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Neurospecificity of phyto-bufadienolides is not related to differences in Na^+/K^+ pump inhibition

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Abstract

The aim of the present study was to investigate the effects of neuro- (cumulative) and cardiotoxic (non-cumulative) bufadienolides originating from plants (phyto-bufadienolides) on the Na^+/K^+ pump current (I_p) in cardiac (rat and guinea pig) and dorsal root ganglion cells (guinea pig), and on Ca^{2+} currents in cardiomyocytes (guinea pig). All bufadienolides tested (non-cumulative drugs: thesiuside, tyledoside C; lanceotoxin B and tyledoside F for the neurotoxic group) were potent blockers of I_p at concentrations in the micro- and submicromolar range. $K_{0.5}$ values for I_p inhibition in dorsal root ganglion neurones were slightly lower compared to cardiomyocytes, but the order of potency was similar in both cell types. Both classes of bufadienolides were equipotent in suppressing I_p, generated by high- and low-affinity pump isoforms. Phenomena related to pump inhibition, as hypercontracture and increase in T-type Ca^{2+} current in cardiomyocytes, were influenced to the same extent. Therefore, from these results, neurospecificity of some bufadienolides could not be explained by differences in Na^+/K^+ pump affinity. © 1997 Elsevier Science B.V.

Keywords: Bufadienolide; Na^+/K^+ pump; Dorsal root ganglion cell; Cardiac cell; Na^+/K^+ ATPase isoform

1. Introduction

Cardiac glycosides are generally known as specific blockers of the Na^+/K^+ pump in various cells. They consist of two groups of steroids: the cardenolides, with an unsaturated, five-membered lactone ring on C-17, and the bufadienolides, with a doubly, unsaturated six-membered lactone ring in C-17. Bufadienolides are the toxic compound in the venom of different *Bufo* species. In amphibia these substances play a role in water and salt regulation (Lichstein et al., 1992). Bufadienolides have been widely used in traditional Chinese medicine as the active compounds in Sensu (Hong et al., 1992) for the treatment of dropsy. Although the bufadienolides, isolated from toad tissues, were extensively studied in search for endogenous digitalis-like factors in mammals (Blaustein, 1993; Eliades et al., 1989; Nicholls et al., 1995), much less information is available on bufadienolides present in a large group of plants growing in arid regions of southern Africa (for review, see Kellerman et al., 1988) and Australia (McKenzie et al., 1989). Ingestion of these plants may cause intoxication in livestock on a large scale. Bufadienolides from representative plants have been isolated, purified and extensively tested in small laboratory animals and in cattle, mainly for veterinary purposes (Naudé and Potgieter, 1971; Anderson et al., 1983, 1987; Kellerman et al., 1988; McKenzie et al., 1989). All plant-derived bufadienolides caused toxicity on the heart when acutely given at high doses. However, when smaller quantities were chronically ingested, only some drugs caused a neurological syndrome known as 'shrinking disease', so called because of the special stance of the animals (Anderson et al., 1983). Cardiac, respiratory and gastro-intestinal symptoms, which were typical for acute poisoning, were minimal or even absent in this case. Because neurotoxicity was found to be related to chronic intake of some bufadienolides, these

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compounds were classified as cumulative (causing primarily neurological symptoms) or non-cumulative compounds (Kellerman et al., 1988). The naturally occurring cardenolides in these regions were apparently non-cumulative, while bufadienolides could be either cumulative or non-cumulative, depending on plant species and the cardiac glycoside involved.

The mechanism of preference, either for the heart or the nervous system, of some bufadienolides is not known. At the cellular level different isoforms of the Na^+/K^+ ATPase occur (for review, see Sweadner, 1989; Levenson, 1994), which are characterized by differences in affinity towards cardiac glycosides. These isoforms are variably expressed in different cell types and may differ between species. In general, the isoforms which are dominantly expressed in neurones bind cardiac glycosides with higher affinity than the cardiac isoform. It is not well known whether these isoforms have a variable specificity for different cardiac glycosides.

