As shown in the table, the plasma concentration of cyclic GMP was 25.7±3.4 pmoles/ml at the onset of atrial fibrillation, which was significantly higher than the 7.8±1.0 pmoles/ml measured the next day after recovery to sinus rhythmus ($p < 0.02$). Thus, under normal conditions cyclic GMP levels are low and increase during fibrillation. However, no significant difference in plasma concentration of cyclic AMP was observed; the concentration of cyclic AMP in plasma changed from 23.8±1.5 pmoles/ml with the onset of atrial fibrillation to 18.0±2.1 pmoles/ml on recovery to sinus rhythmus.

Mean concentration of plasma cyclic GMP and cyclic AMP in patients with paroxysmal atrial fibrillation on the day of attack as well as the next day on recovery to sinus rhythmus

	No.	Atrial fibrillation	Sinus rhythmus
Cyclic GMP	5	25.7±3.4*	7.8±1.0
Cyclic AMP	5	23.8±1.5	18.0±2.1

* Significantly elevated compared with that on the day of sinus rhythmus ($p < 0.02$).

Experimental studies have emphasized the importance of cholinergic stimulation in initiating and sustaining atrial fibrillation[8,9]. Acetylcholine increases the concentration of cyclic GMP by activating the muscarinic receptor[10-11]. The injection of cholinergic agents caused sharp increases in plasma cyclic GMP in fasted rats[12]. The increase in plasma cyclic GMP induced by cholinergic agents was completely abolished by atropine but not affected by hexamethonium, which shows that the increase was due to stimulation of the muscarinic receptor[13]. In our recent study, the plasma cyclic GMP concentration of dogs with electrically induced atrial fibrillation was significantly elevated after the onset of the arrhythmia, whereas the concentration of cyclic AMP showed no significant changes[14]. Thus, the present results indicate that atrial fibrillations may cause an increased stimulation of the parasympathetic nervous system.

1 Strange, R.C., and Mjos, O.D., Eur. J. clin. Invest. *5* (1975) 147.
2 Broadus, A.E., Kaminsky, N.I., and Hardman, J.G., J. clin. Invest. *49* (1970) 2222.
3 Hardman, J.G., Davis, J.W., and Sutherland, E.W., J. biol. Chem. *224* (1969) 6354.
4 Honma, M., and Ui, M., Eur. J. Pharmac. *47* (1978) 1.
5 Cailla, H.L., Racine-Weisbuch, M.S., and Dellage, M.A., Analyt. Biochem. *56* (1973) 394.
6 Cailla, H.L., Vanniet, C.J., and Dellaage, M.A., Analyt. Biochem. *70* (1976) 195.
7 Honma, H., Satoh, T., Takezawa, J., and Ui, M., Biochem. Med. *18* (1977) 257.
8 Burn, J.H., Vaugham Williams, E.M., and Walker, J.H., J. Physiol., Lond. *128* (1955) 277.
9 Scherf, D., and Chick, F.B., Circulation *3* (1951) 764.
10 George, W.J., Polson, J.B., O'Toole, A.G., and Gordenberg, N.O., Proc. natl Acad. Sci. USA *66* (1970) 398.
11 George, W.J., Wirkerson, R.D., and Kadwitz, P.J., J. Pharmac. exp. Ther. *184* (1973) 228.
12 Ui, M., Homma, M., Kunitada, S., Okada, F., Ide, H., Hata, S., and Satoh, T., in: Advances in cyclic nucleotides research, vol. 12, p. 25. Eds P. Hamet and H. Sands. Raven Press, New York 1980.
13 Honma, M., and Ui, M., Eur. J. Pharmac. *47* (1978) 1.
14 Unpublished data.

0014-4754/83/111290-02$1.50+0.20/0

Effects of temperature on anion distribution in perfused rat, guinea-pig and hamster ventricle

D.D. Macchia and P.W. Bankston[1]

Indiana University School of Medicine, Northwest Center for Medical Education, Departments of Physiology and Anatomy, 3400 Broadway, Gary (Indiana 46408, USA), March 9, 1983

Summary. Ventricular tissue from rats, guinea-pigs and hamsters were found to be more anion permeable when perfused and superfused with Ringer's solution at 22 °C rather than 36 °C. When the perfusate temperature was 36 °C the anion permeability of the in vitro rat and guinea-pig ventricle approximated that found in situ. Further, the anion permeability of the rat and guinea-pig heart was found not to be influenced by the absence of plasma proteins in the perfusate.

