Biochemical Pharmacology, Vol. 44, No. 8, pp. 1529–1534, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION OF SODIUM PUMP BY BEPRIDIL

AN *IN VITRO* AND MICROCALORIMETRIC STUDY

H. KOVACIC, P. GALLICE and A. CREVAT*
Laboratoire de Biophysique, UFR de Pharmacie, 27 Bd Jean Moulin, F-13385 Marseille Cedex 5, France

(*Received* 19 *March* 1992; *accepted* 23 *July* 1992)

Abstract—The effects of diltiazem, verapamil, bepridil, nicardipine and nifedipine were studied *in vitro* on Na^+,K^+-ATPase from dog kidney (EC 3.6.1.37). Except diltiazem, all the drugs tested showed an inhibitory effect on Na^+,K^+-ATPase activity in a dose-dependent manner. Among these drugs bepridil is far more effective than the others ($IC_{50} \approx 10^{-4}$ M). Competition studies showed that bepridil acted in a non-competitive manner with the ATP–Mg^{2+} complex and in a partially competitive manner with K^+. Since ouabain acted similarly under the same experimental conditions, we tested the interaction of bepridil and ouabain on Na^+,K^+-ATPase. With low doses of ouabain, the enzyme inhibition corresponded to a potentiated synergy of the two drugs. We then studied the action of bepridil on the sodium pump activity of intact red blood cells by an *ex vivo* microcalorimetric technique. At 10^{-5} M bepridil caused a significant decrease in sodium pump activity (33 ± 8%).

Classically, calcium antagonist (CA†) drugs act by blocking calcium entry through slow calcium channels, thus leading to a decoupling between excitation and contraction. Among the side-effects of these drugs, hydro-electrolytic disorders have been described; particularly a natriuretic effect [1–3]. The cellular mechanism of this action is not yet entirely elucidated [4, 5] but a perturbation of the ATP-dependent ion pumps has been evoked. Studies performed to test the hypothesis have shown discordant results. Findings showed either a stimulation or no effect on Na^+,K^+-ATPase [6–8]. In the case of bepridil, action on Ca^{2+}-ATPase has been proved [9] but no study on the sodium pump has been performed.

The aim of the present study was to evaluate the effect of some CAs and particularly bepridil on Na^+,K^+-ATPase by using an *in vitro* technique and an *ex vivo* microcalorimetric method. We chose one CA molecule among each of the classical clinical classes of these drugs [10]: drugs with predominantly vascular effects such as dihydropyridines, drugs with predominantly cardiac effects such as verapamil and diltiazem, and drugs with a complex pharmacological profile such as bepridil.

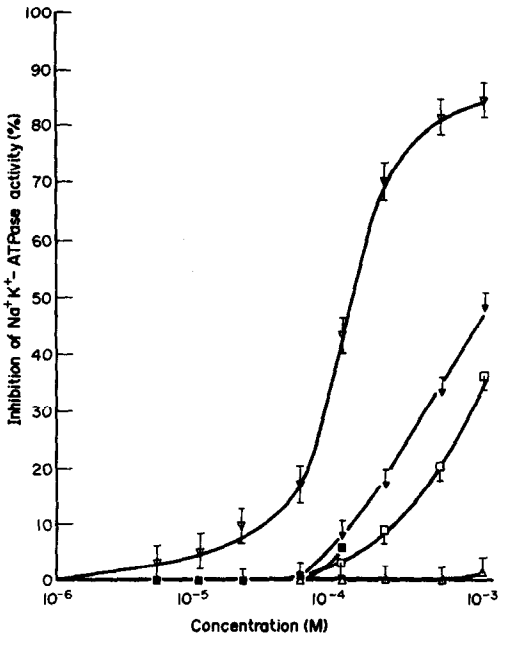

Fig. 1. Inhibitory effect (%) of CA on Na^+,K^+-ATPase activity. (▽) Bepridil, (▼) nicardipine, (△) diltiazem, (■) nifedipine and (□) verapamil. Each point corresponds to the mean value ± SD of triplicate determinations.