In this study, we investigated the effect of representative drugs of both classes of bufadienolides on the Na^+/K^+ pump current in cardiac myocytes and dorsal root ganglion neurones, on L- and T-type Ca^{2+} currents in cardiac cells, and on the development of hypercontracture in cardiac myocytes in conditions of a partially blocked Na^+/K^+ pump. Non-cumulative drugs studied were thesiuside, isolated from *Thesium lineatum* (Anderson et al., 1987), and tyledoside C, from *Tylecodon grandiflorus* (Anderson et al., 1983); representative drugs of the neurotoxic, cumulative group investigated were: lanceotoxin B from *Kalanchoe lanceolata* (Anderson et al., 1984) and tyledoside F from *Tylecodon grandiflorus* (Anderson et al., 1983). All these compounds are present in plants which frequently cause poisoning of sheep and cattle (Kellerman et al., 1988). Na^+/K^+ pump currents were studied in ventricular myocytes and neuronal cells of the guinea pig (an 'ouabain-sensitive' species), and in cardiac cells of young rats (an 'ouabain-insensitive' species) in which low- (predominantly) and high-affinity isoforms are expressed. Ca^{2+} currents and the development of hypercontracture during pump inhibition were investigated in guinea-pig cardiac myocytes.

We found that all representative drugs from either class suppress the Na^+/K^+ pump current, generated by different pump isoforms, and increase the T-type Ca^{2+} current. These findings on the cellular level could not be related to the preferential effect some drugs have either on the heart or on the nervous system.

2. Materials and methods

2.1. Isolation of single cells

Guinea pigs (Duncan-Hartley; about 250 g) and rats (Sprague-Dawley; about 120 g) were killed by cervical dislocation. Ventricular myocytes were enzymatically isolated from the hearts during a Langendorff perfusion with Ca^{2+}-poor solution at 35°C as described in detail (Bielen et al., 1992). The isolated cells were transferred to culture dishes where the Ca^{2+} concentration of the bathing solution was increased stepwise to 1.8 mM. Cells were kept at room temperature and were used on the day of isolation. Dorsal root ganglion cells from the guinea pig were prepared by means of a procedure described by Delree et al. (1989); cells were grown on coverslips in dishes and kept in primary culture for 1–2 days at 37°C in an incubator.

Na^+/K^+ pump measurements were carried out on cardiac ventricular myocytes and dorsal root ganglion neurones of the guinea pig and on cardiac ventricular cells of the rat. The effect of bufadienolides on Ca^{2+} currents was studied in guinea-pig ventricular myocytes.

2.2. Solutions

Three different media were used for internal perfusion via the patch pipettes. For measurement of the Na^+/K^+ pump current, two solutions were used which mainly differed in the Na^+ concentration. Patch pipette solution A with 30 mM Na^+ contained (mM): 90 Cs-aspartate; 15 NaOH; 5 NaCl; 10 EGTA; 5 $MgCl_2$; 5 Na_2-ATP; 5 glucose; 40 HEPES (adjusted to pH 7.2 with CsOH at 35°C). The composition of the patch pipette solution B with 100 mM Na^+ was (mM): 35 Cs-aspartate; 15 NaOH; 75 Na-aspartate; 15 EGTA; 5 $MgCl_2$; 5 Na_2-ATP; 5 glucose; 20 HEPES (pH 7.2 with CsOH). A concentration of 100 mM was chosen in some experiments to minimize changes of the subsarcolemmal Na^+ concentration when activation of the Na^+/K^+ pump was varied (Bielen et al., 1991). Unless otherwise mentioned, solution A was used for measuring I_p. The intracellular pipette medium used in the experiments for determining T- and L-type Ca^{2+} currents (solution C) was of the following composition (mM): 125 CsCl; 2 $MgCl_2$; 3 Mg-ATP; 5 EGTA; 1 $CaCl_2$; 10 glucose; 10 HEPES (pH adjusted to 7.2 with CsOH).