The isolated perfused heart preparation is used extensively to investigate the effects of various chemical and physical perturbations on cardiac function and metabolism[2-4]. Presently, the preparation is gaining popularity as a mean of investigating cellular ion fluxes under conditions where the tissues are not subject to dissection and interruption of the normal electrophysiological activity of the whole heart[5]. The reliability of this model for estimating in situ ion fluxes rests on the assumptions that the heart tissue is adequately perfused so as to avoid ischemic conditions and the solutions used to perfuse the heart do not alter the ionic permeability properties of the cellular membranes. Recently, it has been shown by Macchia and colleagues[6,7], that isolated skeletal muscles of toad incubated in oxygenated Ringer's solution have anion permeability properties which are significantly larger than in situ muscles or muscles incubated in toad plasma. Further, it has been shown that the plasma fraction responsible for maintaining anion permeabilities in vitro similar to those found in situ is plasma albumin. Since recent studies by Polimeni and Page[5], using isolated rat hearts perfused with a protein-free Ringer's solution, have reported cellular Cl efflux values significantly larger than reported by other investigators using isolated dissected heart preparations[8], anion permeability changes in the perfused heart due to the absence of plasma albumin or other plasma fractions could possibly explain these rapid Cl fluxes. Additionally, the integrity of the heart vasculature may be affected by perfusion with non-blood solutions. Isolated heart preparations perfused with Ringer's solution may therefore be subject to changes in cellular membrane permeability which could possibly yield ion flux measurements which are far from that which

exist in situ. Further, changes in vascular integrity could possibly influence interstitial fluid composition or impede adequate ventricular perfusion, thereby influencing cellular membrane permeability or at least suggest disruption of the myocardial tissue integrity. Finally, since many mammalian hearts are perfused with solutions at 36–39 °C we decided to investigate the effect of temperature on the anion permeability of these tissue.

We have therefore examined the anion permeability and tissue morphology of the isolated heart preparation in an effort to assess the usefulness of this model to obtain reliable estimates of in situ ion flux measurements.

Rats, guinea-pigs and hamsters weighing 200–300 g were heparinized (200 USP E) and allowed to sit for 1 h before they were anesthetized with ether and the hearts excised. The hearts were handled in a manner described by Polimeni and Page[5]. Briefly, the excised hearts were placed in 2 °C Ringer's solution and a cannula inserted into the aorta and clipped in place with a small hemostatic clip. The hearts were immediately perfused via the coronary circulation by gravity flow at a pressure of 80 cm H_2O. Perfusion was aided by the spontaneous contraction of the heart. The hearts were attached to a cannula and placed in a Ringer's bath maintained at either 22 or 36 °C. The bath solution was pumped up to the perfusate reservoir and recycled through the heart. The volume of solution recirculated ranged from 200 to 350 ml. The composition of both perfusion and superfusion solutions were (in mM): Na 126.2, Cl 113.5, K 5.9, Mg 0.8, Ca 3.0, H_2PO_4 1.2, ethylenediaminetetraacetate 0.5 and HCO_3 25 (PH = 7.4). The solution also contained 25 mM glucose and was oxygenated by bubbling it with 95% O_2 and 5% CO_2. Following 90 min of perfusion, the hearts were removed, the left ventricle dissected away from the heart, placed in a glutaraldehyde fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.07 M of phosphate buffer (pH = 7.2 at 4 °C) and cut into small strips. The ventricular strips were then placed in fresh fixative for 4 h (4 °C), postfixed in OSO_4 and processed routinely for transmission electron microscopic examination of the ventricular tissue and vascular morphology. A 2nd group of hearts were perfused via the coronary circulation while being superfused by the bath solution for 120 min with Ringer's (22 or 36 °C) containing approximately 2μ Ci/ml $^{35}SO_4$. Following the in vitro perfusion, the hearts were removed, the left ventricle isolated and analyzed for its $^{35}SO_4$ content according to methods described by Macchia et al.[9]. From the equilibrium distribution of $^{35}SO_4$, the sulfate space was determined for these tissues. Since this large impermeant anion is thought to distribute itself homogenously throughout the extracellular (EC) compartment of cardiac tissue (following the equilibrium distribution), the volume with which $^{35}SO_4$ distributes itself (sulfate space) would necessarily equal the extracellular volume (ECV). When tissues are studied under conditions which cause an increase in anion permeability the $^{35}SO_4$ will enter the tissue cells and the apparent sulfate space would increase and no longer be a good estimate of ECV. In order to measure the ECV accurately under conditions in which the tissue anion permeability changes, we elected to use morphometric space measurements according to the methods of Polimeni[10] and Page[11]. This procedure involves the preparation of tissue sections, photographing them at a magnification of X750, and determining relative ECV by 'point counting'. ECV determined by this procedure have previously been shown to be identical with the sucrose space in toad and rat skeletal and cardiac muscles[12]. The fraction of the tissue weight which is water, fH_2O, was determined from the tissue wet and dry weights according to Macchia et al.[9]. The table gives the mean sulfate space (± SEM) for isolated rat, guinea-pig and hamster hearts following 120 min of perfusion and superfusion with 22 °C protein-free Ringer's. As can be seen, the mean sulfate spaces (in g EC H_2O/g wet wt) of 0.232, 0.243 and 0.245 were found to be significantly larger than the morphometric spaces of 0.093, 0.085 and 0.084 for rat, guinea-pig and hamster, respectively. The table also gives the mean sulfate space for isolated rat, guinea-pig and hamster hearts following 120 min of perfusion and superfusion with 36 °C protein-free Ringer's. As shown in the table, the mean sulfate space for rat (0.096) and guinea-pig (0.094) hearts were not statistically different from the morphometric space of these tissues. The sulfate space of hamster hearts perfused-superfused with 36 °C Ringer's (0.142) was observed to be significantly smaller than when the hearts were perfused with 22 °C Ringer's (0.245), but was still found to be approximately 2 times larger than the morphometric space. It is interesting to note here that the morphometric space measurements for the animals studied are significantly less than the in situ morphometric space measurements of 0.20–0.24 reported earlier from our laboratory[9,13]. We interpret this observation as cellular swelling in hearts perfused with solutions of either 22 or 36 °C. The idea of cellular swelling is further supported by the difference observed in the fH_2O of perfused animal heart tissues (0.825–0.853) and the in situ fH_2O-values of 0.77–0.78 reported earlier. This along with the significantly smaller perfused heart morphometric space measurements (as compared to the in situ values) would suggest a significant increase in cellular water content. Absence of a difference in ECV (as measured morphometrically) of hearts perfused with 22 vs 36 °C Ringer's along with a concommittant large change in sulfate space would suggest that at 22 °C the larger impermeant anion is entering the cellular compartment of these tissues as a result of a change in the tissue anion permeability. Here, an increase in anion permeability of the sarcolemma would necessarily result in an increase in the apparent sulfate space. In spite of the difference in ECV observed in in vitro vs in situ hearts, at 36 °C the close agreement between the 2 space measurements for rat and guinea-pig was interpreted as a maintenance of the tissue anion permeability.

