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European Journal of Pharmacology 351 (1998) 313–322

ejp

Mechanisms of amino acid release from the isolated anoxic/reperfused rat heart

Dekun Song [a], Michael H. O'Regan [b], John W. Phillis [a,*]

[a] *Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201, USA*
[b] *Biomedical Sciences, School of Dentistry, University of Detroit Mercy, 8200 W. Outer Drive, Detroit, MI 48219, USA*

Received 18 December 1997; revised 14 April 1998; accepted 17 April 1998

Abstract

Loss of amino acids into the coronary artery perfusate, which is exacerbated during anoxic stress, may have important implications for the ability of hearts subjected to ischemia or anoxia to recover function during reoxygenation. This work investigates the mechanisms underlying the amino acid efflux. Rat Langendorff heart preparations were used to study amino acid loss into coronary artery perfusates during anoxia or anoxia/reoxygenation sequences. Coronary flow rates, heart rates and intra-aortic pressures were recorded. Changes in myocardial amino acid concentrations were equated with amino acid levels in collected anoxic perfusate. With the exception of taurine, the differences in amino acid levels between normoxic and anoxic hearts were smaller than the amounts lost into the coronary perfusates, indicating ongoing replenishment of most amino acids during the anoxic episode. Fifteen-minute periods of exposure to low oxygen levels ($P0_2$ 18–20 mmHg) resulted in large percentage increases in perfusate amino acid levels which returned slowly towards control levels upon reoxygenation. Anion channel blockers, anthracene-9-carboxylic acid, furosemide, and 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid (SITS), depressed anoxia-elicited increases in amino acid release. Phospholipase inhibition with quinacrine, 4-bromophenacyl bromide and 7,7-dimethyl-eicosadenoic acid (DEDA) depressed the anoxia-evoked release of amino acids. Combined applications of SITS and DEDA exhibited additive effects, virtually abolishing anoxia-evoked release of all the amino acids. The protein kinase C inhibitor, chelerythrine chloride, and the protein tyrosine kinase inhibitors, genistein and lavendustin A, inhibited anoxia-evoked amino acid release. Polyunsaturated fatty acids, arachidonic and linoleic, reduced anoxia-evoked amino acid release whereas monosaturated (oleic) and saturated (stearic) acids were ineffective. The glutamate transport inhibitor, dihydrokainate, depressed anoxia-evoked glutamate and aspartate release. These results suggest that at least three possible mechanisms for the anoxia-evoked amino acid efflux including (a) diffusional release through volume activated anion channels, (b) leakage across myocyte plasma membranes as a consequence of phospholipase activation and (c) reversal of Na^+ dependent high-affinity transporters.

Keywords: Heart; Anoxia; Amino acid; Anion channel blocker; Phospholipase; Fatty acid; Ischemic; Injury; (Rat)

1. Introduction

The α-amino acids glutamate, aspartate, glutamine and alanine, together with the β-amino acid taurine, constitute a very significant proportion (67%) of the free amino acid pool in the rat heart (DuRuisseau et al., 1957; Pisarenko et al., 1983; Weisner et al., 1988; Chapman et al., 1993). Glutamate, aspartate and taurine, unlike other amino acids in the heart, are present at substantially higher concentrations than in the plasma (Llovera et al., 1993; Reichel et al., 1995; Suleiman et al., 1997). The concentration gradients (intracellular vs. plasma) for glutamate ($\times$83), aspartate ($\times$120) and taurine ($\times$93) in human hearts are similarly greater than those for other amino acids, including alanine ($\times$7), valine ($\times$1.2) and leucine ($\times$1.8) (Chapman et al., 1993; Suleiman et al., 1997).

Aspartate and glutamate play an important metabolic role in the ischemic myocardium. Deamination of glutamate to α-ketoglutarate provides an alternative pathway for entry of substrate into the tricarboxylic acid cycle. Aspartate can also enter the tricarboxylic acid cycle via its conversion to oxaloacetate to yield adenosine triphosphate and NADH (Pisarenko et al., 1983). Consequently, investi-

* Corresponding author. Tel.: +1-313-577-6745; fax: +1-313-577-5494; e-mail: jphillis@med.wayne.edu

0014-2999/98/$19.00

gations into the availability and metabolism of these two dicarboxylic amino acids has burgeoned in recent years, as a potential means of providing energy-yielding substrates during periods of myocardial ischemia and reoxygenation, such as those encountered during heart transplantation and coronary bypass surgery. Recognition of their importance has led to the development of glutamate- and/or aspartate-enriched cardioplegic solutions for myocardial protection in such cardiac procedures (Rosenkranz et al., 1984; Pisarenko et al., 1995; Svedjeholm et al., 1995).

Glutamate and aspartate levels are depleted in human hearts subjected to cardioplegic arrest followed by reoxygenation (Suleiman et al., 1997). Although such reductions in amino acid concentration have been primarily attributed to metabolism, they might also be partly due to loss into the extracellular space. Increases in perfusate levels of glutamate and aspartate have, in fact, recently been observed in isolated rat Langendorff heart preparations exposed to anoxia or ischemia (Song et al., 1996).

The concentration of taurine in mammalian hearts is particularly high, amounting to about 50–60% of the total free amino acid pool, although taurine is not incorporated into proteins and is metabolized slowly (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 (Kramer et al., 1981) and can adversely affect the outcome of myocardial ischemia (Pion et al., 1989). Taurine is released into perfusates from isolated anoxic or ischemic hearts (Crass and Lombardini, 1978; Song et al., 1996). Cardiac levels of taurine decrease during hypoosmotic, and increase in hyperosmotic, stress leading to the suggestion that taurine may function as an osmoregulator in the mammalian heart (Thurston et al., 1981; Huxtable, 1992).

The potential importance of transmembrane fluxes and loss of the metabolically and osmoregulatory significant amino acids, glutamate, aspartate, and taurine, from arrested hearts warranted a further study of the mechanisms that result in depletion of intracellular amino acids. In this paper, we describe the effects of anoxia on endogenous amino acid loss from an isolated rat Langendorff heart preparation. Anoxic stress was used in preference to ischemia as it allows amino acid efflux to be studied during the actual period of exposure to oxygen-deprivation, rather than at the onset of reoxygenation (an inevitable consequence of ischemic, no-flow, conditions). Experimental objectives included a comparison of amino acid levels in freshly excised hearts vs. those perfused for 20 min with anoxic buffer, as well as an evaluation of the roles that second messenger systems, phospholipases, anion channels, and amino acid transporters may play in the anoxia-induced efflux of amino acids from cardiac cells. A preliminary communication on these experiments was presented at the recent International Union of Physiological Sciences meeting in St. Petersburg (Phillis et al., 1997a).