The bath solution contained (mM): 138 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose; 10 HEPES (pH adjusted to 7.4 at 35°C with NaOH). For I_p measurements superfusion solutions contained, in addition to the components in the bath solution, 2 mM $BaCl_2$ in order to diminish K^+ conductances. In most experiments carried out on cardiac myocytes 5 mM $NiCl_2$ was added as well, in order to inhibit Na^+/Ca^{2+} exchange. For measuring zero pump current KCl was omitted from the superfusion solution. In the superfusion medium used to measure the L-type Ca^{2+} current 20 mM NaCl was replaced by 20 mM CsCl. For T-type Ca^{2+} current measurements the superfusion medium contained (mM): 118 Tris Cl; 0.5 $MgCl_2$; 5.4 $CaCl_2$; 5 glucose; 10 HEPES (pH adjusted to 7.4 at 35°C), 20 CsCl; 3 4-aminopyridine. Experiments for determining effects on Ca^{2+} currents were carried out at 35°C.

2.3. Drugs

The bufadienolides used in this study (thesiuside, lanceotoxin B, tyledoside C and F) were isolated and purified by the Veterinary Research Institute, Onderstepoort, Pretoria. All bufadienolides were added to the superfusion media from a dimethyl sulfoxide (DMSO) stock solution (4×10^{-2} M). At dilutions used in this paper DMSO does not influence the Na^+/K^+ pump current (Gadsby and Nakao, 1989). Ouabain (Sigma, St. Louis, MO, USA) was added from an aqueous stock solution (10^{-2} M).

2.4. Experimental procedure and whole cell recording

Ventricular myocytes were pipetted into a perfusion bath installed on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, Germany). Dorsal root ganglion cells were put into the bath adhered to coverslips. Membrane currents were measured by means of single-electrode whole-cell recording. A Plexiglas ring was pressed to the bottom of the dish reducing the volume of the dish to about 0.3 ml. The cells were superfused at 2 ml min^{-1} with solutions prewarmed to 35°C. Test solutions were applied close to the cell surface under study by means of a multibarrelled pipette (tip diameter about 150 μm). Gravitational release of the solutions was controlled by a command valve. The clamped membrane potential and the corresponding membrane current was measured by means of a Dagan 8800 Total Clamp (Dagan, Minneapolis, MN, USA) voltage clamp amplifier. For I_p measurements currents were registered on a pen recorder (Watanabe, Tokyo, Japan). For the measurement of Ca^{2+} currents clamp protocols were controlled, and data were acquired by a DOS-based 386 microcomputer programmed with pClamp 5.5. I_p was identified as the outward current which could be activated by K_o^+ and blocked by cardiac glycosides. I_p was measured at a holding potential of −20 mV. The L-type Ca^{2+} current was elicited by applying voltage steps to +20 mV from a holding potential of −45 mV. The T-type current was separated from the L-type current by clamping the membrane potential to −30 mV alternatively from a holding potential at −90 and −50 mV. The T-type Ca^{2+} current was defined as the difference between the currents at −30 mV elicited from the two holding potentials. Test pulses of 100 ms were delivered at a frequency of 0.1 Hz. The resistance of the patch pipettes filled with the solutions mentioned above varied between 2 and 4 MΩ.

The membrane capacitances, derived from the capacitive charge flowing during small hyperpolarizing voltage pulses, was 113 ± 3 pF (n = 130) and 125 ± 3 pF (n = 189) for rat and guinea-pig ventricular myocytes, respectively (Hermans et al., 1995) and 33 ± 2 pF (n = 50) for dorsal root ganglion cells.

