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# Protein Kinase Inhibitors Attenuate Cardiac Swelling-induced Amino Acid Release in the Rat

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**Abstract**

Rat Langendorff heart preparations have been used to study the efflux of cardiac amino acids into coronary artery perfusates during brief (5-min) periods of exposure to hyposmotic stress (70 mM NaCl). Coronary flow rates, heart rates and intra-aortic pressures were recorded. Amino acid levels were measured by high-performance liquid chromatography.

Hyposmotic stress caused marked percentage increases in taurine, glutamate and aspartate levels in the coronary perfusate, with smaller increases in phosphoethanolamine, glycine and alanine and non-significant increases in serine and glutamine. Amino acid levels declined during reperfusion with isosmotic Krebs–Henseleit bicarbonate buffer. Inhibition of protein kinase C with chelerythrine chloride (5 $\mu$M) depressed the osmotically-induced release of aspartate, glutamate, taurine and glycine. The protein tyrosine kinase inhibitor, genistein, reduced the anisosmotic efflux of aspartate, glutamate, taurine and phosphoethanolamine. Lavendustin A, another inhibitor of tyrosine kinase, depressed the osmotically evoked release of aspartate, glutamate and taurine.

These studies demonstrate the involvement of protein kinase C and tyrosine kinases in the efflux of amino acids from the osmotically challenged rat heart and imply that these enzymes are involved in the mechanisms responsible for volume regulation by cardiac cells.

An ability to respond to changes in the osmolarity of their internal or external environment by rapid and efficient volume-regulatory mechanisms is an essential element of cell survival (Chamberlin & Strange 1989). Because plasma membranes are relatively freely permeable to water, cells exposed to anisosmotic gradients will rapidly undergo volume changes as a consequence of the influx or efflux of water, depending on the relative imbalances between internal and external osmotic forces. Cells placed in a hyposmotic medium will gain water, swell and would, in the absence of compensatory adjustment mechanisms, be at risk from lysis and death. Such increases in volume can trigger a rapid adaptive response which enables the cells to lose osmolytes, including inorganic ions and amino acids, thus restoring their volume towards the original dimensions. This process is termed a regulatory volume decrease.

Like other animal cells, cardiac cells must constantly regulate their volume when exposed to anisosmotic media (Thurston et al 1981; Drewnowska & Baumgarten 1991). Cardiac cell swelling occurs during myocardial ischaemia–reperfusion, and disruption of volume regulation during anoxia or ischaemia could be a significant factor in the development of myocardial injury and pathology. Mammalian heart cells express a swelling-activated chloride current that seems to be involved in cell-volume regulation (Hagiwara et al 1992; Sorota 1992; Tseng 1992; Vandenberg et al 1994). The swelling-activated chloride current in myocytes is inhibited by a variety of chloride-channel blockers including tamoxifen, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) and anthracene-9-carboxylic acid (Hagiwara et al 1992; Vandenberg et al 1994). Amino acid efflux from

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hyposmotically stressed rat hearts is also inhibited by chloride-channel blockers, including SITS and arachidonic acid (Song et al 1998), suggesting that these channels might provide a diffusional pathway for the amino acids.

A transient delay between the initiation of cell swelling and the activation of chloride channels suggests the involvement in the process of signal-transduction pathways (Hagiwara et al 1992; Tseng 1992). Cell stretch has been shown to activate several second-messenger pathways in cardiac myocytes including tyrosine kinases, protein kinase C and phospholipases (Sadoshima & Izumo 1993). Furthermore, Sorota (1995) has demonstrated that tyrosine kinase inhibition by genistein prevents the activation of cardiac swelling-induced chloride currents.

We have previously reported the inhibition of amino acid efflux from the hyposmotically challenged rat heart by the phospholipase $A_2$ inhibitors 7,7-dimethyleicosadienoic acid and 4-bromophenacylbromide (Song et al 1998). The experiments reported here were designed to evaluate the potential involvement of tyrosine kinases and protein kinase C in the swelling-induced efflux of amino acids from the isolated perfused rat heart.

