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Amino acid release during volume regulation by cardiac cells: Cellular mechanisms

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Abstract

Mechanisms of amino acid efflux during volume regulation in hypoosmotically treated isolated rat hearts were studied by collecting the coronary artery perfusate and analysis by high pressure liquid chromatography. Hypoosmotic stress resulted in marked percentage increases in perfusate taurine, aspartate and glutamate levels, smaller increases in phosphoethanolamine, glycine and alanine and non-significant increases in serine and glutamine. Amino acid levels declined during reperfusion with isosmotic perfusate. The anion channel blocker 4-acetamido-4′-isothiocyanostilbene-2:2′-disulfonic acid (SITS, 500 μM) significantly reduced hypoosmotic release of taurine, aspartate, glutamate and glycine. Furosemide reduced hypoosmotically-evoked releases of taurine, glycine, alanine and phosphoethanolamine. The polyunsaturated amino acids, arachidonic and linoleic also reduced amino acid efflux. Phospholipase A_2 inhibition with 7,7-dimethyleicosadienoic acid (DEDA, 2 μM) reduced osmotically-evoked releases of taurine, aspartate and glutamate. 4-Bromophenacyl bromide (1 μM) inhibited osmotically-evoked release of glutamate and glycine. Combined applications of SITS + DEDA markedly reduced osmotically evoked release of all eight amino acids. Glutamate and aspartate effluxes were not inhibited by the glutamate transport inhibitor dihydrokainic acid (1 mM). These results indicate that the hypoosmotic stress, by inducing cell swelling, can initiate an amino acid efflux as part of a regulatory volume decrease. An opening of anion-permeant channels and phospholipase activation appear to be involved in the regulatory volume decrease phenomenon. © 1998 Elsevier Science B.V.

Keywords: Hypoosmotic stress; Regulatory volume decrease; Anion channels, stretch activated; Phospholipase; Amino acids; Fatty acid; Taurine; Glutamate

1. Introduction

Animal cells possess an ability to respond to changes in their external osmolarity by rapid and efficient volume regulatory mechanisms (Chamberlin and Strange, 1989), involving transmembrane fluxes of osmotically active solutes. As plasma membranes are relatively freely permeable to water, cells exposed to anisosmotic solutions will rapidly undergo volume changes as a consequence of influxes or outflows of water depending on the relative osmotic strength of the external solution. Cells placed in a hypoosmotic medium will gain water and swell. The increase in cell volume causes cells to exhibit adaptive responses which allow them to lose osmolytes, including inorganic ions (Na^+, K^+ Cl^-) and amino acids, thus restoring cell volume towards its original dimensions. This process has been called a regulatory volume decrease.

Cell swelling frequently occurs during pathophysiological situations, such as anoxia or ischemia, in which as a consequence of metabolic failure cells lose K^+, are depolarized, and then gain Na^+ and Cl^- together with osmotically obligate water. Regulatory volume decrease may be an important protective mechanism in that it preserves cells from lysis.

The α-amino acids glutamate, aspartate, glutamine and alanine, together with the β-amino acid taurine, account for a very significant proportion (67%; DuRuisseau et al., 1957; Pisarenko et al., 1983; Weisner et al., 1988; Chapman et al., 1993) of the free amino acid pool in the rat heart. Glutamate, aspartate and taurine, unlike the other amino acids in the rat heart, are present at substantially higher concentrations than in the plasma (Chapman et al., 1993; Llovera et al., 1993; Reichel et al., 1995) with ratios for glutamate, aspartate and taurine of 200:1, 140:1 and 1300:1, respectively.

The concentration of taurine in the rat heart is particularly high, amounting to circa 46% of the total free amino

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acid pool (DuRuisseau et al., 1957) even though taurine is not incorporated into proteins and is only slowly metabolized (Huxtable, 1992). Although taurine does not presently have a well defined physiological role in the heart it is clearly necessary for normal cardiac function, as its depletion is associated with the development of cardiomyopathy and can adversely affect the outcome of myocardial ischemia (Kramer et al., 1981; Pion et al., 1989). Cardiac levels of taurine decrease during hypoosmotic, and increase in hyperosmotic, stress leading to the suggestion that its primary function may be to serve an osmoregulatory function in the mammalian heart (Crass and Lombardini, 1978; Thurston et al., 1981; Atlas et al., 1984; Huxtable, 1992).

Glutamate and aspartate, which are lost from ischemic and anoxic rat heart cells (Song et al., 1996), are substrates for a variety of metabolic reactions (Pisarenko et al., 1983). These amino acids are depleted in human hearts subjected to cardioplegic arrest followed by reperfusion (Suleiman et al., 1997) and in cultured chick heart cells during volume regulation in hypoosmotic solutions (Rasmusson et al., 1993). Recognition of their importance has led to the development of glutamate- and/or aspartate-enriched cardioplegic solutions for myocardial preservation during heart transplantation and coronary bypass surgery (Pisarenko et al., 1995; Rosenkranz, 1995; Svedjeholm et al., 1995).

