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# MECHANISM OF INHIBITION OF SODIUM- AND POTASSIUM-DEPENDENT ADENOSINE TRIPHOSPHATASE BY THE ISOQUINOLINE DERIVATIVE BIIA: A SPECIFIC INTERACTION WITH SODIUM ACTIVATION*

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**Abstract**—An isoquinoline derivative, 3-benzylamino-5,6-dihydro-8,9-dimethoxy-imidazo-(5,1-a)-isoquinoline hydrochloride, BIIA, with positive inotropic and antiarrhythmic actions, reversibly inhibited the $Na^+$, $K^+$-ATPase of deoxycholate and NaI treated microsomes from guinea-pig heart, brain and kidney. The inhibition was pH dependent, increasing with increasing pH and the concentration of the highly lipid soluble, unprotonated molecule. BIIA inhibited $Na^+$, $K^+$-ATPase in a concentration range of 1–100 μmoles/l, in a manner uncompetitive with respect to ATP and $K^+$, and competitive with respect to $Na^+$. $K^+$PNPPase of the same preparation was also inhibited by BIIA, albeit at higher concentrations. This inhibition was competitive with $K^+$. Affinity for substrate, as measured with [$^{14}$C]-ATP, was increased and labelling from $AT^{32}P$ decreased competitive with $Na^+$. BIIA decreases the affinity of the enzyme for ($Mg^{2+}$ + $P_i$) supported ouabain binding, higher concentrations of BIIA also affect ($Na^+$ + ATP) supported binding. It is suggested that BIIA enters a hydrophobic environment of the $Na^+$, $K^+$-ATPase and interacts at or near the $Na^+$-activation sites, inhibiting the formation of the phosphorylated intermediate.

The observation of the positive inotropic action of BIIA on atrial and ventricular myocardium of the guinea-pig [1, 2] led us to investigate the effects of this substance on the isolated $Na^+$, $K^+$-stimulated, $Mg^{2+}$-dependent adenosine triphosphatase ($Na^+$, $K^+$-ATPase; EC 3.6.1.3) and the accompanying $K^+$-activated *p*-nitrophenyl phosphatase ($K^+$-PNPPase; EC 9.6.1.7) of the same species. The fact that BIIA inhibited the $Na^+$, $K^+$-ATPase competitive with $Na^+$, indicated a different mechanism of inhibition to that of cardiac glycosides [2–4].

The investigation of the mechanism of action of a number of $Na^+$, $K^+$-ATPase inhibitors has been of great help in efforts to understand the complex reaction sequence of this enzyme [4]. There are few reagents which specifically interact with the activation by one cation. Phlorizin [5] and deuterium oxide [6] have been found to decrease the affinity of the enzyme specifically for $Na^+$. Subsequently Canessa *et al.* [7] reported an inhibition of the $Na^+$, $K^+$-ATPase by harmaline, a psychotomimetic, indole derivative, which was competitive towards $Na^+$ but not towards $K^+$. Further investigations by Robinson [8] showed, however, that a simple competitive inhibition did not exist, not only did the extent of inhibition not approach zero as the concentration of $Na^+$ was increased, but the apparent $V_{max}$ was decreased by harmaline, due to a parallel decrease in affinity for $K^+$. In the light of these experiences it was considered necessary to investigate the specificity of the interaction of BIIA with the $Na^+$-activation kinetics of the $Na^+$, $K^+$-ATPase, especially as this enzyme may, as is discussed for cardiac glycosides [9, 10] be the receptor mediating positive inotropic action.

## MATERIALS AND METHODS

*Enzyme preparation.* $Na^+$, $K^+$-ATPase containing microsomes were obtained from guinea-pig heart as described by Akera *et al.* [11] and from kidney and brain according to Skou *et al.* [12] and Borsch-Galetke *et al.* [13] respectively, followed by NaI treatment according to Nakao *et al.* [14].

*Enzyme assays.* $Na^+$, $K^+$-ATPase was assayed by continuously measuring the oxidation of NADH in a Zeiss spectrophotometer at 340 nm and 37°, using a procedure first described by Fritz and Hamrick [15]. A routine assay contained (mmoles/l): 3 $Na_2ATP$, 5 $MgCl_2$, 100 NaCl, 10 KCl, 50 imidazole–HCl (pH 7.4), 0.25 NADH, 0.34 phosphoenolpyruvate, 5 units each of lactate dehydrogenase and pyruvate kinase and 20–50 μg enzyme protein. The reaction was started with the addition of NADH and ATP. $Na^+$, $K^+$-ATPase activity was calculated by subtracting that activity seen in the presence of 1 mmole/l ouabain from the total activity.