## Materials and Methods

*Chemicals*

Chelerythrine chloride, genistein and lavendustin A were obtained from Sigma (St Louis, MO). Genistein and lavendustin A were first dissolved in dimethylsulphoxide and then diluted to a final concentration of 0·01% v/v with Krebs–Henseleit bicarbonate buffer (KHB). At this concentration dimethylsulphoxide did not affect amino acid release. Other reagents were of analytical grade or better.

*Isolated rat heart preparation*

Rats (Sprague–Dawley, 250–300 g; Charles River) were anaesthetized with intraperitoneal pentobarbital sodium (50 mg $kg^{-1}$). Heparin (1000 international units $kg^{-1}$) was administered via a femoral vein to prevent microemboli formation after excision of the heart.

The Langendorff hearts were prepared by aortic cannulation, suspended in a water jacket at 38°C and perfused retrogradely via the aorta. Perfusion of the isolated heart with Krebs–Henseleit bicarbonate buffer was performed essentially as described by DeLeiris et al (1984) by means of a constant pressure (75 cm $H_2O$) perfusion system. The KHB (mM: NaCl, 118·0; KCl, 4·7; $CaCl_2$, 2·9; $MgSO_4$, 1·2; $KH_2PO_4$, 1·2; $NaHCO_3$, 25; glucose, 11) was equilibrated with 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 connected to the aortic cannula. Coronary flow rate was recorded with a calibrated drop-counter placed beneath the water jacket.

Hearts were perfused for 20 min to enable stabilization at a flow rate of approximately 5 mL $min^{-1}$. 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 hyposmotic perfusate in which the NaCl concentration had been reduced to 70 mM. Further perfusate samples were collected after 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 perfusate samples were centrifuged at 1200 *g* and stored at −20°C. High-performance liquid chromatographic analysis of perfusate amino acid content was conducted within a few hours using previously published procedures.

Results obtained with three kinase-inhibiting agents are presented in this report. The procedure was the same as for the control group except that drugs were administered in the perfusate for 20 min before collection of the initial two basal samples. The heart was then exposed to hyposmotic perfusate containing the appropriate substance. Drugs were perfused for the rest of the experiment in regular KHB with the collection of perfusate samples as described above. The drugs were chelerythrine chloride (5 $\mu$M; n = 4), a highly specific inhibitor of protein kinase C (PKC), genistein (1 $\mu$M; n = 4), a selective inhibitor of protein tyrosine kinases, and lavendustin A (0·5 $\mu$M; n = 4), another potent inhibitor of tyrosine kinases.

*Statistical analysis*

Statistical differences between amounts of amino acids released from control and drug-treated hearts were analysed by analysis of variance and Scheffe's test with contrasts between the control group and each treatment group (SPSS statistical package). $P < 0.05$ was accepted as denoting statistical significance.

## Results

Basal heart rates for the control preparations were 239 ± 4·3 (s.e.m.) beats $min^{-1}$. Exposure to the hyposmotic perfusate elicited a reduction in the frequency of contraction (173 ± 8) with little change in strength. Heart rates recovered (235 ± 4·5) during reperfusion with regular KHB.

Perfusate flow rates fell from $5{\cdot}48 \pm 0{\cdot}1$ mL min$^{-1}$ to $4{\cdot}79 \pm 0{\cdot}1$ mL min$^{-1}$ during exposure to anisosmotic perfusate and returned to control levels after reperfusion with regular KHB.

Figures 1 and 2 illustrate the basal and hyposmotically evoked levels of several amino acids in the control hearts. Aspartate and glutamate were present at low concentrations (approx 250 nmol L$^{-1}$) with serine, phosphoethanolamine, taurine, glycine, alanine and glutamine (approx 7500 nmol L$^{-1}$) at increasingly higher levels. Osmotic stress resulted in pronounced increases in aspartate, glutamate and taurine levels in the perfusate with smaller increases in the levels of the other amino acids. After a return to regular KHB, amino acid release declined rapidly.