The potential significance of amino acid osmolyte fluxes for volume regulation during anoxic/ischemic stress and the longer term effects of the depletion of these compounds for myocyte recovery during reperfusion warranted a further study of the mechanisms that result in amino acid efflux. As cell swelling, which occurs during anoxic/ischemic stress, can be replicated with less damaging consequences by exposing cells to a hypoosmotic environment, we have adopted this technique to study the mechanisms underlying swelling-induced amino acid efflux from cells in the isolated perfused rat heart.

2. Materials and methods

Male Sprague–Dawley rats (250–300 g) were anesthetized with intraperitoneal injections of pentobarbital sodium (50 mg/kg). Heparin (1000 IU/kg) was administered via a femoral vein to prevent the possible formation of microemboli during excision and cannulation. The heart was then rapidly excised, suspended in a water jacket at 38°C, and perfused via retrograde cannulation of the aorta. Perfusion of the isolated heart with Krebs–Henseleit bicarbonate buffer (KHB) was carried out essentially as described by DeLeiris et al. (1984) using a constant pressure (75 cm H_2O) perfusion system. The KHB (mM concentration: NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.9; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.0), was equilibrated with a gas mixture of 95% O_2 + 5% CO_2 and maintained at 37°C. Heart rate and intra-aortic pressures were recorded on a Grass polygraph via a pressure transducer. Coronary flow rate was recorded with a calibrated drop counter placed beneath the waterjacket.

Hearts were perfused for 20 min to allow for stabilization at a flow rate of circa 5 ml/min. Two basal cardiac perfusate samples were collected at 5 min intervals. Flow to the hearts was then switched for 5 min to a reservoir containing a hypoosmotic perfusate in which the NaCl concentration had been reduced to 70 mM. Perfusate samples were collected at 2.5 and 5 min, after which perfusion with regular KHB was initiated and two more perfusate samples were collected at 5 min intervals. The cardiac perfusate samples were centrifuged at $1200 \times g$ and stored at −20°C. High pressure liquid chromatography (HPLC) assays for perfusate amino acid contents were conducted within a few hours using previously published procedures (Phillis et al., 1994).

Results with four groups of drugs are presented in this report. The procedures for the drug treatment groups were the same as for the control group, except in that drugs were administered in the perfusate for 20 min prior to the collection of the initial two basal samples. The heart was then exposed to hypoosmotic perfusate containing the appropriate substance. Drugs were perfused for the rest of the experiment, in the regular KHB with collection of perfusate samples as described above. The drug groups tested included: (1) the anion channel blockers; 4-acetamido-4′-isothiocyanostilbene-2:2′-disulfonic acid (SITS; Sigma, 500 μM); furosemide (Sigma, 500 μM); (2) the phospholipase inhibitors; 4-bromophenacyl bromide (Sigma, 1 μM); 7,7-dimethyleicosadienoic acid (DEDA, Biomol Research Laboratories, 2 and 20 μM); (3) fatty acid derivatives; arachidonic acid (Sigma, 1 μM); linoleic acid (Sigma, 1 μM); stearic acid (Sigma, 1 μM); oleic acid (Sigma, 1 μM); and (4) the glutamate transport inhibitor dihydrokainic acid (Sigma, 1 mM). A co-eluting factor in the SITS preparation prevented measurements of alanine in the cardiac perfusates. Fatty acids and DEDA were initially dissolved in dimethylsulfoxide (DMSO), which was subsequently diluted to a final concentration of 0.01% v/v in the KHB. At this concentration DMSO did not affect amino acid release.

Statistical differences between amino acid releases from control and drug-treated hearts were analyzed by analysis of variance (ANOVA) and Scheffe's test with contrasts between the control group and each treatment group (SPSS statistical package). A probability of < 0.05 was accepted as denoting a significant difference.

3. Results

Basal heart rates for the control preparations were 239 ± 4.3 (S.E.M.) beats/min. Exposure to the hypoosmotic solution caused a reduction in the frequency (173 ± 8), but

Table 1
Perfusate levels of amino acids released by the heart (nmol/l ± S.E.M.)