2. Materials and methods

The experiments fell into two groups. In group I, amino acid levels were measured in freshly excised hearts ($n = 7$) and compared with those in hearts ($n = 8$) perfused by the Langendorff technique with anoxic (pO_2 18–20 mmHg) Krebs–Henseleit buffer (KHB) for 20 min. Perfusate from these hearts was collected and assayed to measure amino acid loss during anoxia. Group II involved a study of the effects of five groups of pharmacological agents on amino acid release into coronary perfusates during a 15-min period of anoxia followed by reoxygenation.

All of the rats (Sprague–Dawley, 250–300 g) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Heparin (1000 IU/kg) was administered via a femoral vein to prevent the formation of microemboli following excision.

The Langendorff hearts were prepared by aortic cannulation, suspended in a water jacket at 38°C, and perfused retrogradely via the aorta. Perfusion with Krebs–Henseleit bicarbonate buffer (KHB) was carried out using a constant pressure (75 cmH_2O) perfusion system. The KHB (in mM concentrations): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.9; $MgSO_4$, 1.2; K H_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.0, was equilibrated with a gas mixture of 95% O_2 + 5% CO_2 (pH 7.4 ± 0.01) and maintained at 37°C. Anoxia-inducing KHB was prepared by gassing with 95% nitrogen, 5% carbon dioxide for several hours. Heart rate and intra-aortic pressure changes were recorded on a Grass polygraph via a pressure transducer attached to a sidearm of the aortic cannula. Coronary flow rate was recorded with a calibrated drop counter placed beneath the waterjacket.

2.1. Group I experiments

Cardiac amino acid levels were analyzed either immediately following excision or following 20 min of perfusion with oxygen-depleted KHB. All of the 20 min perfusate was also collected for amino acid analysis. Hearts were chilled in cold KHB, blotted and rapidly weighed. A left ventricular sample of approximately 100 mg was sectioned from the apex of each heart (either immediately after removal from the chest or following 20 min of perfusion with oxygen-depleted KHB). Each ventricular sample was placed in a chilled glass homogenizer with 1 ml KHB and pulverized by 15 strokes. After centrifugation ($8000 \times g$, 20 min) the supernatant was removed. One milliliter of 0.2 M perchloric acid was added to the supernatant which was recentrifuged ($8000 \times g$, 20 min). The supernatant was neutralized to pH 7.3 by the addition of 2 ml of 0.5 M $NaHCO_3$. After further centrifugation ($3000 \times g$, 10 min), supernatant amino acid content was determined by high-pressure liquid chromatography (HPLC) using previously published procedures (Song et al., 1996). Coronary perfusate amino acid concentrations were also measured by HPLC assay.

2.2. Group II experiments

Hearts to be used in the pharmacological studies were perfused for 20 min during which four basal cardiac coronary effluent perfusate samples were collected at 5-min intervals. Flow to the hearts was then switched to a reservoir containing oxygen-depleted KHB. Perfusate samples were collected at 5, 10 and 15 min, after which, the flow of normal KHB was resumed. Further samples were collected at 2, 5, 10, 20, 30 and 40 min. Collected cardiac perfusate samples were centrifuged at $1200 \times g$ and stored at -20°C until assayed for amino acid content.

Results with five groups of drugs are presented. For the drug treatment groups, the procedures were the same as described above for the control anoxia group, except in that drugs were administered in the perfusate after two basal samples had been collected. After a 15-min equilibration interval, two further perfusate samples were collected and the heart was then exposed to the anoxic challenge. Drugs were perfused for the rest of the experiment, with collection of perfusate samples at intervals as described above. The drug groups tested included: (1) anion channel blockers; 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid (SITS; Sigma, 500 μM); anthracene-9-carboxylic acid (ACA, Sigma, 500 μM); furosemide (Sigma, 500 mM); (2) phospholipase inhibitors; quinacrine (Sigma, 10 μM); 4-bromophenacyl bromide (BPB, Sigma, 1 μM); 7,7-dimethyleicosadienoic acid (DEDA; Biomol Research Laboratories, 2 μM); (3) a protein kinase C inhibitor, chelerythrine chloride (Sigma, 5 μM), and protein tyrosine kinase inhibitors, genistein (Sigma, 1 μM) and lavendustin A (Sigma, 0.5 μM); (4) fatty acid derivatives; arachidonic acid (Sigma, 1 μM); linoleic acid (Sigma, 1 μM); stearic acid (Sigma, 1 μM); oleic acid (Sigma, 1 μM) and (5) a glutamate transport inhibitor, dihydrokainic acid (Sigma, 1 mM).

Fatty acids, DEDA and lavendustin A 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. The presence of a co-eluting factor in the anthracene-9-carboxylic acid, SITS and chelerythrine preparations prevented measurements of alanine in these perfusates.

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.

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University Animal Investigation Committee.

3. Results

Basal heart rate for the Langendorff preparations was 221 ± 27 beats/min. A 15-min exposure to anoxic conditions was associated with a cessation or marked reduction

Table 1
Heart rates and incidence of post-anoxic fibrillation of isolated rat hearts exposed to 15 min of oxygen-depleted KHB

	No. of hearts	Basal (beats/min)	Anoxic for 15 min (beats/min)	Reoxygenation for 20 min (beats/min)	Fibrillation (%)
Control	7	220 ± 27	100 ± 17 ($n = 4$)[c]	112 ± 22 ($n = 3$)[d]	100
Anthracene-9-Carboxamide	5	219 ± 18	119 ± 30	197 ± 15[b]	0
Furosemide	4	234 ± 19	136 ± 37	191 ± 20[b]	25
SITS	4	230 ± 18	153 ± 14[a]	193 ± 21[b]	0
Quinacrine	7	209 ± 15	163 ± 36[a] ($n = 6$)	186 ± 41[a]	0
Bromophenacyl bromide	8	232 ± 24	101 ± 12 ($n = 6$)	172 ± 32[a]	37.5
DEDA 20 μM	5	231 ± 16	176 ± 31[b]	201 ± 26[b]	0
DEDA 2 μM	5	236 ± 18	174 ± 20[b]	217 ± 17[b]	0
DEDA + SITS	5	226 ± 9	186 ± 11[b]	209 ± 26[b]	0
Chelerythrine	7	226 ± 21	125 ± 18	197 ± 34[b]	28.6
Lavendustin A	5	237 ± 16	147 ± 47	210 ± 24[b]	20
Genistein	6	238 ± 20	132 ± 35	222 ± 26[b]	33.3
Arachidonic acid	5	237 ± 10	145 ± 37 ($n = 4$)	189 ± 23[b]	20
Linoleic acid	4	239 ± 25	162 ± 54	175 ± 49[a]	0
Stearic acid	5	242 ± 17	104 ± 25 ($n = 2$)	154 ± 30[a]	80
Oleic acid	5	229 ± 21	97 ± 20 ($n = 2$)	156 ± 24[a]	80
Dihydrokainate	6	233 ± 13	99 ± 11	197 ± 31[b]	16.7