Since little or no differences existed between the sulfate space and the morphometric space for rat and guinea-pig hearts perfused at 36 °C we considered the absence of plasma proteins from the perfusate of no consequence. The large difference between these 2 space measurements for hamster hearts perfused at 36 °C is as yet not understood.

Sulfate and morphometric space measurements and F_{H_2O} of isolated rat, guinea-pig and hamster ventricles perfused and superfused with 22 °C and 36 °C Ringer's

Animal	Temperature (°C)	Sulfate space G EC water / G wet weight	Morphometric space EC volume / Muscle volume	F_{H_2O}
Rat	22	0.232 ± 0.011 (6)	0.093 ± 0.004 (4)	0.832 ± 0.005 (9)
	36	0.096 ± 0.004 (4)	0.082 ± 0.007 (3)	0.841 ± 0.005 (8)
Guinea-pig	22	0.243 ± 0.030 (4)	0.085 ± 0.005 (3)	0.841 ± 0.008 (7)
	36	0.094 ± 0.006 (3)	0.078 ± 0.008 (3)	0.853 ± 0.002 (4)
Hamster	22	0.245 ± 0.014 (4)	0.084 ± 0.006 (4)	0.822 ± 0.010 (4)
	36	0.142 ± 0.001 (3)	0.077 ± 0.006 (3)	0.825 ± 0.002 (3)

Sulfate space and morphometric data are from a separate experimental series of animals. The number of prints processed, number of points counted and total cross-sectional area analyzed (according to methods of Polimeni[10]) were, respectively, 40; 36; 720; and 706 × 10^4 μm^2. Muscles were fixed in Na cacodylate buffer (pH = 7.4), approximately isosmolal with hamster plasma, to which glutaraldehyde (final concentration 2%) was added. Muscles were embedded in paraffin and stained with hematoxylin and eosin. Mean ± SEM (n).

Preliminary studies do not support the idea that plasma proteins absent from the perfusate are responsible for this difference.

Ultrastructurally, the perfused-superfused ventricles of all 3 species did not appear to have been significantly altered by long perfusion times. The endothelial cells lining the continuous capillaries were found to be intact with no separation at the junctions. More importantly, the myocardial cells appeared intact and healthy. The mitochondria were neither swollen nor condensed and the myofibrillar pattern was regular. The absence of blood elements in our micrographs confirm the adequacy of myocardial muscle perfusion via the coronary vasculature.

These results support the view that when rat and guinea-pig hearts are perfused-superfused with a 36 °C Ringer's solution, unlike skeletal muscle[14], the anion permeability of these tissues are not influenced by the absence of plasma proteins in the perfusate. This would confirm the reliability of the Ringer's perfused heart preparation as described by Polimeni and Page[5] for measuring anion fluxes in vitro which approximates close to that in situ.