	Basal	Anisosmotic	
Aspartate	225 ± 47.5	2241 ± 272[a]	(996%)
Glutamate	272 ± 25	2773 ± 317[a]	(1019%)
Serine	425 ± 53	722 ± 81	(169%)
Phosphoethanolamine	475 ± 26	1260 ± 161[a]	(265%)
Taurine	1273 ± 152	16254 ± 2559[a]	(1276%)
Glycine	2067 ± 36	3714 ± 223[a]	(180%)
Alanine	3760 ± 191	8177 ± 56204[a]	(217%)
Glutamine	7506 ± 293	9282 ± 1058	(124%)

Amino acids in rat cardiac perfusates. Values are from 5 hearts perfused with oxygenated KHB. Anisosmotic perfusate levels were recorded after 5 min of exposure to a KHB solution in which NaCl had been reduced to 70 mM.
[a] $P < 0.01$.

with little change in the strength of contraction. Heart rates recovered during reperfusion with regular KHB (235 ± 4.5). Perfusate flow rates fell from 5.48 ± 0.1 ml/min to 4.79 ± 0.1 ml/min during the period of exposure to the anisosmotic perfusate and returned to control levels after reperfusion with regular KHB.

Basal levels of amino acids in the cardiac perfusates are presented in Table 1. The levels of glutamate, aspartate, serine and phosphoethanolamine were the lowest, with taurine, glycine, alanine, and glutamine present at increasingly higher concentrations. Osmotic stress resulted in pronounced percentage increases in aspartate, glutamate and taurine perfusate levels and less, but still significant, increases in phosphoethanolamine, glycine and alanine levels. Serine and glutamine release was non-significantly elevated. Following a return to normal KHB, amino acid release initially declined rapidly, but required 20–30 min to return to basal levels.

3.1. Effects of anion channel blockers

Blockers of anion transport inhibit volume regulation and efflux of osmoregulatory amino acids in cultured astrocytes, glioma cells and brain slices (Kimelberg et al., 1990; Jackson and Strange, 1993; Sanchez-Olea et al., 1993; Law, 1994) suggesting that the diffusional pathway for amino acids may be an anion channel (Pasantes-Morales, 1996). The effects of the anion channel blockers SITS (500 μM; Jackson and Strange, 1993; Leaney et al., 1997; Okada, 1997) and furosemide (500 μM; Kimelberg et al., 1990; Okada, 1997) were therefore ascertained on hypoosmotically-evoked amino acid release from the rat