*Chelerythrine chloride*

This inhibitor of protein kinase C significantly attenuated the osmotically-evoked releases of aspartate, glutamate, taurine and glycine (Figures 1 and 2), but not those of phosphoethanolamine, serine, alanine or glutamine. With the exception of glycine, the level of which was reduced, basal levels of perfusate amino acids were not significantly affected by administration of chelerythrine.

*Lavendustin A*

This inhibitor of tyrosine kinases significantly attenuated the hyposmotically-evoked release of aspartate, glutamate and taurine (Figure 3) but not that of the other amino acids. It did not reduce the basal levels of these compounds in cardiac perfusates.

*Genistein*

Inhibition of protein tyrosine kinases with this agent significantly attenuated the hyposmotically-evoked release of aspartate, glutamate, phosphoethanolamine and taurine (Figure 4) but not that of the other amino acids. Basal levels of glutamate

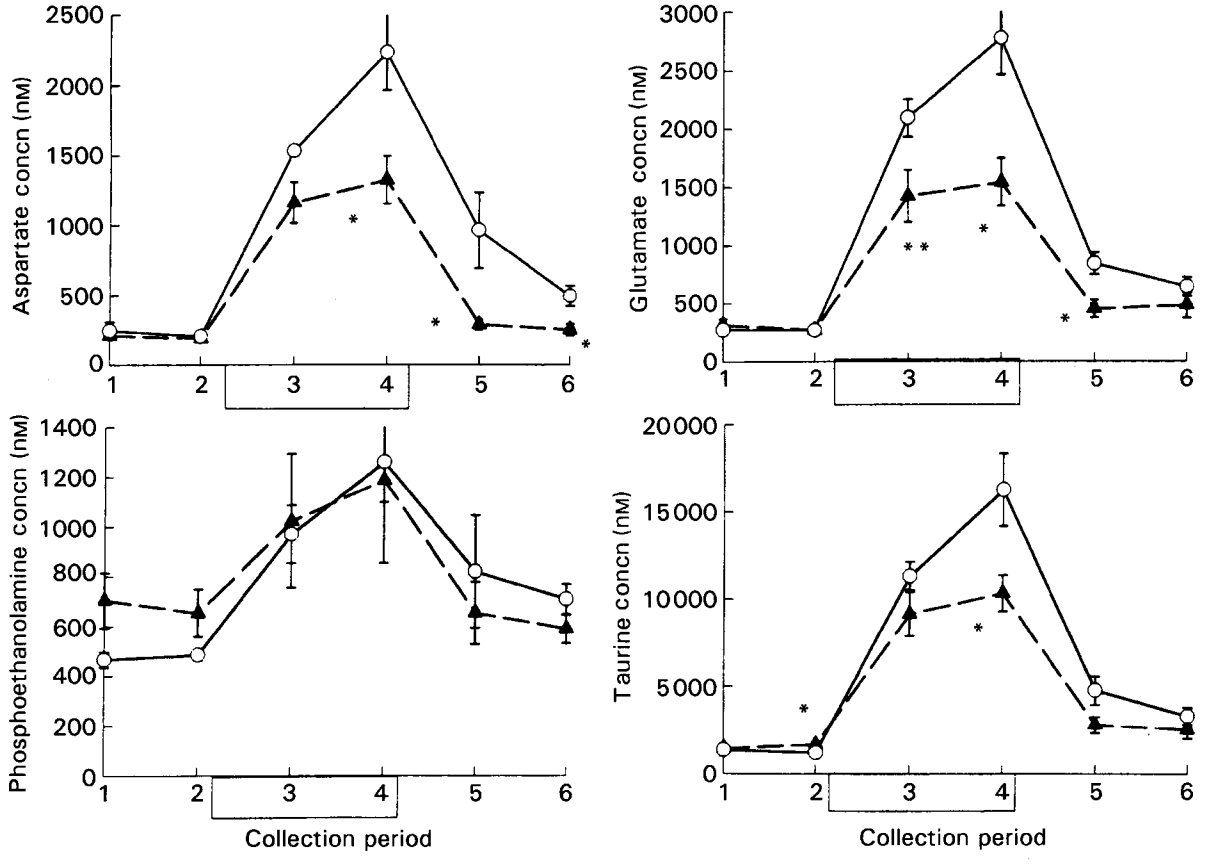


Figure 1. Amino acids in the coronary effluent perfusate from isolated rat heart Langendorff preparations exposed to hyposmotic solutions. Line plots show the time-course of changes in aspartate, glutamate, phosphoethanolamine and taurine before, during and after exposure to modified Krebs–Henseleit bicarbonate buffer containing 70 mM NaCl for a period of 5 min. Perfusates were collected at 5-min intervals before and after hyposmotic stress, and at 2·5-min intervals during the anisosmotic challenge. ○, control hearts; ▲, hearts perfused with chelerythrine chloride (5 μM) for 20 min before and during sample collection. Data are presented as means ± s.e.m. Statistically significant differences between amino acid levels and control values in each sample were determined by analysis of variance and Scheffe's test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