This assay could not be used when altering the

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$Mg^{2+}$ or $K^+$ concentration in the medium, as pyruvate kinase is dependent on both these ions. $Na^+$, $K^+$-ATPase activity was then measured in an incubation medium containing (mmoles/l): 3 $Na_2ATP$, 5 $MgCl_2$, 100 NaCl, 10KCl, 50 imidazole–HCl (pH 7.4) and 20–100 μg enzyme protein. The reaction was started by addition of ATP and allowed to proceed for 10 min. The reaction was stopped by the addition of 0.1 ml 70% TCA solution. Inorganic phosphate was determined according to Fiske and Subbarow [16]. Enzyme protein was measured according to Lowry *et al.* [17]. $K^+$-PNPPase was assayed according to Fujita *et al.* [18] in an incubation medium containing 50 mmoles/l Tris–HCl (pH 7.8), 3 mmoles/l $MgCl_2$, 3 mmoles/s *p*-nitrophenylphosphate and 14 mmoles/l KCl.

[$^{14}$C]-*ATP binding studies.* These were performed with semi-purified kidney $Na^+$, $K^+$-ATPase using a rapid centrifugation technique [19]. 0.5–1.0 mg of enzyme protein was incubated with 1 μmole/l Tris–ATP, containing $1 \times 10^5$ cpm [8-$^{14}$C]-ATP in a 60 mmole/l Tris–EDTA buffer, pH 7.0 for 10 min and then centrifuged at 20,000 *g* for a further 10 min. The precipitate was dissolved in 1 mole/l NaOH, neutralized, added to 10 ml of scintillation cocktail (Unisolve 1®, Werner Zinsser, Frankfurt) and counted in a Packard scintillation counter. Unspecific binding was measured in the presence of 10 mmoles/l Tris–ATP and subtracted from all values.

$Na^+$-*dependent* $^{32}$P-*ATP phosphorylation studies.* $^{32}$P labelling of partially purified kidney $Na^+$, $K^+$-ATPase was performed with [γ-$^{32}$P]-ATP at 0° following a modified procedure of Klodos and Skou [20]. 0.5–1.0 mg of enzyme protein was incubated with (mmoles/l): 50 Tris–HCl (pH 7.4), 5 $MgCl_2$, 1 $Na_2EDTA$, 120 NaCl, 0.5 $P_i$ and 1.25–12.5 μmoles/l ATP containing $1–5 \times 10^5$ cpm [$^{32}$P]-ATP. 10 sec after the addition of the ATP solution the reaction was stopped with 2 ml 5% TCA containing 0.1 mole/l $P_i$, the denatured protein was centrifuged and washed twice with the above mentioned solution. The final precipitate was dissolved in 1 ml 1 mol/l NaOH, neutralized and the radioactivity measured as mentioned above. Unspecific $^{32}$P-binding was taken as that present in the precipitate in the absence of NaCl and presence of 16 mmoles/l KCl.

[$^3$H]-*Ouabain labelling.* Ouabain binding experiments were performed with partially purified kidney $Na^+$, $K^+$-ATPase in two different media, essentially according to Erdmann and Schoner [21]. $Mg^{2+}$ and $P_i$ supported binding was measured in the presence of 50 mmoles/l imidazole–HCl (pH 7.4), 3 mmoles/l $MgCl_2$ and 3 mmoles/l $TrisPO_4$. Na and ATP supported binding was measured in the presence of (mmoles/l): 50 imidazole–HCl (pH 7.4), 100 NaCl, 5 $MgCl_2$, 4 ATP and 1 $Na_2EDTA$. Binding studies were performed in the presence of 20–50 μg enzyme protein and 2.5 nmoles/l[$^3$H]ouabain at 37°. Equilibrium was reached after 10 min incubation. For Scatchard analysis binding was measured in the presence of 0.05–2.5 μmoles/l unlabelled ouabain. The reaction was stopped by rapid cooling and centrifuging at 20,000 *g* for 10 min. The precipitate was dissolved and the radioactivity counted as already described. Specific binding was obtained by subtracting from the total radioactive uptake that amount bound in the presence of 0.1 mmole/l unlabelled ouabain.

Concentrations required for 50 per cent inhibition ($I_{50}$) were calculated from concentration-inhibition curves using a logit transformation [22]. All values are expressed as the mean ± s.e.m.

[8-$^{14}$C]-ATP, [γ-$^{32}$P]-ATP and [$^3$H]ouabain were obtained from NEN Chemicals, Dreieichenheim, FRG.

BIIA was the gift of Dr. Thiemann, Lünen/Westfalen, FRG.