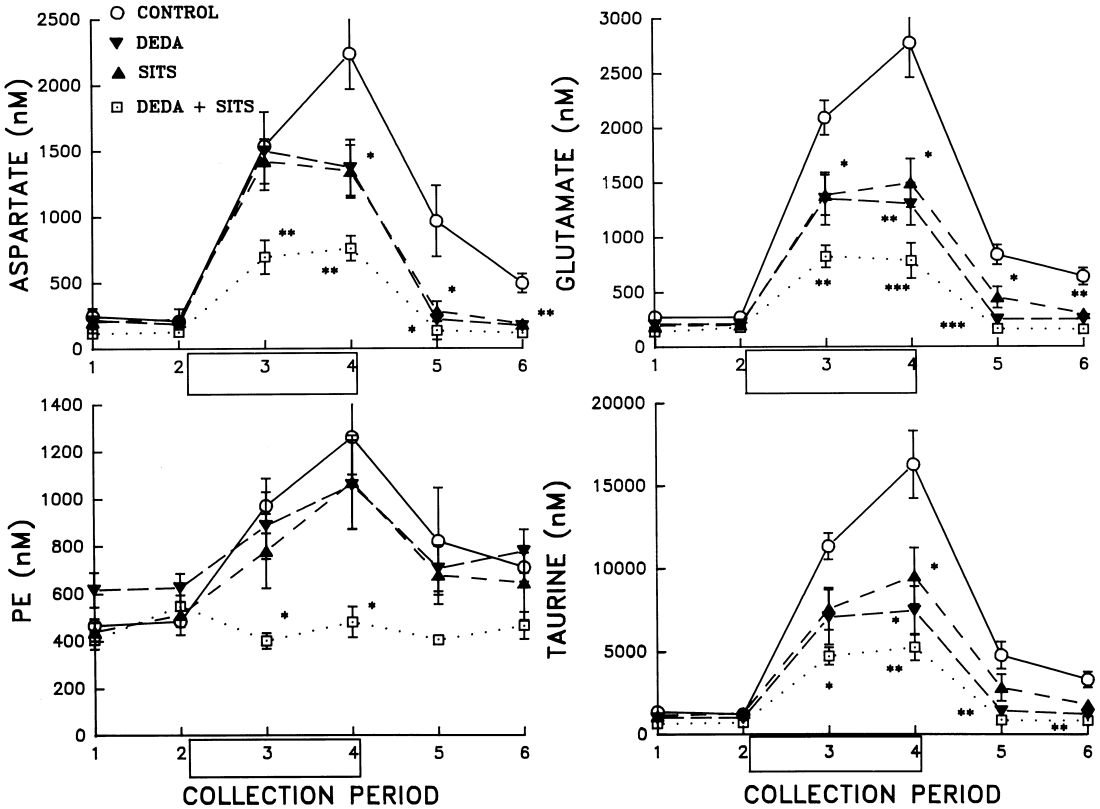

Fig. 1. Amino acids in the coronary effluent perfusate from isolated rat heart Langendorff preparations exposed to hypoosmotic solutions. Line plots show the time course of changes in amino acid levels before, during □, and after exposure to KHB containing 70 mM NaCl for a period of 5 min. Perfusates were collected at 5 min intervals prior to and after hypoosmotic stress, and at 2.5 min intervals during the anisosmotic challenge. ○, control hearts; ▼, hearts perfused with DEDA (2 μM) for 20 min prior to sample collection; ▲, hearts perfused with SITS (500 μM) prior to sample collection; □, hearts perfused with DEDA + SITS. Data are presented as means ± S.E.M. Statistically significant differences in amino acid levels from control values in each sample were determined by ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

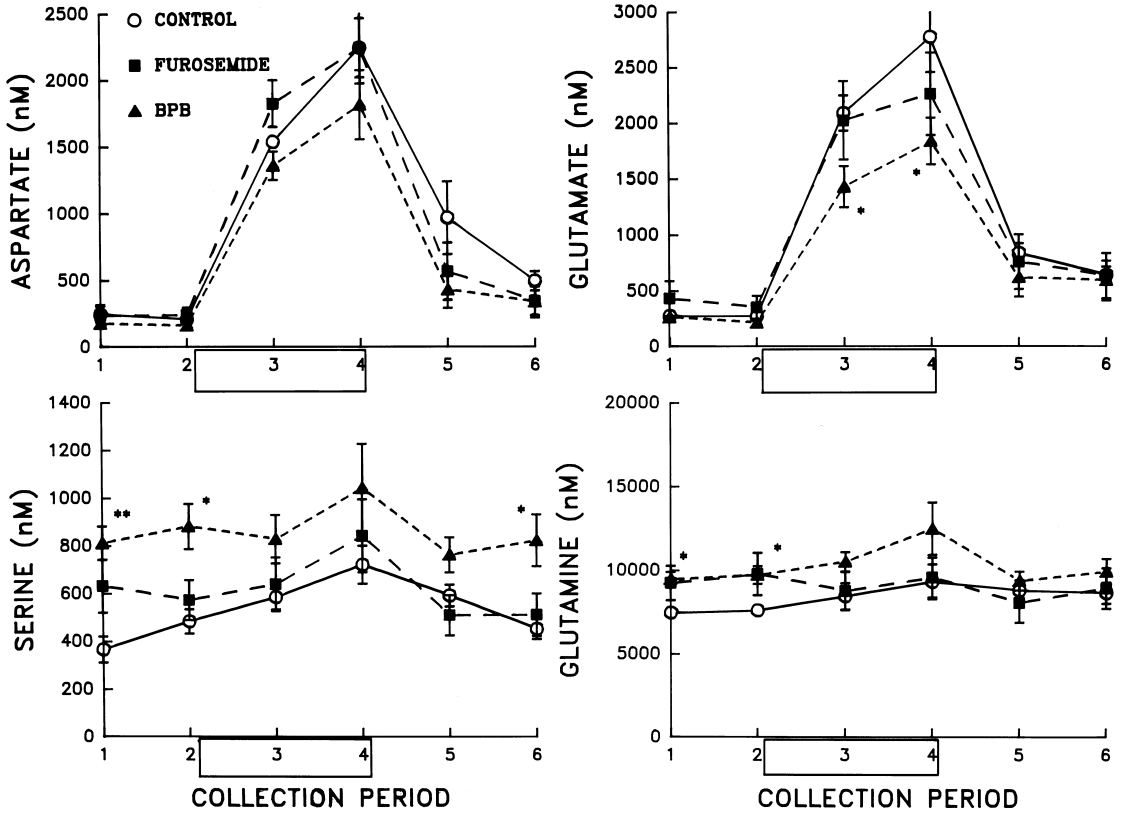

Fig. 2. Effects of furosemide (■, 500 μM) and BPB (▲, 1 μM) on coronary perfusate levels of aspartate, glutamate, serine and glutamine before, during, and after exposure of hearts to hypoosmotic KHB. See legend to Fig. 1 for further details.