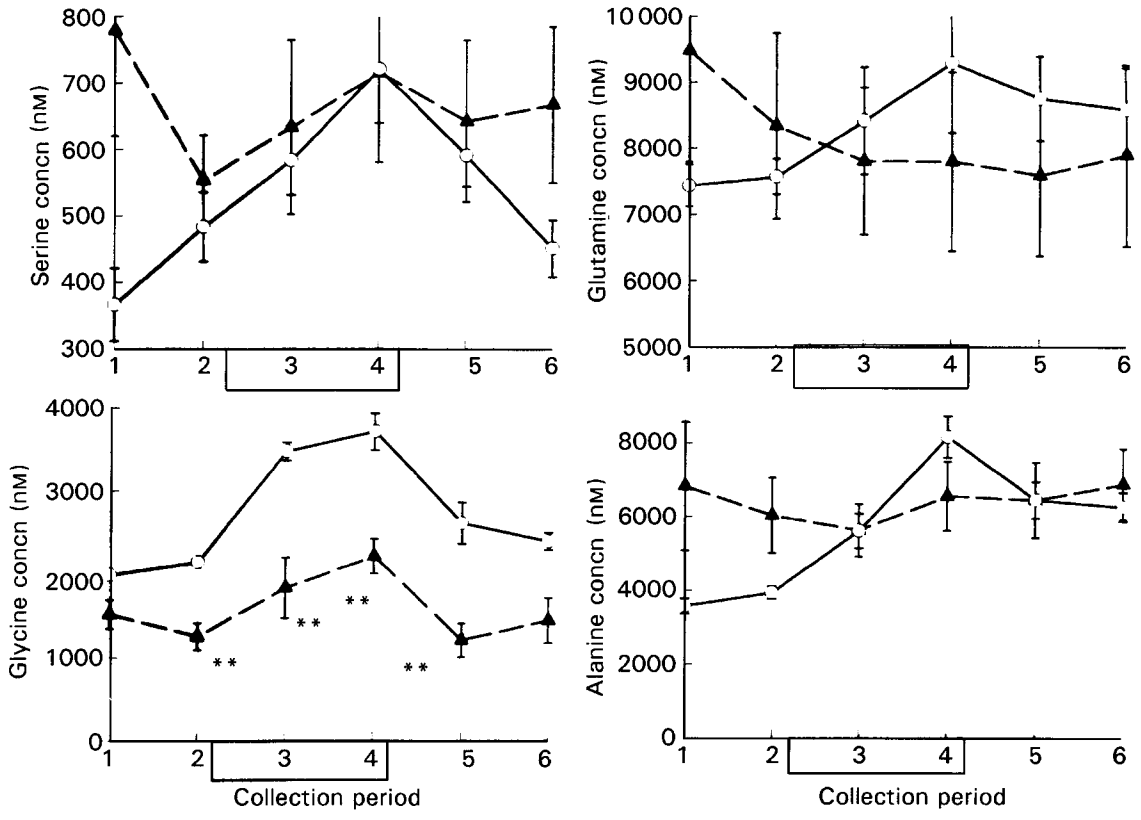


Figure 2. Effects of chelerythrine chloride (5 $\mu$M) on coronary perfusate levels of serine, glutamine, glycine and alanine before, during, and after exposure to hearts to hyposmotic Krebs–Henseleit bicarbonate buffer. Perfusates were collected at 5-min intervals before and after hyposmotic stress, and at 2·5-min intervals during the anisosmotic challenge. ○, control hearts; ▲, hearts perfused with chelerythrine chloride (5 $\mu$M) for 20 min before and during sample collection. Data are presented as means ± s.e.m. Statistically significant differences between amino acid levels and control values in each sample were determined by analysis of variance and Scheffe's test. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

and taurine were also slightly, but significantly depressed.

## Discussion

Amino acid loss during regulatory volume decrease by cardiac cells has been documented by several investigators (Crass & Lombardini 1978; Thurston et al 1981; Visle 1983; Atlas et al 1984). Rasmusson et al (1993) have shown that the loss of organic osmolytes (taurine, glutamate, aspartate, glycine) from chick heart cells during volume regulation in moderately hyposmotic solutions (150 mosmol $kg^{-1}$ $H_2O$) can exceed the loss of solute attributable to $Na^+$ and $K^+$, although the relative contribution of inorganic osmolytes became more pronounced with more severe reductions in extracellular osmolarity (83 mos mol $kg^{-1}$ $H_2O$).

A rapid increase in the rate of efflux of the amino acids aspartate, glutamate, taurine and phosphoethanolamine from the hyposmotically challenged isolated perfused rat heart has also been observed (Song et al 1998). Large increases in the perfusate levels of these amino acids are evident within 2 min of the onset of hyposmotic perfusion and perfusate levels increased even further after 5 min. The initial recovery towards basal levels during reperfusion with isosmotic KHB is also rapid. Efflux of these amino acids might occur via swelling-activated chloride channels in myocyte plasma membranes (Harvey 1996). The amino acids for which the increases in extracellular concentration are most dramatic during regulatory volume decrease are also those with the greatest intracellular/extracellular concentration ratios, suggesting that efflux is by a diffusional mechanism down concentration gradients. Inhibition of efflux by chloride-channel blockers (Song et al 1998) would be consistent with diffusion through swelling-activated chloride channels. In this respect, volume regulation in cardiac cells by amino acid efflux seems to be comparable with that observed in astrocytes, which is also inhibited by chloride-channel blockers (Kimelberg et al 1990; Sanchez-Olea et al 1993). Permeation of glutamate, aspartate and taurine