MATERIALS AND METHODS

Na^+,K^+-*ATPase assay*

Assays on Na^+,K^+-ATPase (EC 3.6.1.37), 90% ouabain inhibitable from dog kidney (Sigma), were prepared by suspending the enzyme in 100 mM Tris–HCl buffer, pH 7.4 (0.28 mg protein/mL). The ATPase assay incubating medium consisted of 700 μL of 100 mM Tris–HCl buffer, pH 7.4 containing 28.6 mM KCl; 142.8 mM NaCl; 28.6 mM $MgCl_2$, 6 H_2O; 1.4 mM EGTA and 4.3 mM ATP disodium salt (vanadate free). CA solutions were then added (200 μL). Controls were done under the same conditions without CA.

After incubation for 5 min at 37°, the reaction was started by rapid addition of 100 μL of ATPase

* Corresponding author.

† Abbreviations: CA, calcium antagonist; EGTA, ethyleneglycolbis (β-aminoethylether)-*N,N,N',N*-tetra-acetic acid; RBC, red blood cell; HP, heat production.

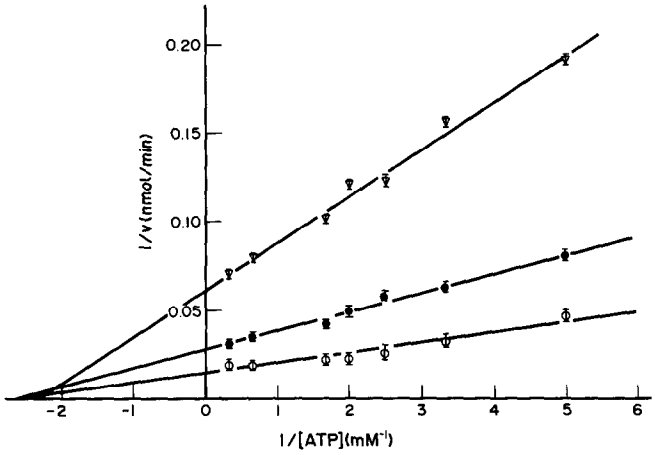

Fig. 2. Lineweaver–Burk plots of the inhibition of Na^+,K^+-ATPase by bepridil as a function of ATP–Mg^{2+} complex concentration. Without (○) and with 0.1 (●) or 0.2 mM (▽) bepridil. Lines were fitted by linear regression. $[ATP]/[Mg^{2+}] = 6.7$. Each point corresponds to the mean value ± SD of triplicate determinations.

preparation and stopped 10 min later by adding 1 mL of cold perchloric acid (1.5 M). After centrifugation at 2000 *g* for 10 min, aliquots (100 μL) of the supernatants were analysed for their inorganic phosphorus content according to Hurst's method [11].

Blanks were done under the same conditions without enzymic suspension. The inhibition percentage induced by CA was calculated as follows:

$$\text{Inhibition} = \frac{\text{Control activity} - \text{Assay activity}}{\text{Control activity}} \times 100.$$

Microcalorimetric assay

Preparation of red blood cells (RBCs). Venous blood (30 mL) from six healthy subjects was collected in sodium heparin tubes. After centrifugation at 600 *g* for 10 min, plasma was collected and recentrifuged for 10 min at 2000 *g* to yield clear plasma. Blood cells were resuspended in 0.9% sodium chloride solution and passed through a column packed with a mixture of α cellulose and microcrystalline cellulose (50:50 w/w) to separate RBCs from leukocytes and platelets, according to Beutler's method [12]. RBCs were then packed by centrifugation at 2000 *g* for 10 min, and 0.5 mL of the RBC pellet for each healthy subject was resuspended in 3.6 mL of the subject's own cleared plasma. Hematocrit value, and counts of leukocytes and platelets were determined on aliquots with a Technicon H1 System apparatus.

