (Thoroed and Fugelli, 1994*b*), but the most prominent role appears to be played by leukotrienes. In the presence of inhibitors of 5-lipoxygenase, which converts arachidonic acid to leukotriene A_4, the volume regulatory efflux of taurine is completely blocked (Thoroed and Fugelli, 1994*b*,*c*). A similar effect on the taurine channel is also seen in the presence of a leukotriene D_4-receptor antagonist (Thoroed and Fugelli, 1994*c*). In addition, inositol phosphates may play a regulatory role, as in Ehrlich ascites tumour cells (Hoffmann *et al.* 1988) and rat liver cells (Dahl *et al.* 1991). Fig. 9 shows that the cellular cyclic AMP level is not elevated under hypo-osmotic conditions in the absence of noradrenaline. Thus, the effect of noradrenaline shown in Figs 1, 2 and 8 is due to an interaction of cyclic AMP with the signal-transduction systems activated by reduction of osmolality. The mechanisms underlying this effect of cyclic AMP and the signal systems involved are still not known.

The 'receptor' initiating the activation of the osmolality-sensitive taurine channel in flounder erythrocytes

One of the most interesting questions regarding cell volume regulation under hypo-osmotic conditions is what triggers the various volume regulatory processes. Hoffmann and Kolb (1991) proposed that the 'volume-receptor' might be stretch-activated channels located in the cell membrane and probably linked to the cytoskeleton, while Parker (1993) suggested that the volume regulatory processes are initiated by reduction of the cytosolic concentration of proteins. The effect of reduction of osmolality on the taurine channel in flounder erythrocytes is a graded response. We have observed this response under hypo-osmotic conditions that cause far less swelling of the cells and dilution of the cytosolic proteins than stimulation by noradrenaline ($1.0\,\mu mol\,l^{-1}$) at normal osmolality (Fugelli and Thoroed, 1986). In addition, there is no correlation between the cell volume and the state of activation of the taurine channel when the values obtained in noradrenaline-containing media are included in the calculations (Fig. 10). According to the hypothesis of Hoffmann and Kolb (1991) and of Parker (1993), the volume regulatory mechanisms should have been strongly activated by $1.0\,\mu mol\,l^{-1}$ noradrenaline at normal osmolality. There could be two explanations of this apparent discrepancy: either steps in the noradrenaline signal-transduction cascade inhibit the volume regulatory mechanism or the 'volume-receptor' is not activated by stretching of the cell membrane or by reduction of the cytoplasmic concentration of proteins. The first alternative is ruled out by the results presented in Figs 1, 2 and 8, which show the accelerating effect of noradrenaline on the efflux of taurine by the osmolality-sensitive taurine channel.

Reduction of osmolality causes influx of osmotically obligated water and hence reduction of the ionic strength of the cytoplasm. Motais *et al.* (1991) suggest that the reduction of the ionic strength is the main factor that triggers the various volume regulatory processes. Flounder erythrocytes swell when stimulated by noradrenaline (Figs 3, 5; Tables 1, 2; Fugelli and Thoroed, 1986). This swelling is due to accumulation of ions, while the cellular content of taurine is not affected (Fugelli and Thoroed, 1986): the cellular ionic strength therefore increases. According to the hypothesis of Motais and co-workers, the taurine channel in these flounder erythrocytes (Fig. 1: △ and ▲) should be less activated by reduction of osmolality than the taurine channel in cells not stimulated by noradrenaline (Fig. 1: ○ and ●). Fig. 1 shows that this is not the case. Thus, the results presented in the present paper and by Fugelli and Thoroed (1986) do not fit the hypothesis of Hoffmann and Kolb (1991), Motais *et al.* (1991) and Parker (1993).

We suggest that the 'volume-receptor' in flounder erythrocytes is located in an organelle. Incubation of flounder erythrocytes in iso-osmotic medium containing urea induces cell swelling and activation of the taurine channel in a similar way to incubation in a medium with low osmolality (Fugelli and Thoroed, 1986; Fig. 10). Membranes are usually highly permeable to water and urea. Thus, exposure of erythrocytes to urea-containing medium or medium with reduced osmolality also induces swelling of organelles. We do not know whether noradrenaline induces increased transport activity in organelles and thereby accumulation of osmolytes and swelling of these structures. The assumptions that the 'volume-receptor' is located in an organelle and that swelling of this organelle is not part of the noradrenergic response of the erythrocytes could explain the negligible stimulatory effect of the noradrenaline-induced cell swelling at normal osmolality on the taurine channel. Many cellular processes are located in organelles, and changes in their structures affect the functions of the cell. Therefore, regulation of the volume of organelles after reduction of extracellular osmolality appears to be of equal importance for the cellular functions as for regulation of the total cell volume.