2.5. Induction of hypercontracture in ventricular myocytes

Cardiac cells may loose their rod-like shape and round up as a consequence of irreversible contracture when they become overloaded with Ca^{2+} secondary to primary Na^+ overload. Intracellular Na^+ accumulation was induced by Na^+/K^+ pump inhibition and the process was accelerated by stimulating the cells electrically at 1 Hz. At least 60 rod-like, well contracting cells were identified in a meshed culture dish on an inverted microscope and the number of cells that remained rod-shaped was counted 10 and 20 min after application of cardiac glycosides.

2.6. Statistics

Data are presented as means ± S.E.M. where possible, and S.E.M. is indicated in the figures only if the size of the data points is exceeded; n indicates the number of cells studied or the number of dishes counted for rod-shaped cells. Statistical analysis was performed using Student's t-test. P values < 0.05 were considered to indicate statistically significant difference.

3. Results

3.1. Inhibition of I_p by bufadienolides in guinea-pig cardiac myocytes

Because bufadienolides isolated from toad skin closely resemble the structure of the cardenolides and are known to inhibit Na^+/K^+ ATPase activity, the first target transport molecule to study was the Na^+/K^+ pump.

Fig. 1 illustrates the procedure to determine the Na^+/K^+ pump current (I_p) and it shows the inhibitory effect of tyledoside C (B) on I_p in a cardiac myocyte and a dorsal root ganglion cell (C). Tyledoside C is a representative drug of the non-cumulative class of compounds. For comparison the effect of ouabain on I_p in cardiac myocytes is included in the figure (A). I_p was measured as the current activated by external K^+. Ba^{2+} (2 mM) was added to the superfusion medium and K^+ was replaced in the internal solution by Cs^+ to block passive K_o^+-sensitive K^+ conductances. In this experimental condition the only K_o^+-sensitive current left is I_p (Bielen et al., 1991, 1992). The Na^+/K^+ pump was continuously activated by superfusing the cells with 5.4 mM K^+. Zero pump current was regularly checked by applying short pulses of K^+-free solution (indicated by the upper trace in the figure). When I_p was reactivated in cardiac myocytes after a short period of pump inhibition in 0 K_o^+, I_p was transiently increased above its steady-state level (see also Fig. 2). The I_p overshoots increased when the preceding 0 K_o^+ periods were prolonged. I_p overshoots are most probably caused by a fast increase in subsarcolemmal $[Na^+]$ during the period of pump suppression, even when inhibition lasts

only for seconds (Bielen et al., 1991). The magnitude of the overshoots depended on the level of I_p activation. Overshoots were very prominent when the pump was fully activated after a long period of pump inhibition. When the pump became progressively suppressed by application of cardiac glycosides, I_p overshoots decreased. Addition of 5 μM ouabain or tyledoside C caused an inward shift of the holding current in the presence of K_o^+ in cardiac myocytes. The difference current in the presence and absence of K_o^+ decreased, indicating an inhibition of I_p. Shortly after the start of I_p suppression, an outward shift of the holding current in 0 K_o^+ became evident. In contrast to ouabain, inhibition of I_p by tyledoside C was not reversible. I_p only recovered to a small extent 7 min after wash-out of the drug and the outward shift of the background current in 0 K_o^+ remained. In the presence of 10 μM tyledoside C I_p decreased further and the outward shift in 0 K_o^+ became more prominent. Reversibility from I_p inhibition by all bufadienolides tested was very poor.