Microcalorimetry. We used an LKB flow Bioactivity monitor 2277 microcalorimeter thermostated at 37°. Thermograms were recorded at a chart speed of 10 cm/hr and at a sensitivity of 30 μW full-scale. Baseline was obtained with subject's own plasma pumped through the microcalorimetric cell at a flow rate of 36 mL/hr. The thermogram was then recorded by pumping, at the same rate, 3.5 mL of RBC suspension preincubated at 37° for 60 min. The steady state in heat production (HP) was reached by continuous pumping in closed circuit (v = 3 mL), then ouabain solution was added at a flow rate of 1 mL/hr for 6 min (final concentration 0.4×10^{-3} M). For bepridil assays, drug was preincubated with RBC suspension for 60 min at 37° and the same experiment was carried out.

RESULTS

Action of CA on Na^+,K^+-ATPase

The effects of CAs (bepridil, diltiazem, nicardipine, verapamil and nifedipine) on Na^+,K^+-ATPase activity studied in a concentration range of 10^{-6}–10^{-3} M are reported in Fig. 1.

Competition of bepridil with ATP

Figure 2 shows the Lineweaver–Burk plot of enzyme inhibition by fixed concentrations of bepridil (0.1 and 0.2 mM) in the presence of increasing ATP concentrations (0.3–3 mM). The $[ATP]/[Mg^{2+}]$ ratio remained constant in all experiments ($[ATP]/[Mg^{2+}] = 6.7$). There is a common intersection on the *x*-axis, showing that the affinity of the ATP–Mg^{2+} complex for the enzyme is not modified in the presence of bepridil.

Competition of bepridil with potassium

Figure 3 shows the Lineweaver–Burk plot of enzyme inhibition by the same concentrations of bepridil as for ATP competition but in the presence of increasing potassium concentration (1–20 mM). For comparison, the insert shows ouabain action (5×10^{-6} M) under the same experimental conditions. There is a common intersection above the *x*-axis and at the left of the *y*-axis as in the case of ouabain. Classically, such curves correspond to a partially competitive inhibition mechanism [13].

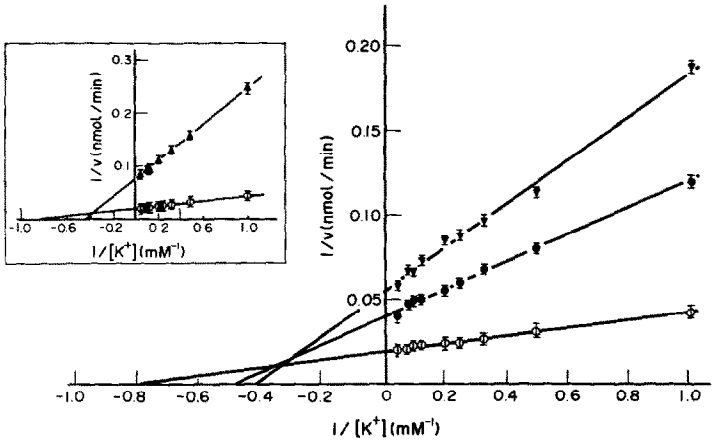

Fig. 3. Lineweaver–Burk plots of the inhibition of Na^+,K^+-ATPase by bepridil as a function of K^+ concentration. Without (○) and with 0.1 (●) or 0.2 mM (▽) bepridil. Inset represents plots obtained with ouabain 5×10^{-6} M (▲) under the same conditions. Lines were fitted by linear regression. The ATP and Mg^{2+} concentrations were 3 and 20 mM, respectively. Each point corresponds to the mean value ± SD of triplicate determinations.

Table 1. Bepridil effect on RBC sodium pump activity from six different healthy subjects

Subjects	1	2	3	4	5	6
Control						
HP (mW/L RBCs)	18.0	13.0	15.0	9.0	15.0	12.5
Bepridil 5×10^{-6} M						
HP (mW/L RBCs)	17.0	12.0	14.0	—	—	—
Inhibition (%)	5.5	7.7	6.7	—	—	—
Bepridil 10^{-5} M						
HP (mW/L RBCs)	10.5	8.0	9.5	6.5	12.0	8.0
Inhibition (%)	41.7	38.5	36.7	38.9	20.0	36.0

Each value represents the mean of three experiments. Methodological error(s) was 1 mW/L RBCs.