The authors are grateful of Dr Øivind Nordby for his indispensable help with the Coulter Counter and of Dr Olav Sand and Dr Ian H. Lambert for their critical comments during the preparation of the manuscript. This work was supported by the Norwegian Research Council for Science and the Humanities and the Fridtjof Nansen Foundation for the Advancement of Science.

References

ANDRÉ, C., VAUQUELIN, G., DE BACKER, J.-P. AND STROSBERG, A. D. (1981). Identification and chemical characterization of β-adrenergic receptors in intact turkey erythrocytes. *Biochem. Pharmac.* **30**, 2787–2795.

ARONSON, P. S. (1985). Kinetic properties of the plasma membrane Na^+–H^+ exchanger. *A. Rev. Physiol.* **47**, 545–560.

BAROIN, A., GARCIA-ROMEU, F., LAMARRE, T. AND MOTAIS, R. (1984*a*). Hormone-induced co-transport with specific pharmacological properties in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol., Lond.* **350**, 137–157.

BAROIN, A., GARCIA-ROMEU, F., LAMARRE, T. AND MOTAIS, R. (1984*b*). A transient sodium–hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol., Lond.* **356**, 21–31.

BORGESE, F., GARCIA-ROMEU, F. AND MOTAIS, R. (1986). Catecholamine-induced transport systems in trout erythrocyte. Na^+/H^+ countertransport or NaCl cotransport? *J. gen. Physiol.* **87**, 551–566.

BORGESE, F., SARDET, C., CAPPADORO, M., POUYSSEGUR, J. AND MOTAIS, R. (1992). Cloning and expression of a cAMP-activated Na^+/H^+ exchanger: evidence that the cytoplasmic domain mediates hormonal regulation. *Proc. natn. Acad. Sci. U.S.A.* **89**, 6765–6769.

CALDWELL, P. C. AND KEYNES, R. D. (1969). The exchange of $^{22}Na^+$ between frog sartorius muscle and the bathing medium. In *Laboratory Techniques in Membrane Biophysics* (ed. H. Passow and R. Stampfli), pp. 63–68. Berlin: Springer-Verlag.

CHAMBERLIN, M. E. AND STRANGE, K. (1989). Anisosmotic cell volume regulation: a comparative view. *Am. J. Physiol.* **257**, C159–C173.

DAHL, S. V., HALLBRUCKER, C., LANG, F. AND HÄUSSINGER, D. (1991). Role of eicosanoids, inositol phosphates and extracellular Ca^{2+} in cell-volume regulation of rat liver. *Eur. J. Biochem.* **198**, 73–83.

FINCHAM, D. A., WOLOWYK, M. W. AND YOUNG, J. D. (1987). Volume-sensitive taurine transport in fish erythrocytes. *J. Membr. Biol.* **96**, 45–56.

FUGELLI, K. AND REIERSEN, L. O. (1978). Volume regulation in flounder erythrocytes. The effect of osmolality on taurine influx. In *Osmotic and Volume Regulation* (ed. C. Barker Jørgensen and E. Skadhauge), pp. 418–432. Copenhagen: Munksgaard.

FUGELLI, K. AND ROHRS, H. (1980). The effect of Na^+ and osmolality on the influx and steady state distribution of taurine and gamma-aminobutyric acid in flounder (*Platichthys flesus*) erythrocytes. *Comp. Biochem. Physiol.* **67**A, 545–551.

FUGELLI, K. AND THOROED, S. M. (1986). Taurine transport associated with cell volume regulation in flounder erythrocytes under aniso-osmotic conditions. *J. Physiol., Lond.* **374**, 245–261.