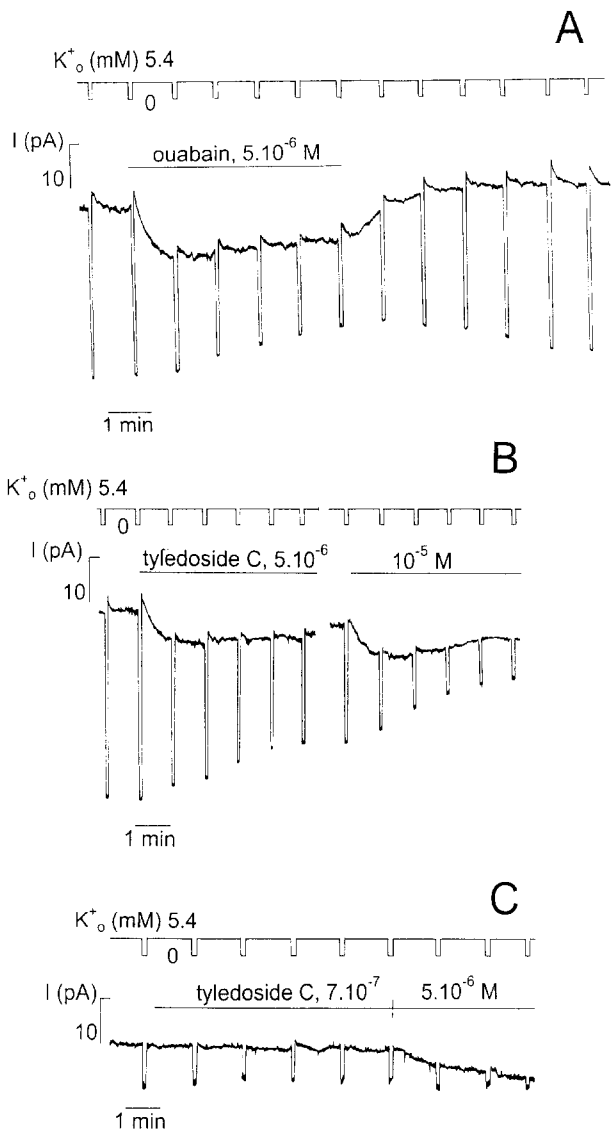

Fig. 1. Phyto-bufadienolides block I_p in cardiac myocytes and in dorsal root ganglion neurones. The effect of the cardenolide ouabain (A) and the non-cumulative bufadienolide tyledoside C (B) on the Na^+/K^+ pump current (I_p) in a cardiac ventricular myocyte, and the effect of tyledoside C on a dorsal root ganglion cell (C) of the guinea pig. The upper trace in each figure represents the K_o^+ concentration (5.4 or 0 mM), the middle trace indicates the presence and the concentration of the cardiac glycosides, the lower trace represents the holding currents measured at a holding potential of −20 mV. The Na^+/K^+ pump current (I_p) was determined as the difference in the holding currents measured in the presence of K_o^+ (5.4 mM $[K^+]_o$; pump activated) and in the absence of K_o^+ (zero I_p). To exclude other K_o^+-sensitive currents except I_p, 2 mM Ba^{2+} was added to all superfusion media and K^+ was substituted by Cs^+ in the internal solution.

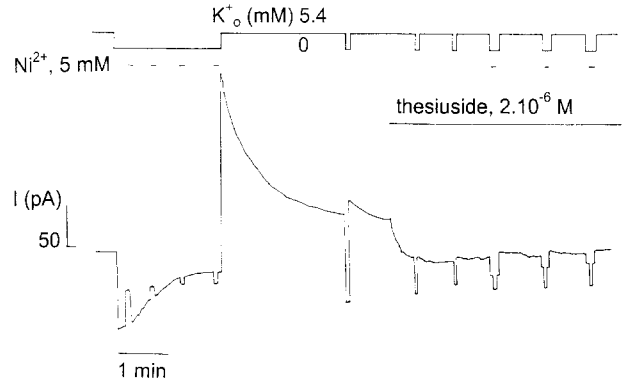

Fig. 2. The outward shift of the holding current in 0 K_o^+ in a guinea-pig myocyte is Ni^{2+} sensitive. Influence of 5 mM Ni^{2+} on the holding current (lower trace) during maximal pump inhibition in 0 K_o^+ or during partial pump suppression in the presence of thesiuside (2×10^{-6} M). Upper trace: $[K^+]_o$; interrupted line: application of Ni^{2+}; full horizontal line: addition of thesiuside.