Mean ± S.D.
Heart rates for drug-treated preparations compared with control group.
[a] $P < 0.05$.
[b] $P < 0.01$. n values after beat frequencies indicate the number of hearts which continued to beat during anoxia and following reoxygenation.
[c] 3 hearts had ceased to beat.
[d] 4 hearts continued to fibrillate.

in the frequency and strength of contractions and an increase in coronary flow rates. Reoxygenation was accompanied by a recovery of contractile activity, albeit not as strongly as before. All of the control hearts initially developed ventricular fibrillation during reoxygenation with subsequent recovery of 3 hearts (see Table 1). Perfusate flow rates increased during anoxia from a basal rate of 4.37 ± 0.17 (S.E.M.) ml/min to 5.95 ± 0.83 ml/min. During reoxygenation the flow rate declined to 4.67 ± 0.15 ml/min at 20 min and 3.76 ± 0.12 ml/min after 40 min.

3.1. Group I. Myocardial amino acid content

Myocardial levels of amino acids in freshly excised left ventricle were (nmol/g wet weight); aspartate, 261 ± 26.8; glutamate, 749 ± 13.9; serine, 102 ± 20.3; glutamine, 981 ± 70.3; glycine, 312 ± 102.7; phosphoethanolamine, 83 ± 9.3; taurine, 4217 ± 229.6; alanine, 260 ± 25. After 20 min of perfusion with oxygen-depleted KHB the levels of most amino acids in the heart were reduced; the exceptions being aspartate, which was non-significantly elevated, and serine-unchanged (Table 2). Only the reductions of taurine and glutamine achieved significance. Amino acid loss into the coronary perfusates is shown in Table 2, where the figures in parentheses represent the percentage of the original heart content recovered in the perfusate.

The amounts of amino acid recovered in the coronary perfusates exceeded the loss of tissue amino acids, taurine being the only compound with near parity of the two values.

3.2. Group II. Anoxia and amino acid levels in cardiac perfusates

Basal levels of amino acids in cardiac perfusates are presented in Table 3. The levels of glutamate, aspartate and phosphoethanolamine were the lowest, with serine, taurine, glycine, alanine, and glutamine being present at increasingly higher concentrations. Anoxic stress resulted in pronounced increases in glutamate, aspartate and taurine levels in cardiac perfusates, with less marked increases in phosphoethanolamine, and smaller increases in the other amino acids (Table 3). Perfusate levels of most amino acids returned slowly towards control levels during the 40 min of reoxygenation. However, aspartate and taurine levels continued to increase during the first (taurine) or first two (aspartate) collections prior to declining.

Table 3
Perfusate levels of amino acids released by the heart (nmol/l ± S.E.M.)

	Basal	Anoxia	Anoxia/Basal ×100
Aspartate	231 ± 15	2530 ± 390	(1095%)
Glutamate	291 ± 18	6536 ± 1057	(2246%)
Glycine	2204 ± 185	5236 ± 823	(238%)
Phosphoethanolamine	483 ± 54	3058 ± 323	(633%)
Serine	713 ± 56	2127 ± 340	(298%)
Alanine	3916 ± 524	16 432 ± 2392	(419%)
Taurine	1279 ± 85	12 803 ± 2226	(1001%)
Glutamine	10 343 ± 805	27 640 ± 4064	(267%)

Amino acids in rat cardiac perfusates.
Basal value (nmol/l) are from 24 hearts perfused with oxygenated KHB. Anoxia represent perfusate levels immediately prior to reoxygenation in 11 nondrug-treated hearts.
Aspartate and taurine levels were even higher in the initial reoxygenation perfusates reaching maximum levels of 3966 ± 792 and 17 329 ± 4620 nM, respectively.

3.3. Effects of anion channel blockers on perfusate amino acids

The effects of the ion channel blockers anthracene-9-carboxylic acid (ACA), 4-acetamido-isothiocyanostilbene-

Table 2
Amino acid contents of rat hearts before and after exposure to 20 min of anoxia compared to amino acid content of coronary perfusate collected during the period of anoxia

Amino acid	Pre-anoxia	Post-anoxia	Difference	Perfusate
Aspartate	282 ± 29	320 ± 15 (+13%)[d]	+38	241 ± 31 (85%)[e]
Glutamate	809 ± 94	672 ± 40 (−17%)	−137	421 ± 63 (58%)
Serine	110 ± 22	110 ± 10 (9)	0	158 ± 15 (143%)
Glutamine	1059 ± 76	828 ± 44 (−22%)[a]	−231	1949 ± 169 (184%)
Glycine	337 ± 111	205 ± 13 (−39%)	−132	352 ± 27 (104%)
Phosphoethanolamine	90 ± 10	82 ± 8 (−9%)	−8	242 ± 18 (269%)
Taurine	4884 ± 248	3237 ± 83 (−34%)[c]	−1647	1700 ± 140 (35%)
Alanine	281 ± 27	227 ± 14 (−19%)	−54	756 ± 115 (269%)

Values for whole hearts are based on estimates for samples of ventricular tissue adjusted for weight of intact heart.
Mean wet weights for pre-anoxic hearts was 1.08 ± 0.03 g: for anoxic hearts 1.26 ± 0.03 g reflecting tissue swelling during the period of anoxia.
Significant reductions in tissue amino acid levels.
[a] $P < 0.05$.
[c] $P < 0.001$.
[d] The numbers in parentheses represent percentage changes from control, pre-anoxic, values.
[e] Numbers in parentheses represent percentage of original content recovered in perfusate, indicating de novo formation of free amino acid during anoxia.
The values are expressed as nmol/heart ± S.E.M.