Bepridil–ouabain interaction

Figure 4 shows dose-dependent curves for the inhibition of Na^+,K^+-ATPase by ouabain (10^{-7}–10^{-4} M) which were obtained without and with 4×10^{-6} or 4×10^{-5} M bepridil. In Fig. 5 ouabain concentration is plotted on the *x*-axis and bepridil concentration on the *y*-axis. Each point of the curve corresponds to a mixture of ouabain and bepridil leading to an inhibition of 40%. The isobole thus plotted must be a straight line in the case of a mere additive effect. The concave line we obtained implies that the two drugs act in a potentiated synergistic manner. For example, with 0.5 μM ouabain, 46 μM bepridil would be necessary to obtain 40% inhibition in the case of an additive effect. As can be seen in Fig. 5, only 25 μM bepridil leads to the same inhibition. For all the above experiments each result represents the mean ± SD of three experiments.

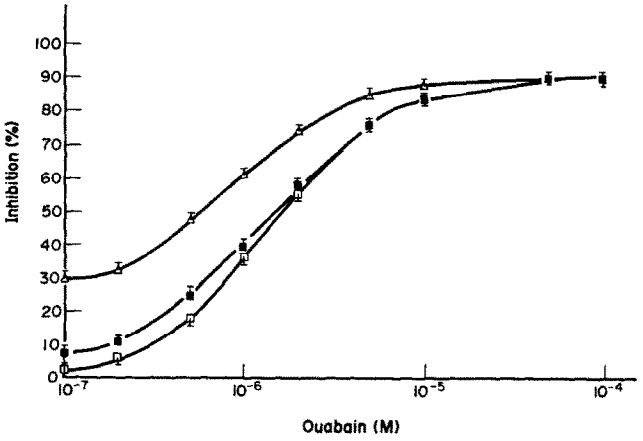

Fig. 4. Effect of bepridil on Na^+,K^+-ATPase inhibition by ouabain. Without (□) or with 4×10^{-6} (■) and 4×10^{-5} M (△) bepridil. Each point corresponds to the mean value ± SD of triplicate determinations.

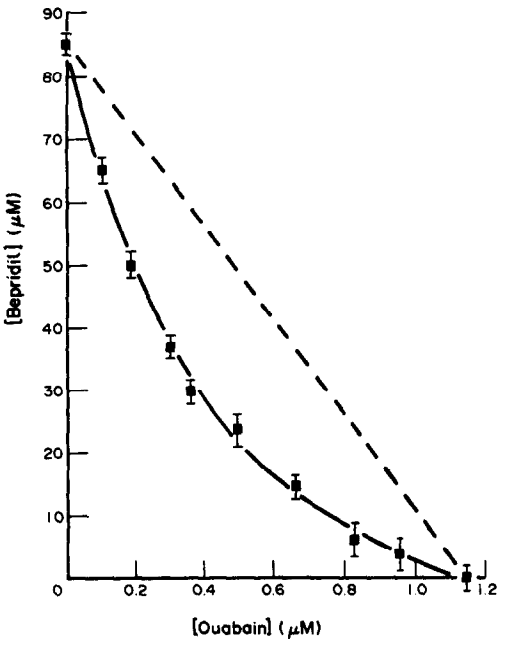

Fig. 5. Loewe isobole for 40% Na^+,K^+-ATPase inhibition. Each point of the curve corresponds to a mixture of ouabain and bepridil leading to a 40% inhibition (dashed line represents the theoretical curve for a mere additive effect).