## RESULTS

### *Enzyme inhibitory effects of BIIA*

$Na^+$,$K^+$-ATPase activities of preparations obtained from guinea-pig heart, kidney and brain, as measured with the continuous spectrophotometric method, were 45.2 ± 5.6, 190.1 ± 16.4 and 171.2 ± 7.8 μmoles $PO_4^{3-}$/(mg protein . hr), respectively (4 preparations). $Na^+$, $K^+$-ATPase from each tissue was inhibited by BIIA to a similar extent (Fig. 1), $I_{50}$ values being 20.3 μmoles/l for heart, 24.1 μmoles/l for kidney and 38.9 μmoles/l for brain enzyme. Guinea-pig kidney $K^+$-PNPPase activity was 35.0 μmoles PNP/(mg protein . hr) and was less sensitive to BIIA with an $I_{50}$ of 170 μmoles/l.

### *Reversibility of inhibition*

The full extent of inhibition was observed upon the addition of BIIA to the medium, when continuously recording the $Na^+$, $K^+$-ATPase activity, a behaviour suggesting a readily attainable true equilibrium and a reversible interaction between drug and enzyme [23]. This latter assumption was confirmed by investigating the reversibility of the inhibition using the dilution method of Peters *et al.* [24]. A 1 : 10 dilution and 30 min incubation demonstrated the inhibition to be 100 per cent reversible.

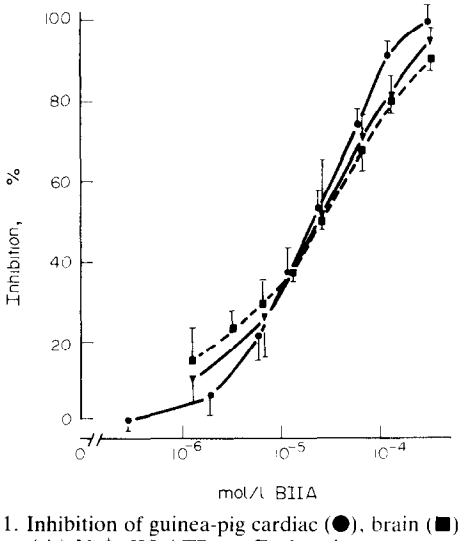


Fig. 1. Inhibition of guinea-pig cardiac (●), brain (■) and kidney (▲) $Na^+$, $K^+$-ATPase. Each point represents the mean of 4 experiments ($n$ = 4). Vertical lines indicate standard error. Standard incubation conditions were used.

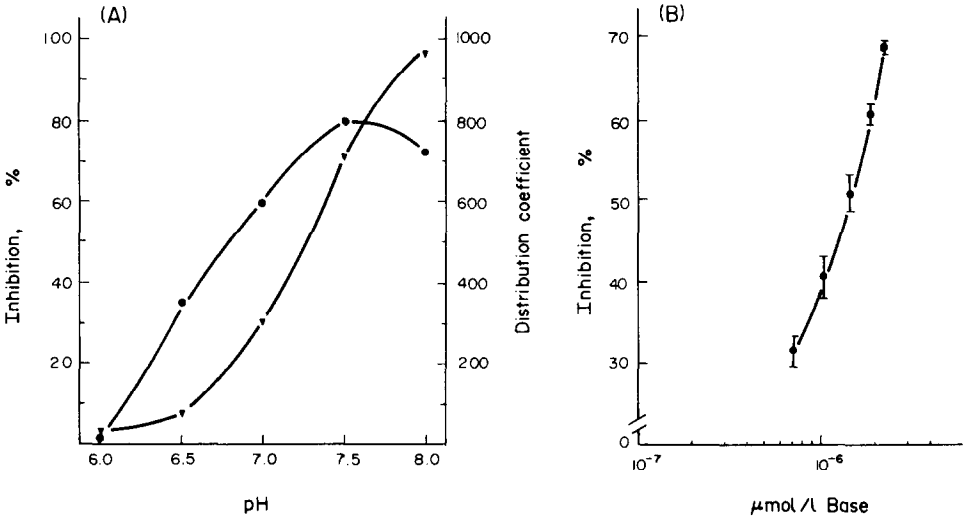


Fig. 2. (A) Inhibition of guinea-pig kidney $Na^+$, $K^+$-ATPase by 30 μmoles/l BIIA as a function of pH (●). Standard incubation conditions were used, except the presence of 100 mmoles/l imidazole to ensure a sufficient buffering capacity. The influence of pH is also seen on the distribution coefficient between octanol and Soerensen buffer (▼) ($n = 4$). (B) Inhibition of guinea-pig kidney $Na^+$, $K^+$-ATPase as a function of the concentration of BIIA free base, as calculated from the $pK_a$, at different pH values (from bottom to top; 6.5, 6.75, 7.0, 7.25 and 7.5). Standard incubation conditions were used, except the presence of 100 mmoles/l imidazole.