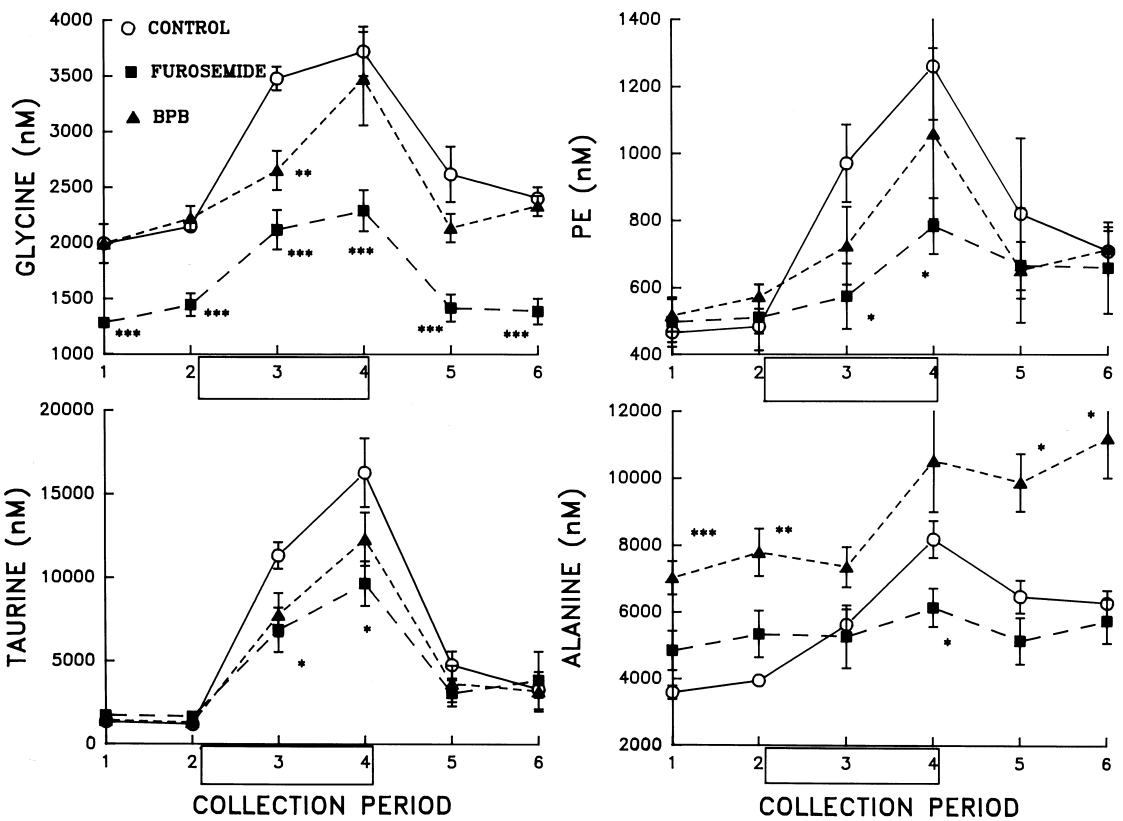

Fig. 3. Effects of furosemide (■, 500 μM) and BPB (▲, 1 μM) on glycine, phosphoethanolamine, taurine and alanine levels in coronary perfusates before, during, and after exposure of hearts to hypoosmotic KHB. See legend to Fig. 1 for further details.

heart. SITS caused significant reductions in the release of aspartate, glutamate, taurine and glycine (Fig. 1). Furosemide (500 μM) significantly reduced the basal release of glycine and the anisomotically evoked efflux of taurine, glycine, alanine and phosphoethanolamine (Figs. 2 and 3). It did not affect the efflux of glutamate and aspartate.

3.2. Phospholipase inhibition

Phospholipase A_2 and C inhibitors depress the ischemia-evoked release of aspartate and glutamate from the rat cerebral cortex (Phillis and O'Regan, 1996) and anoxia-evoked release from the isolated perfused rat heart (Phillis et al., 1997). In the present experiments, DEDA (2 μM) (Fig. 1) reduced the release of aspartate, glutamate and taurine from hypoosmotically challenged rat hearts. The basal levels of serine, alanine and glutamine were elevated in the 4-bromophenacyl bromide-treated hearts and it reduced the hypoosmotically evoked efflux of glutamate and glycine (Figs. 2 and 3).

A combined application of DEDA (2 μM) and SITS (500 μM) significantly reduced aspartate, glutamate and taurine release and virtually abolished the efflux of phosphoethanolamine (Fig. 1). The effects of the combined application on aspartate, glutamate and phosphoethanolamine were significantly greater than the individual actions of either DEDA or SITS and the effect of the combined agents on taurine efflux was significantly greater than that of SITS. This result tends to suggest that the two agents may act on disparate mechanisms leading to amino acid efflux from cardiac cells.