Table 4
Effects of pharmacological agents on the anoxia-evoked release of amino acids (μM; mean $\pm$ S.E.M.)

	Aspartate	Glutamate	Glycine	PE	Serine	Alanine	Taurine	Glutamine
Control	2.53 $\pm$ 0.39	6.54 $\pm$ 1.06	5.24 $\pm$ 0.82	3.06 $\pm$ 0.32	2.13 $\pm$ 0.34	16.43 $\pm$ 2.39	12.80 $\pm$ 2.23	27.64 $\pm$ 4.06
ACA	0.49 $\pm$ 0.12[b]	1.51 $\pm$ 0.4[a]	2.91 $\pm$ 0.39	1.23 $\pm$ 0.23[b]	0.9 $\pm$ 0.15[b]		6.07 $\pm$ 1.93	6.92 $\pm$ 0.99[a]
Furosemide	0.58 $\pm$ 0.13[b]	0.94 $\pm$ 0.26[a]	2.38 $\pm$ 0.32[a]	1.86 $\pm$ 0.09	0.93 $\pm$ 0.1[b]	9.86 $\pm$ 1.41	3.7 $\pm$ 1.17[a]	18.09 $\pm$ 1.86
SITS	0.31 $\pm$ 0.03[b]	0.41 $\pm$ 0.06[b]	3.16 $\pm$ 0.48	0.95 $\pm$ 0.13[b]	0.92 $\pm$ 0.1[b]		1.68 $\pm$ 0.19[a]	12.43 $\pm$ 1.02[b]
Quinacrine	1.03 $\pm$ 0.24[a]	1.73 $\pm$ 0.31[a]	3.18 $\pm$ 0.54	1.07 $\pm$ 0.18[b]	1.33 $\pm$ 0.38	11.64 $\pm$ 1.8	4.7 $\pm$ 1.45[a]	11.31 $\pm$ 1.18[b]
BPB	1.3 $\pm$ 0.33	2.33 $\pm$ 0.62[a]	3.08 $\pm$ 0.88	1.4 $\pm$ 0.26[b]	1.2 $\pm$ 0.35	10.97 $\pm$ 1.0	4.61 $\pm$ 1.74[a]	13.02 $\pm$ 1.65[a]
DEDA 20 μM	0.47 $\pm$ 0.07[b]	1.04 $\pm$ 0.26[b]	4.61 $\pm$ 0.57	2.15 $\pm$ 0.42	1.29 $\pm$ 0.14	20.08 $\pm$ 2.02	3.24 $\pm$ 0.71[a]	18.48 $\pm$ 3.35
DEDA 2 μM	0.74 $\pm$ 0.38[a]	1.18 $\pm$ 0.65[b]	3.03 $\pm$ 0.23[a]	1.25 $\pm$ 0.19[b]	1.09 $\pm$ 0.1[a]	13.04 $\pm$ 1.97	3.37 $\pm$ 1.42[a]	14.15 $\pm$ 1.72[a]
DEDA + SITS	0.17 $\pm$ 0.02[c]	0.22 $\pm$ 0.04[c]	2.44 $\pm$ 0.17[a]	0.76 $\pm$ 0.19[c]	0.65 $\pm$ 0.06[a]		1.04 $\pm$ 0.09[b]	4.12 $\pm$ 1.0[b]
Arachidonic	1.06 $\pm$ 0.57	1.45 $\pm$ 0.9[a]	2.83 $\pm$ 0.34	1.52 $\pm$ 0.16[a]	1.11 $\pm$ 0.22[a]	10.35 $\pm$ 1.86	4.18 $\pm$ 1.22[a]	15.56 $\pm$ 3.21
Linoleic	0.56 $\pm$ 0.11[a]	0.85 $\pm$ 0.15[a]	3.33 $\pm$ 0.40	1.75 $\pm$ 0.29[a]	1.69 $\pm$ 0.23	13.95 $\pm$ 2.97	2.19 $\pm$ 0.24[a]	16.24 $\pm$ 1.52
Stearic	2.45 $\pm$ 0.81	4.14 $\pm$ 0.83	8.44 $\pm$ 1.48	4.9 $\pm$ 1.17	2.58 $\pm$ 0.57	24.17 $\pm$ 4.04	15.19 $\pm$ 2.54	32.87 $\pm$ 7.8
Oleic	1.03 $\pm$ 0.38[a]	2.88 $\pm$ 1.33	5.9 $\pm$ 1.78	2.81 $\pm$ 0.79	2.65 $\pm$ 1.35	14.5 $\pm$ 3.22	5.32 $\pm$ 1.51	27.51 $\pm$ 9.17
Dihydrokainate	1.35 $\pm$ 0.48	2.34 $\pm$ 0.93[a]	3.77 $\pm$ 0.45	1.98 $\pm$ 0.34	1.36 $\pm$ 0.22	11.14 $\pm$ 1.29	8.46 $\pm$ 2.34	13.92 $\pm$ 1.66
Genistein	1.2 $\pm$ 0.58	1.62 $\pm$ 0.47[a]	1.82 $\pm$ 0.11[b]	0.94 $\pm$ 0.19[c]	0.7 $\pm$ 0.09[b]	8.2 $\pm$ 1.28[a]	8.29 $\pm$ 2.55	9.31 $\pm$ 1.11[b]
Lavendustin A	1.04 $\pm$ 0.32[a]	1.63 $\pm$ 0.45[a]	2.0 $\pm$ 0.29[a]	0.95 $\pm$ 0.22[c]	1.13 $\pm$ 0.19[a]	10.14 $\pm$ 1.02	4.39 $\pm$ 0.87[a]	9.12 $\pm$ 0.9[b]
Chelerythrine	1.2 $\pm$ 0.2[a]	2.54 $\pm$ 0.4[a]	2.21 $\pm$ 0.12[a]	1.02 $\pm$ 0.05[b]	0.93 $\pm$ 0.1[b]		9.87 $\pm$ 1.81	12.04 $\pm$ 1.1[a]

Values are from perfusate samples obtained during the final 5 min of anoxia.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ compared to the control group.