*Lipid solubility, degree of ionization and effectivity of BIIA*

As a correlation has been found between lipid solubility and potency to inhibit the $Na^+$, $K^+$-ATPase *in vitro* by certain drugs [25], the distribution coefficient of BIIA between octanol and buffer was measured according to the method described by Borchard and Drouin [26] and was found to be dependent on pH (Fig. 2(A)). Not only does the distribution coefficient increase with increasing pH, reaching values several times higher than those for local anaesthetics measured with the same system [26], but an almost parallel increase in BIIA's capacity to inhibit the $Na^+$, $K^+$-ATPase is observed with increasing alkalinity. The dissociation constant ($pK_a$) for the BIIA molecule was determined according to Armstrong and Barlow [27]. It can be assumed that BIIA exists in acid milieu as the positively charged molecule seen in Fig. 3. The $pK_a$ value for BIIA was 7.01, suggesting that at pH 7.0 half the molecules are in the protonated form. As the extent of inhibition and lipophility of BIIA increases with increasing pH, it can be seen that the lipophilic, unprotonated form is responsible for the inhibition. Fig. 2B demonstrates the concentration response relationship between the amount of base, as calculated from the $pK_a$ value and the extent of inhibition of the $Na^+$, $K^+$-ATPase. In order to investigate the effect of pH on the enzyme kinetics and thus the interaction between BIIA and the $Na^+$, $K^+$-ATPase the influence of pH on the $K_m$ for ATP was investigated in the pH range 6.5 to 7.5. The $K_m$ value was not significantly altered remaining 0.39 ± 0.02 mmole/l at the five pH values measured (see Fig. 2(B)).

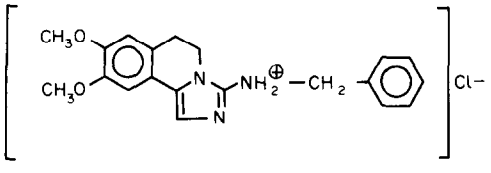


Fig. 3. Chemical structure of BIIA (benzylamino-5,1-*a*-isoquinoline-hydrochloride, mol.wt 371.5).

*Effects of* $Mg^{2+}$ *and ATP*

At a constant ATP concentration the inhibition of the $Na^+$, $K^+$-ATPase by BIIA was not significantly affected by a concentration range of 1–15 mmoles/l $MgCl_2$.

The effect of BIIA on the kinetics of ATP hydrolysis is shown in Fig. 4, in the form of a representative double reciprocal plot. BIIA caused a parallel upward shift of the control line, decreasing $K_m$ and $V_{max}$ by the same factor, typical of an uncompetitive inhibition. This is also reflected in a decrease in $I_{50}$ from 48.8 μmoles/l at 0.5 mmole/l ATP to 24.1 μmoles/l BIIA at 3 mmoles/l ATP.

*Effect of varying the* $Na^+$ *and* $K^+$ *concentrations*

The effect of varying the $Na^+$ concentration on the inhibition of the $Na^+$, $K^+$-ATPase by BIIA is shown as a representative double reciprocal plot in Fig. 5. BIIA is a simple competitive inhibitor of $Na^+$ activation, as the extent of inhibition approaches zero as the $Na^+$ concentration is increased. The apparent affinity of $Na^+$ for the enzyme was decreased reflected by the observation that the concentration for half maximal activation ($K_{0.5}$) increased from 4.6 mmoles/l to 22.7 mmoles/l in the presence of 10 μmoles/l BIIA. The apparent $V_{max}$ was not affected. The degree of sigmoidicity measured as the slope of the Hill plot [28], an expres-

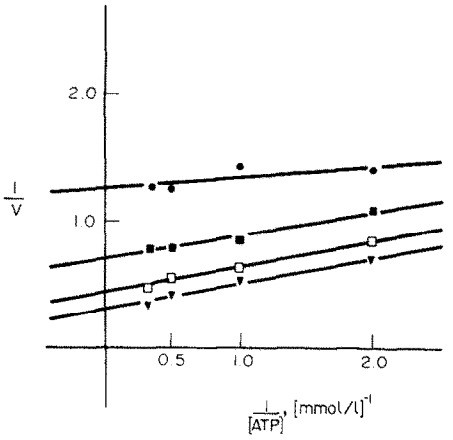


Fig. 4. Lineweaver–Burke plot of the effect of ATP concentration on guinea-pig kidney $Na^+$, $K^+$-ATPase activity in the absence (▼) and in the presence of (□) 6.7, (■) 27.0 and (●) 67.0 μmoles/l BIIA. Standard incubation conditions were used, except that the ATP concentration was varied from 0.5–3 mmoles/l ($n = 4$).