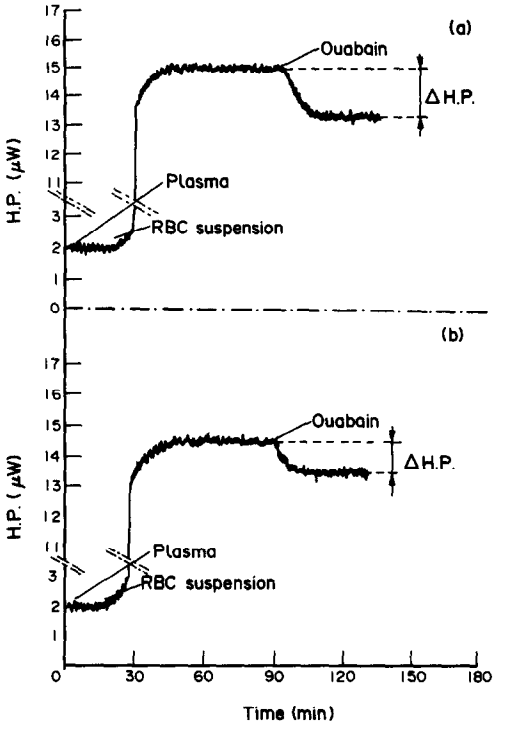

Fig. 6. Microcalorimetric assay for bepridil inhibitory effect on living RBC sodium pump activity. (a) A typical thermogram from one determination of the HP of an RBC sample in plasma. The samples are introduced at the times indicated by arrows. RBC suspension = 3.5 mL. Ouabain final concentration, 4×10^{-4} M. HP represents HP due to sodium pump. (b) The same experiment after incubation with bepridil (10^{-5} M).

Microcalorimetric assay

Figure 6 shows a typical recording of HP without (a) and with (b) bepridil (10^{-5} M). The difference between steady-states obtained before and after ouabain addition corresponds to the sodium pump activity (ΔHP) which is expressed as mW/L RBCs [14]. From these recordings we measured the action of two doses of bepridil on the sodium pump (Table 1). The methodological error(s) in ΔHP determination was calculated from the expression [14]:

$$s = (\Sigma d^2/2N)^{1/2}$$

where d is the difference between two calorimetric determinations on the same RBC subject and N is the total number of subjects studied. Under our experimental conditions, $s = 1$ mW/L RBCs.

DISCUSSION

The CA drugs we studied exerted various effects on isolated Na^+,K^+-ATPase activity in the concentration range tested. Among the CA tested, the effect on Na^+,K^+-ATPase seems to be related to clinical classification. Drugs with predominantly cardiac effects exerted no inhibitory action (diltiazem) or did so at very high concentration ranges (verapamil: inhibition 50% > 10^{-3} M). Drugs with a complex pharmacological profile like bepridil were more effective than the other drugs ($K_a \approx 10^4$ M^{-1}). Our results agree with those of others since flunarizine, which is also in this group, was reported to inhibit Na^+,K^+-ATPase from the cerebral cortex in the same dose/effect ratio [8]. Finally, among drugs with predominantly vascular effects such as dihydropyridines, nicardipine seems to act in a concentration range intermediate between the two other classes. Nifedipine had the same behavior as nicardipine up to 10^{-4} M but because of its poor solubility it was impossible for us to study it under our experimental conditions at concentrations higher than 10^{-4} M. As bepridil exerted the most potent effect, we sought to specify some points about its mechanism of action. Competition between bepridil and the ATP–Mg^{2+} complex (Fig. 2) was performed with a constant [ATP]/[Mg^{2+}] ratio leading to a concomitant change in free Mg^{2+} concentration. Consequently, the effect observed might be due at least in part to this change. Indeed, high concentrations of free Mg^{2+} partially inactivate Na^+,K^+-ATPase as shown by Cantley and Josephson [15]. However, under our experimental conditions the inhibitory effect due to free Mg^{2+} would remain constant as calculated from Cantley and Josephson's results. Therefore, in our experiment the change in free Mg^{2+} concentration intervened but in a constant manner. Under these conditions, we can assert that bepridil acts in a non-competitive manner with the

ATP–Mg^{2+} complex since the curve intersects on the x-axis. The graphs in Fig. 3 show, for bepridil and ouabain, an intersection corresponding to a partially competitive inhibition with K^+. For ouabain such a result has been obtained previously [16, 17]. Thus, it seems that bepridil acts like this cardiac glycoside on Na^+,K^+-ATPase. For the same effect, the concentrations of bepridil were about 100-fold higher than those of ouabain (ouabain $IC_{50} \approx 1.4 \times 10^{-6}$ M, bepridil $IC_{50} \approx 10^{-4}$ M), reflecting a lesser affinity of bepridil for Na^+,K^+-ATPase.