1 Acknowledgments. Ms Joyce Craig is to be thanked for assistance in the preparation of the electron micrographs. This work was supported by an award from the American Heart Association, Indiana Affiliate and the NIH (AM 27148) made to Dr Donald Macchia. P.B. was supported in part by funds from the Lake County Medical Center Development Agency and an award from the American Heart Association, Indiana Affiliate.

2 Bergmann, S.R., Clark, R.E., and Sobel, B.E., Am. J. Physiol. *236* (1979) H644.
3 Bunder, R., Haddy, F.J., Querengasser, A., and Gerlach, E., Pflügers Arch. *353* (1975) 317.
4 Fallen, E.L., Elliott, W.C., and Gorlin, R., J. appl. Physiol. *22* (1967) 836.
5 Polimeni, P.I., and Page, E., Am. J. Physiol. *238* (1980) C169.
6 Macchia, D.D., Physiologist *24* (1981) 88.
7 Golnick, P.L., Anderson, W.M., and Macchia, D.D., Physiologist *24* (1981) 88.
8 Carmeliet, E., and Verdonck, F., J. Physiol., Lond. *265* (1977) 193.
9 Macchia, D.D., Polimeni, P.I., and Page, E., Am. J. Physiol. *237* (1979) C125.
10 Polimeni, P.I., Am. J. Physiol. *227* (1974) 676.
11 Page, E., Am. J. Physiol. *235* (1978) C147.
12 Page, E., Polimeni, P.I., and Macchia, D.D., Ann. N.Y. Acad. Sci. *341* (1980) 524.
13 Macchia, D.D., and Didelot, M.J., Fedn Proc. *41* (1982) 1524.
14 Macchia, D.D., Am. J. Physiol. *11* (1982) C207.

0014-4754/83/111291.03$1.50 + 0.20/0

The effect of bisphosphonates on glycolysis in cultured calvaria cells and their homogenate

R. Felix and H. Fleisch

Department of Pathophysiology, University of Bern, Murtenstrasse 35, CH-3010 Bern (Switzerland), February 18, 1983

Summary. Rat calvaria cells previously cultured for 7 days in the presence of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) or dichloromethylenebisphosphonate (Cl_2MBP), showed a decrease in the glycolytic pathway. When glycolysis was analyzed under anaerobic conditions, this effect was not observed. The inhibition by the bisphosphonates occurred to a similar degree regardless of whether lactate production was measured in whole cells or in cell homogenates. Bisphosphonates added directly to the homogenate had no inhibitory effect. Thus, the effect is not a direct one and is unlikely to be due to a soluble mediator in the cytoplasm.

Bisphosphonates are compounds which contain a P–C–P bond and are thus related to pyrophosphate, but are resistant to metabolic destruction. They inhibit mineral formation and dissolution in vitro and prevent ectopic calcification and resorption of bone in vivo[2–5]. These effects have been made use of clinically. Thus, 1-hydroxyethylidene-1,1-bis-phosphonate (HEBP) has been found to decrease the development of ectopic ossification after total hip replacement[6] and in paraplegia[7]. Furthermore, various bisphosphonates have proved useful in the management of Paget's disease, a disease in which bone turnover is increased[8–10] and in tumoral bone disease[11,12].

Initially, these in vivo effects were mainly attributed to physico-chemical interaction of the bisphosphonates with calcium phosphate crystals. In recent years, however, it has been shown that bisphosphonates also influence cellular metabolism[13]. Recently, it has been demonstrated that HEBP and dichloromethylenebisphosphonate (Cl_2MBP) are taken up by calvaria cells in culture, where intracellular concentrations of up to three times that in the medium were measured[14,15]. 75% of the accumulated bisphosphonate is in the cytosol, and apart from other effects[13] these compounds decrease the production of lactate[14]. The mechanism by which they act on the glycolytic pathway is as yet unknown. To elucidate the mechanism of action, the effect of HEBP and Cl_2MBP on the production of lactate under anaerobic conditions was studied. Furthermore, the rate of glycolysis was measured in homogenates of cells treated with these bisphosphonates.

Materials and methods. The bisphosphonates 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) and dichloromethylenebisphosphonate (Cl_2MBP) were provided by the Procter & Gamble Co., Cincinnati, USA.

Cell culture. Calvaria cells of 1-day-old rats were isolated and cultured in petri dishes with a diameter of 5.5 cm (Corning) or in 24-well tissue culture cluster dishes 3542, diameter 1.6 cm (Costar, Cambridge, USA) as previously described[14]. The medium was changed on days 1, 4 and 7 (day of plating: day 0). The bisphosphonates were added from days 1–8. To determine lactate production, cells were incubated in 95% air, 5% CO_2 or in 95% N_2, 5% CO_2 at days 7–8 for 16 h[14]. Medium was then collected, protein precipi-