sion of the cooperative allosteric activation of this enzyme by $Na^+$ [29], was not significantly altered by BIIA (plots not shown), remaining 1.2 ± 0.1 in eight experiments, with concentrations of 1–30 μmoles/l BIIA. The effect of varying the $K^+$ concentration on the inhibition of the $Na^+$, $K^+$-ATPase by BIIA is

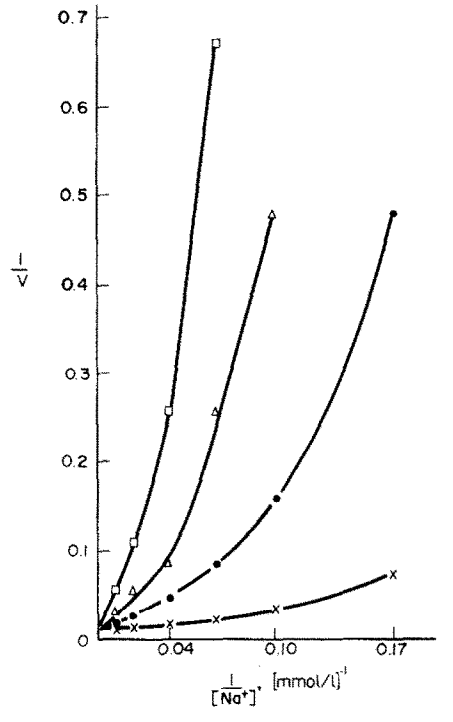


Fig. 5. Lineweaver–Burke plot of the effect of varying the $Na^+$ concentration on guinea-pig kidney $Na^+$, $K^+$-ATPase in the absence (×) and in the presence of (●) 3, (△) 10 and (□) 30 μmoles/l BIIA. Standard incubation conditions were used except that the NaCl concentration was varied from 10–100 mmoles/l ($n = 4$).

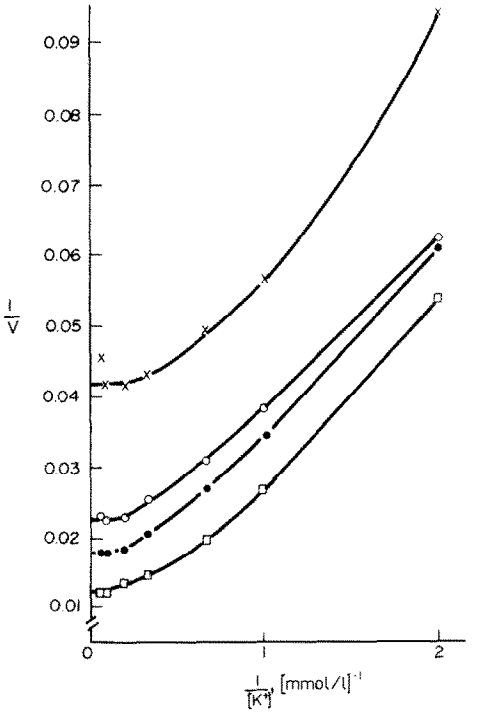


Fig. 6. Lineweaver–Burke plot of the effect of varying the $K^+$ concentration on guinea-pig kidney $Na^+$, $K^+$-ATPase activity in the absence (□) and in the presence of (●) 3, (○) 10 and (×) 30 μmoles/l BIIA. Standard incubation conditions were used, except that the KCl concentration varied from 0.5–20 mmoles/l ($n = 4$).

shown in Fig. 6 as a representative Lineweaver–Burke plot. As with ATP activation kinetics, BIIA causes a parallel upward shift of the control line, typical of an uncompetitive interaction. This effect is only seen, however at suboptimal $K^+$ concentrations. Increasing the $K^+$ concentration above 10 mmoles/l had no further effect on the potency of BIIA. Reducing the $K^+$ concentration from 10 to 2 mmoles/l increased the $I_{50}$ from 24.1 to 92.3 μmoles/l BIIA. No change in cooperativity of activation was observed. The effect of varying $K^+$ concentration on the inhibition of the $K^+$-PNPPase was also investigated. The representative Lineweaver–Burk plot in Fig. 7 shows that, in agreement with other investigations [30], activation of $K^+$-PNPPase by $K^+$ is also a cooperative phenomenon. BIIA inhibited the $K^+$-PNPPase competitive with $K^+$, without altering $V_{max}$ and increasing the degree of cooperativity very slightly, from $n = 1.8$ in the absence to $n = 2.1$ in the presence of 100 μmoles/l BIIA.

### [$^{14}$C]-*ATP binding studies*

In the absence of $Mg^{2+}$ and $Na^+$, [$^{14}$C]-ATP binding was increased by BIIA in a dose-dependent manner. In the presence of 1 μmole/l ATP, the semi-purified $Na^+$, $K^+$-ATPase bound 62 ± 8 pmoles ATP per mg protein ($n = 4$). The addition of 0.3, 30 and 300 μmoles/l BIIA caused binding to increase by 66 ± 18, 202 ± 15 and 303 ± 34 per cent respectively.