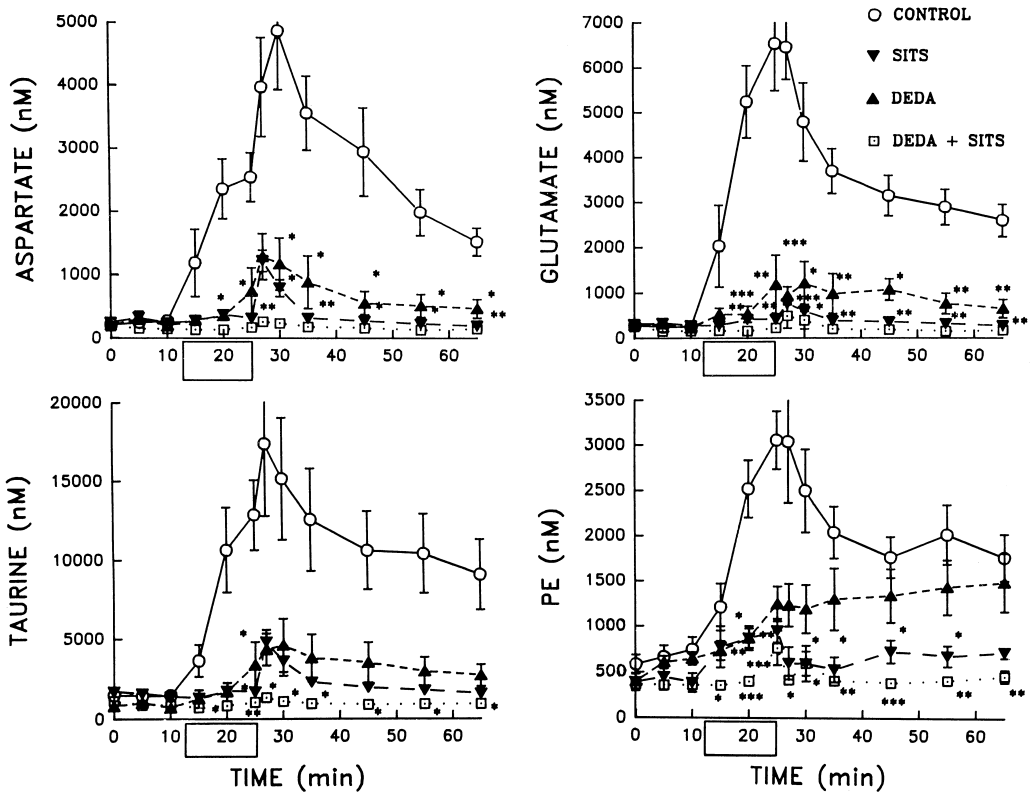

Fig. 1. Amino acids in the coronary effluent perfusate from isolated rat heart Langendorff preparations. Line plots show the time course of changes in aspartate, glutamate, taurine and phosphoethanolamine levels before, during (big rectangle), and after 15 min periods of anoxia. In each instance, the initial amino acid value represents the mean of the first two collections. SITS (500 μM; ▼), DEDA (2 μM; ▲) and SITS + DEDA (□). Data are presented as the means $\pm$ S.E.M. Statistically significant changes in amino acid levels from control values at each time point were determined by ANOVA. $*$ $P < 0.05$; $**$ $P < 0.01$; $***$ $P < 0.001$.

2:2′-disulfonic acid (SITS) and furosemide on anoxia-evoked amino acid release were examined. Anthracene-9-carboxylic acid (500 μM) significantly reduced the anoxia-evoked release of glutamate, aspartate, glutamine, serine and phosphoethanolamine (Table 4). Anoxia-evoked releases of the other amino acids were not significantly reduced.

Furosemide (500 μM) significantly depressed the anoxia-evoked releases of glutamate, aspartate, glycine, serine, and taurine. Efflux of other amino acids tended to be lower than in the controls (Table 4). SITS (500 μM) significantly reduced the anoxia-evoked release of aspartate, glutamate, taurine, phosphoethanolamine, serine and glutamine (Fig. 1, Table 4). None of the hearts preincubated with anthracene-9-carboxylic acid or SITS fibrillated and 1 of the 4 furosemide-treated hearts fibrillated briefly during reoxygenation (Table 1). Heart rates in the SITS-treated preparations, although reduced, were significantly higher during anoxia than those of the control animals (Table 1).

3.4. Effects of phospholipase inhibitors

The non-selective phospholipase A_2 inhibitors quinacrine (10 μM) and 4-bromophenacyl bromide (BPB; 1 μM) significantly reduced anoxia-evoked effluxes of aspartate, glutamate, glutamine, taurine and phosphoethanolamine (Fig. 2, Table 4). None of the quinacrine and 3/8 of the BPB-exposed hearts fibrillated briefly during reoxygenation (Table 1).

7,7-Dimethyleicosadenoic acid (DEDA), which preferentially inhibits the calcium-independent secretory type of phospholipase A_2 was tested at concentrations of 2 and 20 μM. It significantly, and equieffectively, reduced anoxia-evoked release of glutamate, aspartate and taurine at both concentrations, whereas only the lower concentration also reduced the efflux of glutamine, glycine, phosphoethanolamine and serine (Table 4). Indeed, at 20 μM, the efflux of glycine during reoxygenation became significantly enhanced by DEDA. Alanine efflux, though not significantly reduced by either concentration, was also elevated above control levels during reoxygenation following exposure to 20 μM DEDA. At both concentrations, DEDA prevented the hearts from fibrillating during reoxygenation. Heart rates in the quinacrine- and DEDA-treated preparations were sustained at significantly higher rates than in the control hearts during anoxic stress (Table 1).

The effects of a combined application of DEDA (2 μM) and SITS (500 μM) on anoxia-elicited release of amino acids are illustrated in Fig. 1. Anoxia-induced efflux of all amino acids was virtually abolished, suggesting an additive effect of the two compounds. None of the hearts fibrillated during reoxygenation.