FUGELLI, K. AND ZACHARIASSEN, K. E. (1976). The distribution of taurine, gamma-aminobutyric acid and inorganic ions between plasma and erythrocytes in flounder (*Platichthys flesus*) at different plasma osmolalities. *Comp. Biochem. Physiol.* **55**A, 173–177.

GUIZOUARN, H., BORGESE, F., PELLISSIER, B., GARCIA-ROMEU, F. AND MOTAIS, R. (1993). Role of protein phosphorylation and dephosphorylation in activation and desensitization of the cAMP-dependent Na^+/H^+ antiport. *J. biol. Chem.* **268**, 8632–8639.

HAAS, M. (1989). Properties and diversity of (Na–K–Cl) cotransporters. *A. Rev. Physiol.* **51**, 443–457.

HOFFMANN, E. K. AND KOLB, H.-A. (1991). Mechanisms of activation of regulatory volume responses after cell swelling. *Adv. comp. env. Physiol.* **9**, 140–185.

HOFFMANN, E. K., LAMBERT, I. H. AND SIMONSEN, L. O. (1988). Mechanisms in volume regulation in Ehrlich ascites tumor cells. *Renal Physiol. Biochem.* **11**, 221–247.

HOFFMANN, E. K., SIMONSEN, L. O. AND LAMBERT, I. H. (1993). Cell volume regulation: intracellular transmission. *Adv. comp. env. Physiol.* **14**, 187–248.

JENNINGS, M. L. (1989). Structure and function of the red blood cell anion transport protein. *A. Rev. Biophys. biophys. Chem.* **18**, 397–430.

KAISER, G., WIEMER, G., KREMER, G., DIETZ, J. AND PALM, D. (1978). Identification and quantification of β-adrenoceptor sites in red blood cells from rats. *Naunyn-Schmiedeberg's Arch. Pharmac.* **305**, 41–50.

KREGENOW, F. M. (1973). The response of duck erythrocytes to norepinephrine and an elevated extracellular potassium. Volume regulation in isotonic media. *J. gen. Physiol.* **61**, 509–527.

LAMBERT, I. H. (1994). Eicosanoids and cell volume regulation. In *Cellular and Molecular Physiology of Cell Volume Regulation* (ed. K. Strange), pp. 273–292. Boca Raton: CRC Press.

LANDS, A. M., ARNOLD, A., MCAULIFF, J. P., LUDUENA, F. P. AND BROWN, T. G., JR (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature* **214**, 597–598.

LEITE, M. V. AND GOLDSTEIN, L. (1987). Ca^{2+} ionophore and phorbol ester stimulate taurine efflux from skate erythrocytes. *J. exp. Zool.* **242**, 95–97.

MADELIAN, V., MARTIN, D. L., LEPORE, R., PERRONE, M. AND SHAIN, W. (1985). β-receptor-stimulated and cyclic adenosine 3′,5′-monophosphate-mediated taurine release from LRM55 glial cells. *J. Neurosci.* **5**, 3154–3160.

MAHÉ, Y., GARCIA-ROMEU, F. AND MOTAIS, R. (1985). Inhibition by amiloride of both adenylate cyclase activity and the Na^+/H^+ antiporter in fish erythrocytes. *Eur. J. Pharmac.* **116**, 199–206.

MARGALIT, A., SOFER, Y., GROSSMAN, S., REYNAUD, D., PACE-ASCIAK, C. R. AND LIVNE, A. A. (1993). Hepoxilin A_3 is the endogenous lipid mediator opposing hypotonic swelling of human intact platelets. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2589–2592.

MARTIN, D. L., MADELIAN, V. AND SHAIN, W. (1990). Osmotic sensitivity of isoproterenol- and high-$[K^+]_o$-stimulated taurine release by cultured astroglia. In *Taurine: Functional Neurochemistry, Physiology and Cardiology* (ed. H. Pasantes-Morales, D. L. Martin, W. Shain and R. M. Del Rio), pp. 349–356. New York: Wiley-Liss.

MCCARTY, N. A. AND O'NEIL, R. G. (1992). Calcium signaling in cell volume regulation. *Physiol. Rev.* **72**, 1037–1061.

MINNEMAN, K. P., HEDBERG, A. AND MOLINOFF, P. B. (1979). Comparison of *beta* adrenergic receptor subtypes in mammalian tissues. *J. Pharmac. exp. Ther.* **211**, 502–508.