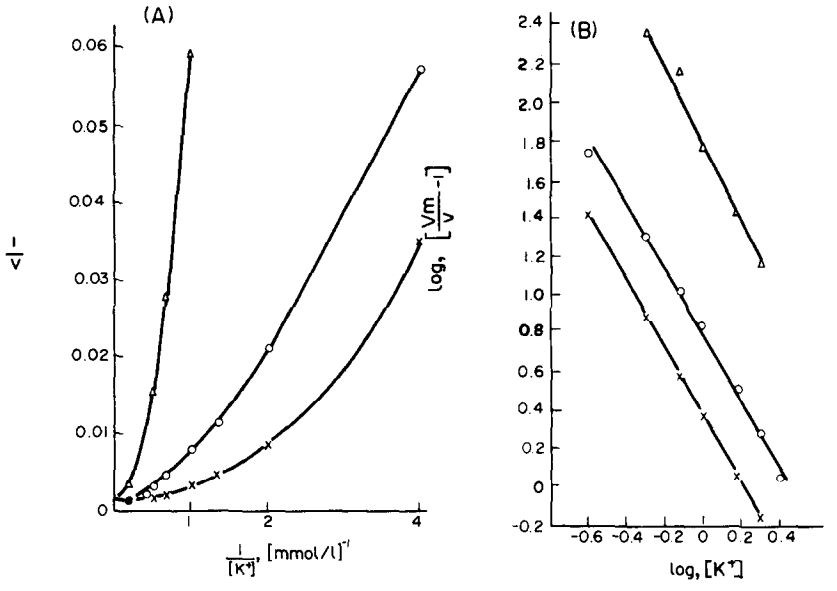


Fig. 7. (A)Lineweaver–Burke plot of the effect of varying the $K^+$ concentration on the activity of guinea-pig kidney $K^+$-PNPPase in the absence (×) and the presence of (○) 10 and (△) 100 μmoles/l BIIA. Standard incubation conditions were used, except that KCl concentration varied between 0.25 and 5 mmoles/l. (B) A Hill plot of the same data as in (A) ($n = 4$).

This increase in binding was no longer evident in the presence of 30 mmoles/l $Na^+$. A $Na^+$ concentration above 70 mmoles/l was itself inhibitory. The addition of 5 mmoles/l $Mg^{2+}$ resulted in a complete loss of specific binding.

*Effects on the* $Na^+$ *dependent* $^{32}P$*-phosphorylation*

The $Na^+$-dependent phosphorylation was inhibited by BIIA in a dose-dependent manner. The $I_{50}$ for the inhibition was 1.0 μmole/l, at an ATP concentration of 12.5 μmoles/l. The inhibition increased with increasing ATP concentrations (data not shown) and was competitive with $Na^+$ as can be seen from a typical double reciprocal plot in Fig. 8.

*Effects on* [$^3$H]*ouabain binding*

$I_{50}$ values for the inhibition of [$^3$H]ouabain binding of 50 nmoles/l [$^3$H]ouabain were 10.2 ± 2.5 in

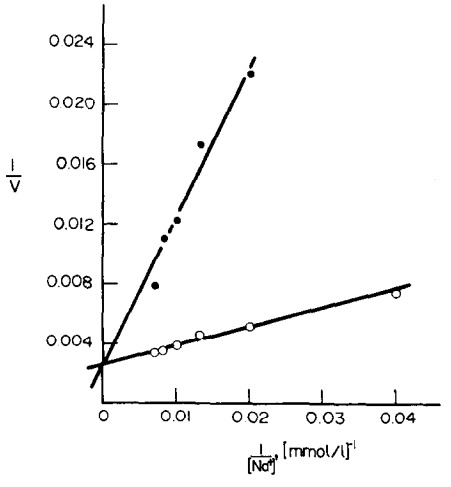


Fig. 8. Lineweaver–Burke plot of guinea-pig kidney $Na^+$, $K^+$-ATPase, $Na^+$-dependent $^{32}P$ phosphorylation as a function of the $Na^+$ concentration in the absence (○) and presence of (●) 5.7 μmoles/l BIIA. $V$ = pmoles $PO_4^{3-}$ bound/(mg protein . 10 sec). Standard incubation conditions were used, except that the NaCl concentration was varied from 25–125 mmoles/l in the presence of 12.5 μmoles/l ATP ($n = 4$).