3.3. Fatty acids

The polyunsaturated fatty acids arachidonic and linoleic are potent blockers of regulatory volume decrease and amino acid efflux from cultured rat cerebellar astrocytes (Sanchez-Olea et al., 1995). The monounsaturated (oleic) and saturated (stearic) acids were ineffective. Previous studies have shown that arachidonic acid can regulate anion channels (Anderson and Welsh, 1990), indicating that polyunsaturated fatty acids may act on the same diffusional pathways as the anion channel blockers. Arachidonic acid (1 μM; Sanchez-Olea et al., 1995; Okada, 1997) and linoleic acid (1 μM) significantly reduced the hypoosmotically-evoked release of aspartate, glutamate, taurine (Fig. 4) and glycine. Stearic acid (1 μM) enhanced the hypoosmotically-evoked release of phosphoethanolamine, taurine (Fig. 4), glutamine and alanine. Oleic

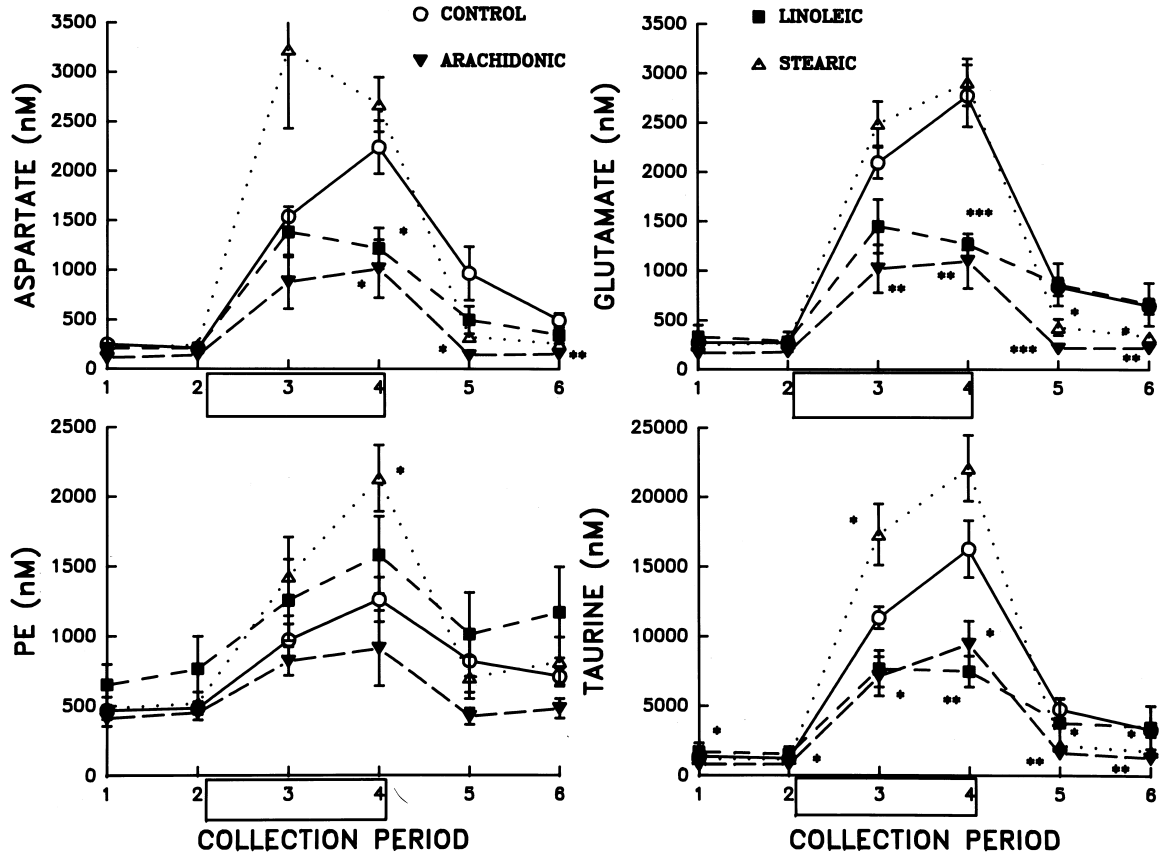

Fig. 4. Effects of fatty acids, arachidonic (▼, 1 μM), linoleic (■, 1 μM), and stearic (△, 1 μM) on efflux of aspartate, glutamate, phosphoethanolamine and taurine from perfused rat hearts exposed to hypoosmotic KHB. See legend to Fig. 1 for further details.