These results led us to study the possibility of an interaction of these drugs. Such an interaction is evidenced by the results shown in Fig. 4. IC_{50} for ouabain was significantly lower in the presence of bepridil. It decreased from 1.4×10^{-6} to 1.2×10^{-6} M, and then to 0.4×10^{-6} M for bepridil concentrations equal to 0, 4×10^{-6} and 4×10^{-5} M, respectively. Moreover, for the above concentrations the percentages of inhibition observed with the mixture of the drugs were higher than those obtained with the mere addition of the effects of each drug taken separately, thus suggesting a potentiated synergistic inhibitory effect. This was confirmed by the curve in Fig. 5 which shows a potentiated synergy (see Results). However, it seems that this is an indirect synergy since the potentiation disappears at high ouabain doses; for instance, with bepridil = 4×10^{-5} M the synergy disappears for concentrations of ouabain greater than 2×10^{-6} M. It is possible that, at high enough concentrations, ouabain displaces bepridil from its action site.

In vitro measurements of Na^+,K^+-ATPase inhibition are often not sufficiently indicative of sodium pump inhibition in the intact cell [18]. Microcalorimetry permits measurements of sodium pump activity in living RBCs [19, 20]. Bepridil induced a significant dose-dependent decrease in sodium pump activity. This result obtained on living cells is in agreement with the above *in vitro* findings. However, in our experiments, living RBCs were incubated with bepridil before measurements were taken, which may explain why bepridil is much more effective on living cells than in *in vitro* experiments. The concentrations used were higher than those observed in plasma from patients treated with the drugs ($\approx 10^{-6}$ M) but, as proved by Cramb and Dow [21] for isolated ventricular myocytes incubated with bepridil, the drug is concentrated 100-fold within the cell. It is always difficult to correlate *in vitro* results with an effect observed *in vivo*, but the concentrations we used to demonstrate the effect of bepridil on the sodium pump are in the range of those observed in myocytes. Therefore, if our *in vitro* results also occur *in vivo*, a pharmacological effect similar to those of digitalic drugs should be observed, which is not the case except for the negative chronotropic effect [22]. The explanation for this discrepancy may be found in the inhibition of Na^+,Ca^{2+} counter transport by bepridil [23] and in the high affinity sites for the drug in the membranes of myocytes and within them [21, 24]. Such an affinity would hinder the binding of bepridil with Na^+,K^+-ATPase ($K_a \approx 10^4\ M^{-1}$) thus explaining why bepridil did not show a more marked ouabain-like action.

Acknowledgements—The authors are grateful to Professor J. C. Sari for his helpful discussion, and H. Bouteille and M. Vidalin for their skilful technical assistance.