MOTAIS, R., GUIZOUARN, H. AND GARCIA-ROMEU, F. (1991). Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. *Biochim. biophys. Acta* **1075**, 169–180.

MUKHERJEE, C., CARON, M. G., COVERSTONE, M. AND LEFKOWITZ, R. J. (1975). Identification of adenylate cyclase-coupled β-adrenergic receptors in frog erythrocytes with (−)-[^{3}H]alprenolol. *J. biol. Chem.* **250**, 4869–4876.

NIKINMAA, M. (1982). Effects of adrenaline on red cell volume and concentration gradient of protons across the red cell membrane in the rainbow trout, *Salmo gairdneri*. *Molec. Physiol.* **2**, 287–297.

NIKINMAA, M. AND HUESTIS, W. H. (1984). Adrenergic swelling of nucleated erythrocytes: cellular mechanisms in a bird, domestic goose and two teleosts, striped bass and rainbow trout. *J. exp. Biol.* **113**, 215–224.

PARKER, J. C. (1993). In defense of cell volume? *Am. J. Physiol.* **265**, C1191–C1200.

PASANTES-MORALES, H. AND SCHOUSBOE, A. (1988). Volume regulation in astrocytes: a role for taurine as an osmoeffector. *J. Neurosci. Res.* **20**, 505–509.

SAGER, G. (1983). β-2 adrenergic receptors on intact human erythrocytes. *Biochem. Pharmac.* **32**, 1946–1949.

SALAMA, A. AND NIKINMAA, M. (1990). Effect of oxygen tension on catecholamine-induced formation of cAMP and on swelling of carp red blood cells. *Am. J. Physiol.* **259**, C723–C726.

SEAMON, K. B. AND DALY, J. W. (1981). Forskolin: a unique diterpene activator of cyclic AMP-generating systems. *J. cyclic Nucleotide Res.* **7**, 201–224.

SIMCHOWITZ, L. AND CRAGOE, E. J., JR (1986). Inhibition of chemotactic factor-activated Na^+/H^+ exchange in human neutrophils by analogues of amiloride: structure–activity relationships in the amiloride series. *Molec. Pharmac.* **30**, 112–120.

STENE-LARSEN, G. (1981). Comparative aspects of cardiac adrenoceptors: characterization of the β_2-adrenoceptor as a common 'adrenaline'-receptor in vertebrate hearts. *Comp. Biochem. Physiol.* **70**C, 1–12.

TETENS, V., LYKKEBOE, G. AND CHRISTENSEN, N. J. (1988). Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **134**, 267–280.

THOROED, S. M. AND FUGELLI, K. (1993). Characterization of the Na^+-dependent taurine influx in flounder erythrocytes *J. comp. Physiol.* B **163**, 307–316.

THOROED, S. M. AND FUGELLI, K. (1994*a*). Free amino compounds and cell volume regulation in erythrocytes from different marine fish species under hyposmotic conditions: the role of a taurine channel. *J. comp. Physiol.* B **164**, 1–10.

THOROED, S. M. AND FUGELLI, K. (1994*b*). The Na^+-independent

taurine influx in flounder erythrocytes and its association with the volume regulatory taurine efflux. *J. exp. Biol.* **186**, 245–268.

THOROED, S. M. AND FUGELLI, K. (1994*c*). The role of leukotriene D_4 in the activation of the osmolality-sensitive taurine channel in erythrocytes from marine fish species. *Acta physiol. scand.* **151**, 27A.

THOROED, S. M., LAMBERT, I. H., HANSEN, H. S. AND HOFFMANN, E. K. (1994). Cell swelling stimulates the Ca^{2+}-sensitive, cytosolic phospholipase A_2 in Ehrlich ascites tumor cells. *Acta physiol. scand.* **151**, 25A.

WHELER, G. H. T. AND KLEIN, D. C. (1979). Cyclic AMP-induced release of [^{14}C]taurine from pinealocytes. *Biochem. biophys. Res. Commun.* **90**, 22–27.

WHELER, G. H. T. AND KLEIN, D. C. (1980). Taurine release from the pineal gland is stimulated *via* a β-adrenergic mechanism. *Brain Res.* **187**, 155–164.