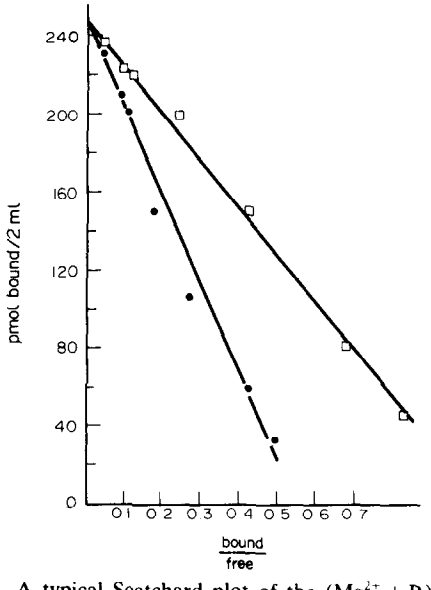


Fig. 9. A typical Scatchard plot of the ($Mg^{2+}$ + $P_i$) supported [$^3$H]ouabain binding to guinea-pig kidney $Na^+$, $K^+$-ATPase in the absence (□) and the presence of (●) 10 μmoles/l BIIA. Incubation conditions were as described under Methods ($n = 4$).

($Mg^{2+}$ + $P_i$) medium and 98.7 ± 3.1 μmoles/l BIIA ($Na^+$ + ATP) medium, ($n$ = 4).

Figure 9 shows two typical Scatchard plots for the equilibrium binding of ouabain to semi-purified kidney $Na^+$, $K^+$-ATPase, in the presence of $Mg^{2+}$ and $P_i$ and in the absence and presence of 10 μmoles/l BIIA. The number of binding sites ($B_{max}$) and the dissociation constants ($K_d$) were estimated from the intercept and the slope of the linear regression line fitted by the least squares method.

The affinity of the enzyme for ($Mg^{2+}$ + $P_i$)-supported ouabain binding was significantly reduced in the presence of 10 μmoles/l BIIA, as measured in the increase of $K_d$ from 1.02 ± 0.15 μmoles/l to 2.10 ± 0·.38 μmoles/l ouabain ($n$ = 8, $P < 0.005$). $B_{max}$ was not significantly altered by the same concentration of BIIA (870 ± 60 pmoles/mg protein without and 845 ± 110 pmoles/mg protein with 10 μmoles/l BIIA). 10 μmoles/l BIIA did not significantly affect ($Na^+$ + ATP)-supported binding, the $K_d$ was 1.09 ± 0.07 in the absence and 1.12 ± 0.09 μmoles/l ouabain in the presence of drug. Neither was $B_{max}$ affected, being 711 ± 42 in the absence and 733 ± 45 pmoles/mg protein in the presence of BIIA, ($n$ = 8).

## DISCUSSION

The digitalis-like properties of BIIA, e.g. changes in EGC, incidence of arrhythmias and increase in basal tension in isolated heart preparations, encouraged us to investigate the effects of this drug on the $Na^+$, $K^+$-ATPase *in vitro*. BIIA was found indeed to inhibit this enzyme in a concentration range at which it produced a positive inotropic effect *in vivo*. The mechanism of inhibition of the $Na^+$, $K^+$-ATPase is however a completely different one to that of cardiac glycosides [2, 3].

BIIA has been found to inhibit the $Na^+$, $K^+$-ATPase competitive with $Na^+$ and uncompetitive with ATP and $K^+$. In contrast the interaction of ouabain with this enzyme is promoted by $Na^+$ and competitive with $K^+$ at low concentrations and mixed competitive and noncompetitive at higher concentrations [31]. It can therefore be postulated that the receptor for BIIA on the $Na^+$, $K^+$-ATPase may be different from that of cardiac glycosides.

The interaction of BIIA with the $Na^+$, $K^+$-ATPase is of great interest, due to its specific interaction with the $Na^+$ activation at μmolar concentrations. Apart from BIIA there are very few compounds that decrease the affinity of the $Na^+$-site for $Na^+$, whilst increasing that of the $K^+$-site for $K^+$. Substances comparable with BIIA are phlorizin [5] and deuterium oxide [6]. Both of these are however less potent in their overall inhibition of the enzyme. They indeed increase the affinity of the enzyme for $K^+$, but this in contrast to BIIA, results in an activation of the $K^+$-PNPPase activity. Phlorizin, like BIIA, decreases the affinity of the enzyme for $Na^+$, but not in a simple competitive manner as it converts the positive cooperativity of the $Na^+$ sites into a negative one [5].