REFERENCES

1. Ene MD, Williamson PJ, Roberts CJC and Waddell G, The natriuresis following oral administration of the calcium antagonist—nifedipine and nitrendipine. *Br J Clin Pharmacol* **19**: 423–427, 1985.
2. Young MA, Watson RDS, Stallard TJ and Littler WA, Calcium channel blockers, are they diuretics? *Br J Clin Pharmacol* **20**: 95s–98s, 1985.
3. Leonetti G and Zanchetti A, Renal effects of calcium antagonists in systemic hypertension. *J Hypertens* **3** (Suppl 3): S537–541, 1985.
4. Zanchetti A, Role of calcium antagonists in systemic hypertension. *Am J Cardiol* **59**: 130b–136b, 1987.
5. Romero JC, Roij L, Granger JP *et al.*, Multiple effects of calcium entry blockers on renal function in hypertension. *Hypertension* **10**: 140–151, 1987.
6. Pan M and Janis RA, Stimulation of Na,K-ATPase of isolated smooth muscle membrane by the Ca^{2+} channel inhibitors, nimodipine and nitrendipine. *Biochem Pharmacol* **33**: 787–791, 1984.
7. Kloke HJ, Swarts HGP, Sluiter HE, Huysmans FTM and de Pont JJHM, Effects of dihydropyridine calcium antagonists on rabbit renal Na,K-ATPase activity *in vitro*. *Eur J Pharmacol* **147**: 119–123, 1988.
8. Palmer GC, Palmer SJ and Christie-Pope BC, Protective action of calcium channel on Na,K-ATPase in gerbil cerebral cortex following ischemia. *J Neurosci Res* **19**: 252–257, 1988.
9. Lamers JMJ, Verdouw DP and Mas-Oliva J, The effects of felodipine and bepridil on calcium stimulated calmodulin binding and calcium pumping ATPase of cardiac sarcolemma before and after removal of endogenous calmodulin. *Mol Cell Biochem* **78**: 169–176, 1987.
10. Singh BN, Baky S and Nademance K, Second generation calcium antagonists: search for greater selectivity and versatility. *Am J Cardiol* **55**: 214–221, 1985.
11. Hürst R, The determination of nucleotide phosphorus with a stannous chloride-hydrazine sulfate reagent. *Can J Biochem* **42**: 287, 1964.
12. Beutler CW, The removal of leucocytes and platelets from whole blood. *J Lab Clin Med* **88**: 328–333, 1976.
13. Metzler DE, Enzymes: the protein catalysts of cells. In: *Biochemistry, The Chemical Reactions of Living Cells*, pp. 301–352. Academic Press, New York, 1977.
14. Levin K, Determination of heat production from erythrocytes in normal man and in anemic patients with flow microcalorimetry. *Scand J Clin Lab Invest* **32**: 55–65, 1973.
15. Cantley LC and Josephson L, A slow interconversion between active and inactive states of the Na,K-ATPase. *Biochemistry* **15**: 5280–5287, 1976.
16. Matsui H and Schwartz A, Kinetic analysis of ouabain-K^+ and Na^+ interaction on a Na^+,K^+-dependent adenosine triphosphate from cardiac tissue. *Biochem Biophys Res Commun* **25**: 147–152, 1966.
17. Post L and Sen LK, Sodium and potassium-stimulated ATPase. In: *Methods in Enzymology* (Eds. Estabrook RW and Pullman ME), Vol. 10, pp. 762–768. Academic Press, New York, 1967.
18. Swaminathan R, Red blood cell sodium transport and phosphate release in uremia. *Nephron* **37**: 142, 1984.
19. Monti M, Hedner P, Ikomi-Kumm J and Valdemarsson

S, Erythrocyte thermogenesis in human obesity: microcalorimetric investigation of sodium–potassium pump and cell metabolism. *Metabolism* **36**: 155–159, 1987.
20. Monti M and Ikomi-Kumm J, Erythrocyte heat production in human obesity. *Metabolism* **34**: 183–188, 1985.
21. Cramb G and Dow JW, Uptake of bepridil into isolated ventricular myocytes. *Biochem Pharmacol* **32**: 227–231, 1983.
22. Remme WJ, Kruijssen HACM, Krauss XH, Hooghenhuyze DCA and Storm CJ, The acute hemodynamic effects of intravenous Bepridil in patients with coronary artery disease. *Rev Med* **24**: 1281–1288, 1983.
23. Garcia ML, Slaughter RJ, King VF and Kaczorowski GJ, Inhibition of sodium calcium exchange in cardiac sarcolemmal membranes vesicles. *Biochemistry* **27**: 2410–2415, 1988.
24. Cramb G and Dow JW, Two site binding of Bepridil and modulation of adenylate cyclase in cardiac sarcolemmal membranes. *Biochim Biophys Acta* **736**: 99–108, 1983.