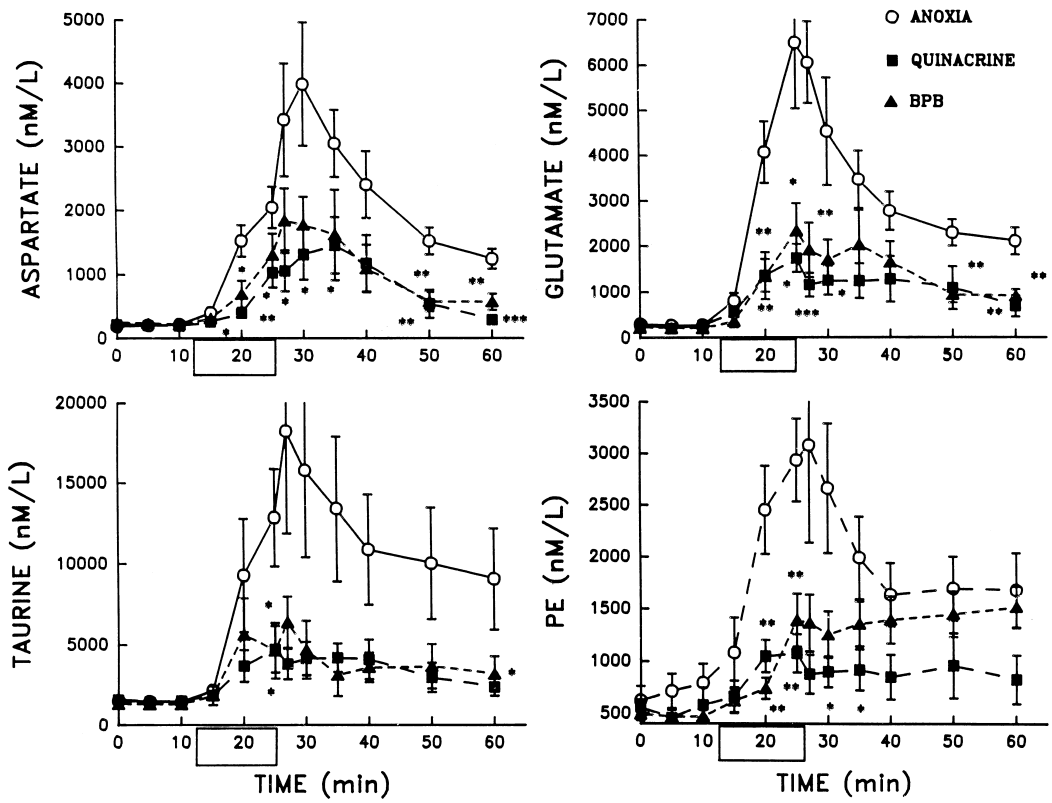

Fig. 2. Effects of phospholipase inhibitors quinacrine (10 μM; ■) and BPB (1 μM; ▲) on anoxia-evoked aspartate, glutamate, taurine and phosphoethanolamine release from the isolated rat heart. See legend to Fig. 1 for further details.

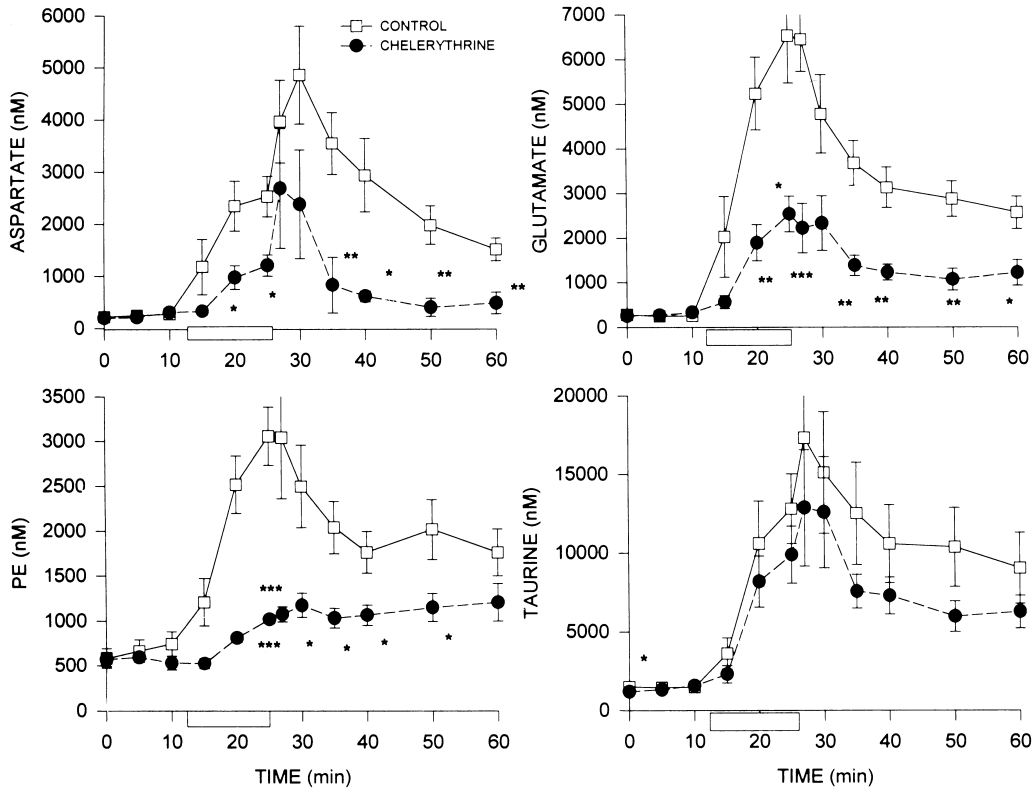

Fig. 3. Effect of chelerythrine chloride (5 μM) on aspartate, glutamate, taurine and phosphoethanolamine from the isolated rat heart. See legend to Fig. 1 for further details.