The extreme lipophilic nature of the unprotonated BIIA molecule, which is supposed to be responsible for the inhibition of the $Na^+$, $K^+$-ATPase, and its interaction with $Na^+$-activation is particularly interesting in the light of Roufogalis' observations [32]. A correlation was found between the lipophility of local anaesthetics and phenothiazine derivatives and their ability to inhibit the $Na^+$, $K^+$-ATPase *in vitro*. The phenothiazines, which were the most lipid-soluble group, interfered with a higher degree of specificity with the $Na^+$ activation sites, which were postulated to lie in deep, hydrophobic areas of the membrane microenvironment. The less lipid-soluble local anaesthetics were, in contrast, found to affect more specifically the $K^+$ activation, probably acting on more superficial areas of the membrane. BIIA may well belong to the former group of substances, with regard to its interaction with the $Na^+$ activation. It has been found to have an octanol: buffer partition coefficient of 975 at pH 8.0 far greater than any local anaesthetics investigated [26].

The possibility that change in pH alters the affinity of the $Na^+$, $K^+$-ATPase for activating ions or substrate and thus the interaction of BIIA with the enzyme may be excluded in the pH range investigated (6.5–7.5). Robinson and Flashner [33] have shown that the $K_{0.5}$ for $Na^+$ and $K^+$ is unaltered and Yoda and Yoda [34] have shown an unaffected level of phosphorylation throughout the above mentioned pH range. Our own investigations have also shown no significant change in the $K_m$ for ATP. Changes in the above mentioned constants at pH values above and below those measured prevented us from comparing percent inhibition with the concentration of free base at more extreme values of pH.

A degree of similarity can be seen between the inhibitory effects of BIIA and those of the thioxanthine derivative, flupentixol [35]. This substance, like BIIA, inhibits the $Na^+$, $K^+$-ATPase in its lipophilic, unprotonated form.

With an $I_{50}$ of 80 μmoles/l (pH 7.4), it is less potent than BIIA, but also inhibits competitive with $Na^+$ and uncompetitive with ATP. Flupenthixol also inhibits the $K^+$-PNPPase, but at concentrations which suggest that this inhibition is responsible for the inhibition of the overall activity. The action of this substance is accordingly not specific for $Na^+$-activation, being competitive in parallel for $K^+$, thus suggesting a different inhibitory mechanism.

BIIA is structurally somewhat unique amongst $Na^+$, $K^+$-ATPase inhibitors, although other isoquinoline derivatives, such as sanguinarine [36], a benzophenanthridine alkaloid and quindonium bromide [37], an azasteroid have been shown to inhibit the $Na^+$, $K^+$-ATPase and possess cardiotonic effects. These substances have however both hydrophobic and hydrophilic properties by virtue of a single iminium cation, contained within an aromatic polycyclic system. BIIA is effective in its uncharged, hydrophobic form and contains nitrogen atoms in an imidazole and tertiary amino group. Cohen *et al.* [38] have investigated a whole series of compounds, with substituted imine and iminium quinoline and isoquinoline skeletons. It was shown that substances sharing several structural features with sanguinarine and BIIA were inactive as $Na^+$, $K^+$-ATPase inhibitors. Sanguinarine inhibits in a manner different to BIIA, uncompetitive with respect to $Na^+$ and $K^+$

and non-competitive with ATP, suggesting a completely different site of action.

Summarizing, BIIA inhibits $Na^+$, $K^+$-ATPase *in vitro* due to an uncommonly specific interaction with $Na^+$-activation, which in turn leads to a dose-dependent reduction in phosphorylation from ATP. Both the inhibition of phosphorylation and inhibition of the overall reaction are increased by increasing ATP concentrations, suggesting that BIIA interacts with the enzyme subsequent to its interaction with substrate [23]. BIIA has also been shown, in these investigations, to increase substrate binding under certain conditions.

The nature of the interaction between BIIA and $K^+$, at suboptimal $K^+$ concentrations, suggests that BIIA is dependent on the activity of the enzyme. At high $K^+$ concentrations the $K^+$-dependent dephosphorylation will be at a maximum [4], and thus the concentration of enzyme in the $E_1$ form, which binds ATP and thus BIIA will be higher.

Further experiments have demonstrated '$Na^+$-like' effects of BIIA, which may be triggered by BIIA's interaction at or near the $Na^+$-activation sites. Firstly its capacity to inhibit the $K^+$-PNPPase at high concentrations, competitive with $K^+$, a property also shown by $Na^+$ under certain conditions [32], although unlike BIIA, $Na^+$ decreases the cooperative interaction of the $K^+$ sites. Secondly the affinity of the enzyme for ouabain was reduced by BIIA in the presence of $Mg^{2+}$ and $P_i$, $Na^+$ also increases the $K_d$ for ($Mg^{2+}$ + $P_i$)-supported binding without altering the number of binding sites [39]. Higher concentrations of BIIA reduce ($Na^+$ + ATP)-supported binding, perhaps due to an inhibition of phosphorylation. The possibility that BIIA also inhibits the phosphorylation of the $Na^+$, $K^+$-ATPase from $P_i$ has yet to be investigated.

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