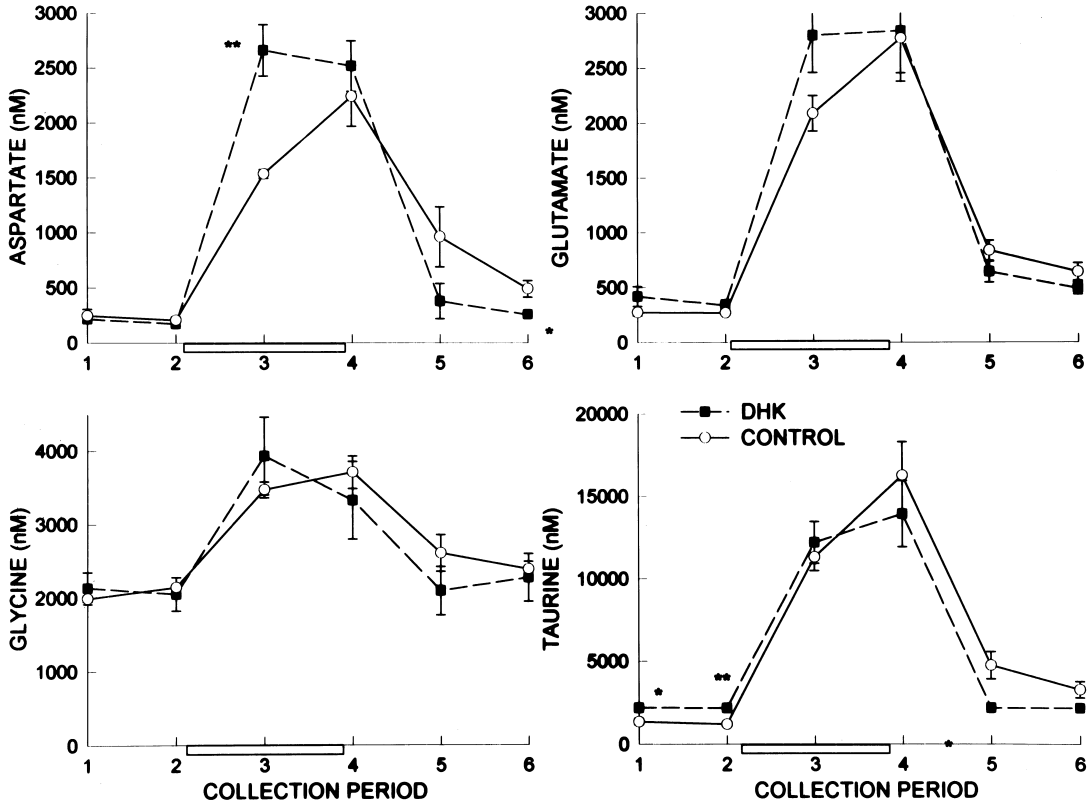

Fig. 5. Lack of effect of dihydrokainic acid (1 mM) on aspartate, glutamate, glycine and taurine levels in coronary perfusates from hypoosmotically challenged rat hearts. See legend to Fig. 1 for further details.

acid (1 μM) elevated the basal levels of all amino acids, except aspartate and taurine, and increased the hypoosmotically-evoked release of all amino acids, except glutamate and taurine.

3.4. Dihydrokainic acid

The non-competitive glutamate transport inhibitor, dihydrokainic acid, was tested for its ability to inhibit hypoosmotically evoked release of glutamate and aspartate. Dihydrokainic acid (1 mM) selectively reduces the anoxia-evoked release of these amino acids from the perfused rat heart (unpublished observations). At this concentration, dihydrokainic acid failed to reduce the hypoosmotically-evoked release of glutamate and aspartate (Fig. 5), indicating that reversal of the glutamate-transporter is unlikely to be involved in this form of amino acid efflux.

4. Discussion

Cardiac ischemia and/or anoxia have recently been demonstrated to precipitate large increases in the efflux of amino acids into the vascular perfusate of isolated rat Langendorff preparations (Song et al., 1996). Elevations in the levels of aspartate, glutamate, glycine, taurine, phosphoethanolamine, serine, alanine, and glutamine were observed, with the percentage increases of aspartate, glutamate and taurine being the most pronounced. A possible contributor to amino acid release from ischemic or anoxic hearts is the process of a regulatory volume decrease by which swollen cardiac cells attempt to regain their normal volume by releasing osmolytes, including taurine, glutamate, aspartate and other amino acids (Rasmusson et al., 1993). A comparable involvement of amino acids in volume regulation by brain cells has recently been reviewed by Pasantes-Morales (1996). Swelling occurs as a result of anoxia-induced depolarization leading to sodium and chloride accumulation together with osmotically obligate water. The regulatory volume decrease response in cardiac cells is rapid with swelling to a peak volume within 3 min of exposure to hypoosmotic solutions (Rasmusson et al., 1993). Return to control solution elicited a rapid shrinkage in these cultured chick heart cells to a volume that was lower than the original. Recovery to the original volume was slow, perhaps due to the loss of solute that was not rapidly replaceable by the cells.