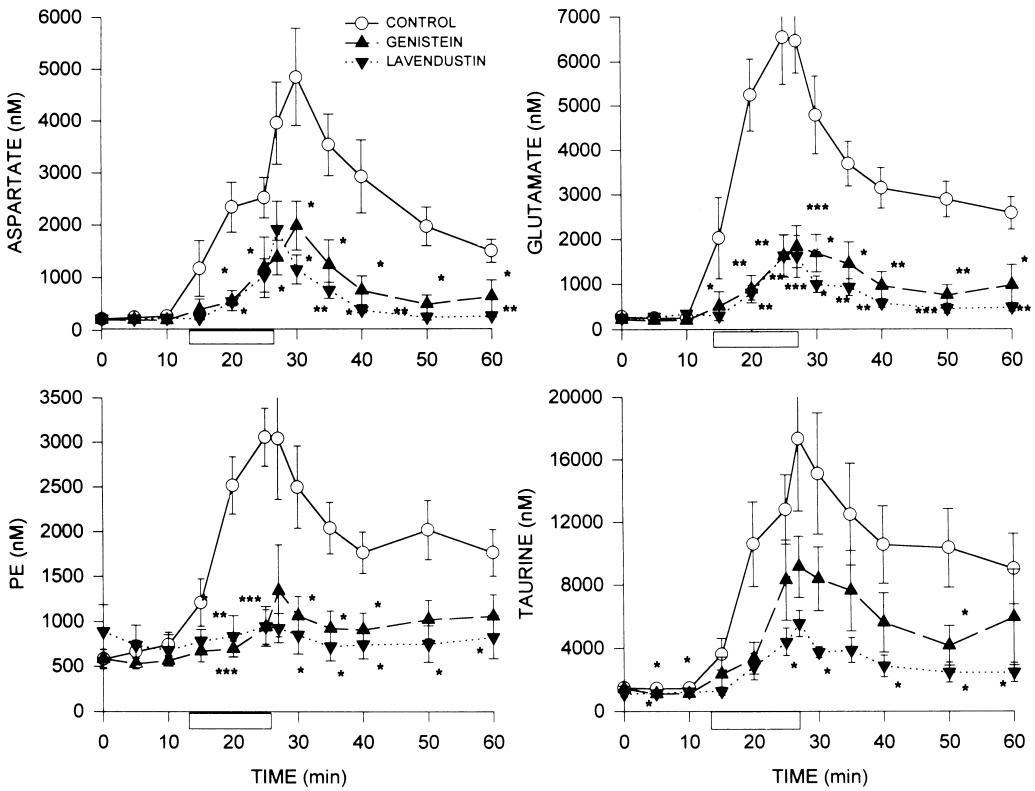

Fig. 4. Effect of genistein (1 μM; ▲) and lavendustin A (0.5 μM; ▼) on aspartate, glutamate, taurine and phosphoethanolamine release from the isolated rat heart. See legend to Fig. 1 for further details.

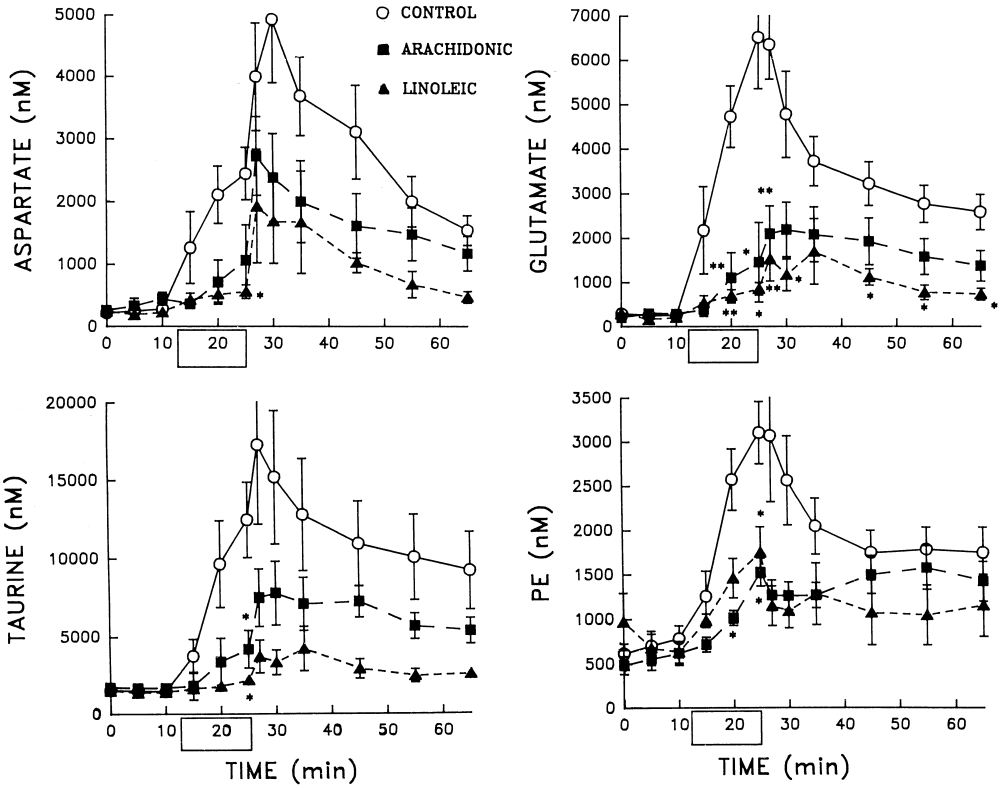

Fig. 5. Effects of polyunsaturated fatty acids (arachidonic ■, 1 μM; linoleic ▲, 1 μM) on aspartate, glutamate, taurine and phosphoethanolamine release from isolated rat heart. See legend to Fig. 1 for further details.

3.5. Inhibition of protein kinase C and protein tyrosine kinase

Chelerythrine (5 μM), a protein kinase C inhibitor, significantly inhibited the anoxia-evoked release of aspartate, glutamate, phosphoethanolamine, glycine, serine, and glutamine, but not that of taurine (Fig. 3, Table 4). The protein tyrosine kinase inhibitors genistein (1 μM) and lavendustin A (0.5 μM) depressed anoxia-evoked releases of glutamate, glycine, phosphoethanolamine, taurine and glutamine (Fig. 4, Table 4). Genistein also reduced the evoked release of alanine, while lavendustin A reduced the anoxia-evoked efflux of aspartate.

3.6. Free fatty acids

The effect of polyunsaturated (arachidonic, linoleic), monounsaturated (oleic) and saturated (stearic) fatty acids on anoxia-evoked efflux of amino acids from the rat heart were examined. Arachidonic acid (1 μM) significantly reduced the efflux of glutamate, phosphoethanolamine, taurine, serine and depressed release of the other amino acids (Fig. 5, Table 4). Linoleic acid significantly reduced anoxia-evoked release of aspartate, glutamate, taurine and phosphoethanolamine (Fig. 5). Fibrillation during reoxygenation occurred in only 1/9 hearts perfused with these acids. Oleic and stearic acids (1 μM) increased the levels of most amino acids during reoxygenation, the exceptions being glutamate and aspartate, and inhibited fibrillation during reoxygenation in only 20% of the hearts tested.

3.7. Glutamate transport inhibition

Dihydrokainic acid is a non-competitive (non-transportable) inhibitor of glutamate and aspartate transport. As such, it is not carried into cells and does not elicit release by heteroexchange with intracellular glutamate and aspartate. Dihydrokainic acid (1 mM) significantly inhibited the anoxia-evoked release of glutamate and depressed that of aspartic acid (Table 4). During reoxygenation, the release of alanine, glycine and serine appeared to be elevated. Other amino acids including taurine were not affected. Dihydrokainic acid suppressed fibrillation during reoxygenation in 5/6 hearts.