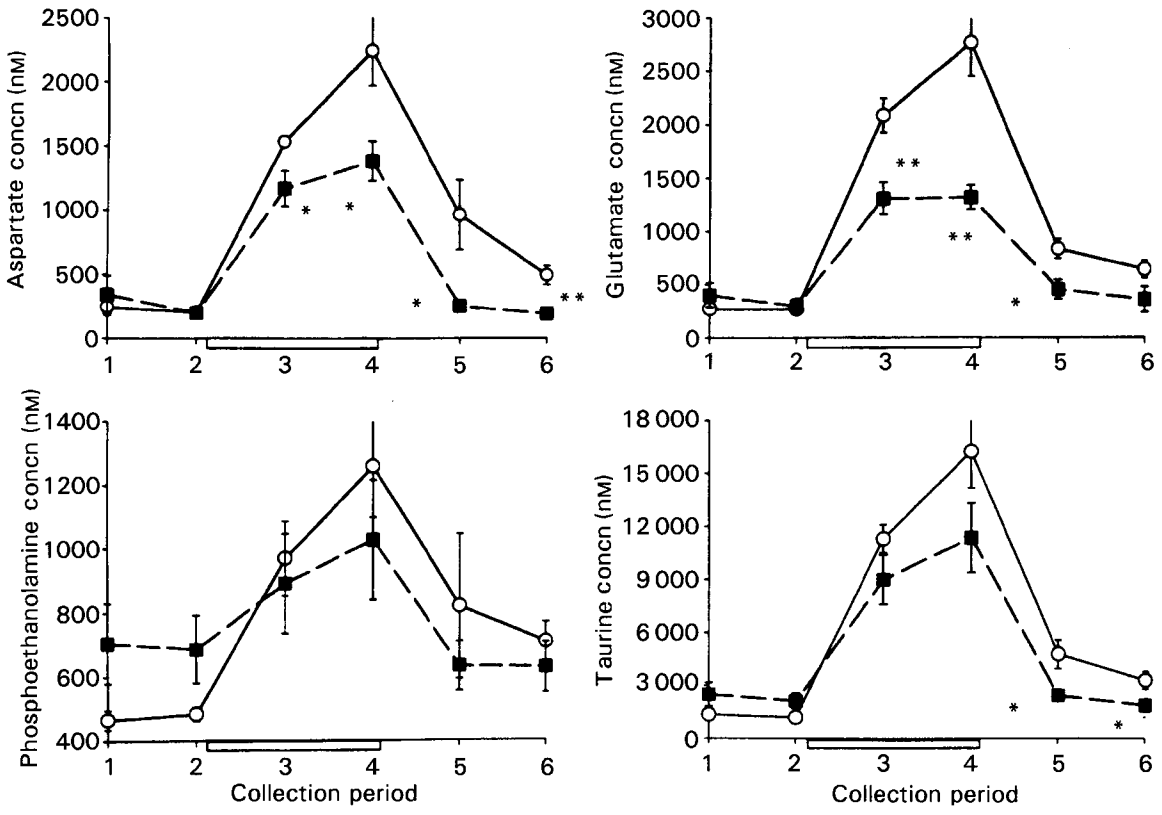


Figure 3. Effects of lavendustin A on aspartate, glutamate, phosphoethanolamine and taurine levels in coronary perfusates before, during and after exposure of hearts to hyposmotic Krebs–Henseleit bicarbonate buffer. ○, Control hearts; ■, hearts perfused with lavendustin A (0·5 $\mu$M) for 20 min before and during sample collection. Perfusates were collected at 5-min intervals before and after hyposmotic stress, and at 2·5-min intervals during the anisosmotic challenge. Data are presented as means ± s.e.m. Statistically significant differences between amino acid levels and control values in each sample were determined by analysis of variance and Scheffe's test. $*P < 0.05$; $**P < 0.01$.

through swelling-activated chloride channels in C6 glioma cells has been reported (Jackson & Strange 1993; Roy 1995).

Protein kinase C seems to be involved in the regulatory volume decrease mechanism in astrocytes (Bender et al 1992, 1993) for which volume decrease was blocked by PKC inhibition or down-regulation. Stretch-evoked activation of protein kinase C in cardiac myocytes has been reported by Sadoshima & Izumo (1993), although definitive evidence for the involvement of PKC in the activation of swelling-induced chloride currents in cardiac cells is currently lacking and studies with non-selective serine-threonine kinase inhibitors such as H7 and H8 have failed to demonstrate any reduction in $Cl^-$ current (Hagiwara et al 1992; Tseng 1992), possibly as a result of inhibition of protein kinase A and protein kinase G as well as of PKC. Inhibition of PKC with the selective inhibitor chelerythrine chloride depressed release of amino acids from hyposmotically challenged rat hearts in the current experiments. This agent also inhibits the ischaemia-evoked release of glutamate and other amino acids from the rat cerebral cortex (Phillis & O'Regan 1996).

The protein tyrosine kinase inhibitor, genistein, inhibits activation of the swelling-induced chloride current in dog atrial myocytes (Sorota 1995) indicating that tyrosine protein phosphorylation might be involved. Genistein also suppressed stretch-induced *c-fos* expression in cardiac myocytes, suggesting that tyrosine kinase activation is required for the stretch response (Sadoshima & Izumo 1993). Inhibition of hyposmotically-evoked amino acid efflux by the two tyrosine kinase inhibitors genistein and lavendustin A, tested in the current experiments, would be consistent with the suggestion that tyrosine kinase-evoked activation of swelling-induced chloride channels (Sorota 1995) is involved in the process of regulatory volume decrease.