The efflux of amino acids from the rat heart occurred rapidly with large increases in perfusate levels being evident 2.5 min after the onset of hypoosmotic perfusion. Levels had increased even further at 5 min. The initial recovery towards basal levels during reperfusion with regular KHB was also rapid, paralleling the time course of volume recovery in cultured chick heart cells.

The pharmacology of regulatory volume decrease has been intensively studied in brain cells, both in vitro and in vivo. Regulatory volume decrease-induced osmolyte efflux in these cells is sensitive to a variety of membrane channel blockers, especially those affecting Cl^- channels (Kimelberg et al., 1990; Sanchez-Olea et al., 1993; Gonzalez et al., 1995), including polyunsaturated fatty acids such as arachidonic and linoleic acids (Sanchez-Olea et al., 1995). The action of arachidonic acid is not a consequence of its metabolic products (Sanchez-Olea et al., 1995). It has been established that the swelling-induced efflux of taurine and glutamate from glial cells is not due to a reversal of Na^+-dependent transporters, but is, rather via a diffusional mechanism down concentration gradients (Kimelberg et al., 1990; Pasantes-Morales et al., 1994). The failure of the glutamate transport inhibitor dihydrokainic acid to reduce hypoosmotically-evoked glutamate and aspartate efflux observed in the present experiments is consistent with this conclusion. Nor do extracellular calcium ions appear to be required for the regulatory volume decrease response to hypoosmotic stress in cultured neurons (Moran et al., 1997). The current interpretation of these data is that regulatory volume decrease involves a diffusional efflux of several osmolytes, including Na^+, K^+, and amino acids through swelling-activated Cl^- channels. Permeation of glutamate, aspartate and taurine through such swelling activated Cl^- channels has recently been demonstrated in C6 glioma cells (Jackson and Strange, 1993; Roy, 1995). Swelling activated Cl^- channels which can be activated by osmotically induced cell swelling have been identified in both atrial and ventricular myocytes (Harvey, 1996). The swelling activated Cl^- current can be blocked by disulfonic stilbene derivatives (Vandenberg et al., 1994), and these channels may be the diffusional pathway for amino acids.

The attenuation of hypoosmotically-evoked release of amino acids from heart cells by anion channel blockers and polyunsaturated fatty acids described in this paper appears to be remarkably similar to that observed during regulatory volume decrease in brain cells, suggesting that the underlying mechanisms are similar.

Bender et al. (1992, 1993 have examined the role of phospholipases and second messengers in volume homeostasis by astrocytes. Changes in cell volume associated with hypoosmotic swelling result in the rapid hydrolysis of phosphatidylinositol to form cellular inositol phosphates. This response was inhibited by the phospholipase C inhibitor U-73122. Furthermore agents which stimulate phosphatidylinositol hydrolysis significantly accelerated regulatory volume decrease. Regulatory volume decrease is blocked by protein kinase C inhibition or downregulation, whereas protein kinase C activation by phorbol 12-myristate 13-acetate facilitated the swelling induced by hypoosmotic stress (Bender et al., 1992), implicating protein kinase C in promoting the response. Protein kinase C can translocate to the plasma membrane (Cardell et al., 1990) where it could activate phospholipase A_2, thus destabilizing the membrane and promoting an efflux of amino acids by diffusion along their concentration gradients. An involvement of phospholipases in regulatory volume decrease, which is suggested by our observation that DEDA and 4-bromophenacyl bromide depressed hypoosmotically-evoked acid release, is not consistent with previous findings that phospholipase A_2 inhibition failed to inhibit the efflux of inositol during hypoosmotic stress of C6 glioma cells (Strange et al., 1993). Resolution of this discrepancy, admittedly involving different osmolytes and cell types, awaits further investigation.

The possibility that phospholipases are involved in the opening of anion permeable channels, and hence that only one diffusional channel is required to account for the release of amino acids has to be considered. However, DEDA and SITS, at concentrations which maximally depressed amino acid efflux when applied separately, had an additive effect when applied together, suggesting that there may be two independent efflux pathways.

In conclusion, multiple mechanisms may be present for the release of amino acids from cardiac cells during hypoosmotic stress, including diffusional efflux via swelling-activated anion selective channels and as a consequence of membrane permeability changes resulting from phospholipase activation by protein kinase C. Although the RVD phenomenon, triggered by cell swelling during pathophysiological crises (such as cardiac arrest) or during cardiac surgery may initially contribute to the preservation of cellular integrity, it is possible that the loss of amino acids may ultimately jeopardize the ability of cardiac cells to resume metabolic activity and protein synthesis. Replacement of lost amino acids may account for the beneficial effects of adding glutamate and aspartate to cardioplegic solutions (Rosenkranz, 1995; Svedjeholm et al., 1995).

Acknowledgements

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