4. Discussion

Cardiac ischemia and/or anoxia induces large increases in the efflux of amino acids into the extracellular space with their subsequent loss into the blood or vascular perfusates (Song et al., 1996). The increases in the rate of amino acid efflux above basal levels are particularly pronounced for aspartate, glutamate and taurine (Table 3), for

which there are large concentration gradients across the plasma membrane. With the exception of taurine, the amounts of amino acid recovered in the coronary perfusates exceeded the fall in tissue amino acid content, implying that they were being replenished during anoxia, presumably by metabolism of proteins or de novo synthesis.

Cellular swelling subsequent to ischemia (Steenbergen et al., 1985) may contribute to increasing extracellular concentrations of amino acids such as aspartate, glutamate and taurine by a process known as regulatory volume decrease. Amino acid efflux during regulatory volume decrease is thought to occur via a diffusional mechanism involving stretch activated, anion permeant, channels, rather than by a reversal of Na^+-dependent transporters (Song et al., 1998). In this context, it is of interest that the Cl^- channel blockers, anthracene-9-carboxylic acid and SITS, are protective against myocardial ischemia/reoxygenation injury (Tanaka et al., 1996). Release of glutamate and aspartate and, to a lesser extent, the other amino acids was inhibited by the anion channel blockers anthracene-9-carboxylic acid, furosemide and SITS. The polyunsaturated fatty acid anion channel inhibitors, arachidonic and linoleic, reduced anoxia-evoked amino acid release whereas the monounsaturated (oleic) and saturated (stearic) fatty acids enhanced anoxia-evoked release of most amino acids. However, only partial inhibition of amino acid release by anion channel blockers was observed in the present experiments, suggesting the likelihood of additional mechanisms of amino acid release from the anoxic heart.

The observation that amino acids with large intracellular/extracellular concentration ratios are preferentially released during anoxia suggests that their efflux is via diffusional channels, the rate of loss being dependent on concentration gradients. The inhibitory effects of the phospholipase inhibitors quinacrine, 4-bromophenacyl bromide and DEDA observed in the present experiments would be consistent with an involvement of phospholipase A_2 in the amino acid efflux phenomenon. Loss of the plasma membrane integrity as a result of enhanced phospholipase activity would allow amino acids to diffuse down their concentration gradients into the extracellular space. The possibility that phospholipase activation contributed to the opening of diffusional anion channels was evaluated by combining SITS and DEDA. Our results appear to show that the actions of the two agents were additive, implying that their effects are exerted on separate mechanisms underlying the release of amino acids. However, other investigators have suggested that phospholipase A_2 activation may be involved in the opening of anion permeant channels in hypoosmotically challenged cells (Margalit et al., 1993; Thoroed et al., 1997).

The role of protein kinases in the signal transduction cascade linking cell swelling to volume regulated chloride currents in cardiac cells has received considerable attention. The protein tyrosine kinase inhibitor genistein inhibited the swelling induced activation of chloride currents in dog atrial myocytes (Sorota, 1995). There is also evidence for tyrosine phosphorylation of a mitogen-activated protein (MAP) kinase in cultured neonatal rat ventricle cells in response to cell stretch (Sadoshima and Izumo, 1993). It has been shown in an intestinal cell line that tyrosine phosphorylation of cellular proteins, including a MAP-kinase-like protein, is increased in response to osmotic stress, and that inhibition of protein tyrosine kinases reduced regulatory volume decreases and anion efflux in these cells (Tilly et al., 1993). The inhibitory effects of genistein and lavendustin A on amino acid efflux described in the present report would be consistent with an involvement of protein tyrosine kinases in the regulatory volume decrease response. We have previously shown that the protein kinase C inhibitor, chelerythrine, attenuates amino acid release from the ischemic cerebral cortex (Phillis and O'Regan, 1996) and suggested that phospholipase A_2 could be activated by this kinase. The inhibitory effects of chelerythrine on amino acid release from the anoxic rat heart observed in the present experiments would be consistent with this interpretation.

Neutral and acidic amino acids are taken up into cells by Na^+-dependent co-transport systems (Collarini and Oxender, 1987). Under the usual physiological conditions these carriers are driven by the sodium gradient to concentrate intracellular glutamate, aspartate and taurine to levels that are approximately $100\times$ higher than those in the plasma. The transporters can potentially be reversed by marked changes in the levels of Na^+ and K^+ across the plasma membrane, such as those that can occur during anoxia or ischemia when depolarization, due to failure of the Na^+/K^+ pump, results in elevated levels of intracellular Na^+ and extracellular K^+. Inhibition of glutamate transport with dihydrokainic acid, reduced glutamate and aspartate release without affecting that of the other amino acids. Dihydrokainic acid failed to inhibit glutamate and aspartate release from hypoosmotically challenged hearts (Song et al., 1998) which would not have been subjected to the massive ionic shifts that occur in anoxic myocytes. Attempts to demonstrate a similar involvement and reversal of the high-affinity, Na^+-dependent, taurine transporter were frustrated by the co-elution of hypotaurine, an inhibitor of the transporter, with taurine during the HPLC assay.

Overall, the data indicates that amino acid release from the anoxic rat heart may involve at least three mechanisms including: (i) a diffusional efflux through stretch activated anion channels; (ii) a diffusional release across plasma membranes after their integrity has been compromised by phospholipase activity; and (iii) as a result of reversal of Na^+-dependent transporters. In all of these respects, amino acid efflux from the anoxic rat heart displays many similarities to ischemia-evoked amino acid release from the rat cerebral cortex where there is also evidence for an involve-

ment of anion channels, phospholipase activity and a reversal of Na^+-dependent membrane amino acid transporters (Phillis and O'Regan, 1996; Phillis et al., 1994, 1996, 1997b).

5. Conclusion

Amino acid release by cardiac cells as a consequence of the activation of diverse mechanisms may represent a protective response to pathophysiological situations, such as anoxia and ischemia, which result in cell swelling. The loss of amino acids during anoxia/ischemia may offer an explanation for the beneficial effects of adding glutamate and aspartate to cardioplegic solutions.

Acknowledgements

Supported by USPHS grant NS26912-09.

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