In conclusion, the data presented in this report support the concept that amino acid efflux during osmotically-evoked regulatory volume decrease in cardiac cells is linked to the activation of protein kinase C and tyrosine kinase. The results are

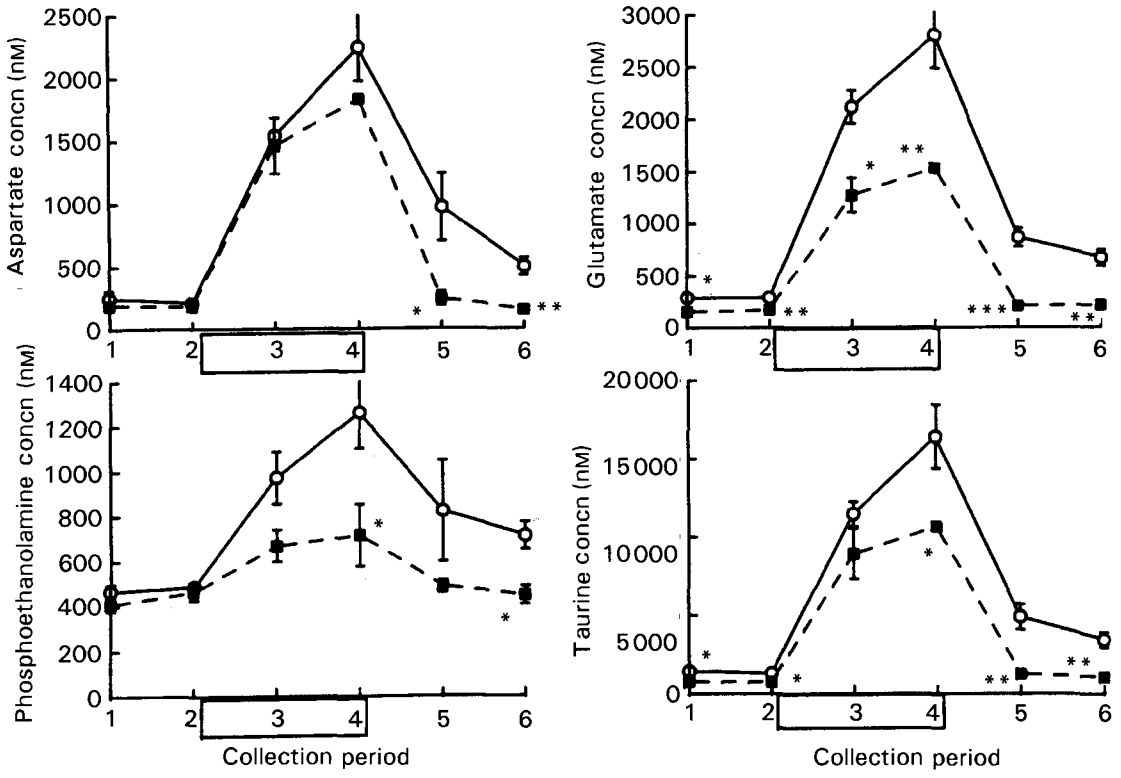


Figure 4. Effects of genistein on aspartate, glutamate, phosphoethanolamine and taurine levels in coronary perfusates before, during and after exposure of hearts to hyposmotic Krebs–Henseleit bicarbonate buffer. ○, control hearts; ■, hearts perfused with genistein (1·0 μM) for 20 min before and during sample collection. Perfusates were collected at 5-min intervals before and after hyposmotic stress, and at 2·5-min intervals during the anisosmotic challenge. Data are presented as means ± s.e.m. Statistically significant differences between amino acid levels and control values in each sample were determined by analysis of variance and Scheffe's test. $*P < 0.05$; $**P < 0.01$.

consistent with suggestions that these enzymes are involved in the opening of swelling-activated chloride channels which might then provide a pathway for the diffusion of amino acids along their concentration gradients.

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