The outward shift of the holding current shortly after inhibition of I_p was most probably caused by a change of Na^+/Ca^{2+} exchange current, secondary to an increase of Na^+ in the subsarcolemmal space during partial inhibition of the pump. The involvement of the Na^+/Ca^{2+} exchanger is illustrated by the effect of Ni^{2+} (5 mM; Fig. 2) on the holding current during inhibition of the pump. Ni^{2+} has been shown to be an effective blocker of Na^+/Ca^{2+} exchange. The current record in Fig. 2 starts with the holding current of a cell superfused with 5.4 mM K_o^+. Upon superfusion with 0 K_o^+ the holding current shifted inwardly. The difference between both currents is equal to I_p in this cell. Shortly after the start with 0 K_o^+ superfusion the current shifted in the outward direction. Application of 5 mM Ni^{2+} in 0 K_o^+ resulted initially in an outward shift of the holding current, and subsequently in an inward shift. The holding current in 0 K_o^+ remained unchanged when Ni^{2+} was added from the start of 0 K_o^+ superfusion. The shift of the Ni^{2+}-sensitive current from inward to outward during pump inhibition is consistent with a rise of $[Na^+]_i$. Reactivation of the pump with 5.4 K_o^+ resulted in a large I_p overshoot which exponentially declined. Concomitantly with pump activation the holding current in 0 K_o^+ shifted inwardly again. Superfusion of the cell during pump acti-

vation with the non-cumulative bufadienolide thesiuside (2 μM) resulted in a decline of I_p and a rise in background current in 0 K_o^+. Addition of 5 mM Ni^{2+} during the 0 K_o^+ pulses shifted the holding current back to its initial level at the start of 0 K_o^+ superfusion. This finding indicates that changes in Na^+/K^+ pump activity in cardiac myocytes are associated with changes in subsarcolemmal $[Na^+]$ influencing the Na^+/Ca^{2+} exchange. Although 5 mM Ni^{2+} block the exchange current completely, it does not prevent the changes of internal Na^+ caused by transient inhibition and activation of the Na^+/K^+ pump. The estimation of the percentages of inhibition of I_p by different bufadienolides was not influenced, however, by the presence or absence of Ni^{2+}. To prevent hypercontracture of the myocytes and loss of seals during pump inhibition, most experiments were performed in the presence of 5 mM Ni^{2+}.

The percentage inhibition of I_p as a function of the bufadienolide concentration could be fitted for each bufadienolide by a sigmoid curve obeying the Hill equation:

$$\%\ \text{inhibition of}\ I_p = \frac{100 \times [\text{drug}]^h}{(K_{0.5})^h + [\text{drug}]^h}$$

where $K_{0.5}$ is the concentration for half-maximal inhibition of I_p, and h the slope factor of the curve. Fig. 3 shows the experimental data and the curves, obtained by least-square non-linear regression, for the cumulative, neurotoxic bufadienolide lanceotoxin B and for the non-cumulative compound, thesiuside. The percentages of I_p inhibition were measured 4 min after addition of the drugs. Only two concentrations at the maximum were tested cumulatively on each individual cell. Because of the high effectivity of these drugs in inhibiting I_p and the slow reversibility, the perfusion bath was cleaned and new cells were pipetted into the bath before testing another pair of concentrations. The $K_{0.5}$ values for lanceotoxin B and for thesiuside in guinea-pig cardiac cells amounted to 5.1×10^{-7} and 4.8×10^{-7} M, respectively; the slopes of both curves were 1.0 and 0.9 for lanceotoxin B and thesiuside, respectively. The difference between $K_{0.5}$ values and slope factors of both drugs was not statistically significant. Fig. 4 illustrates the concentration–response relation for the cumulative tyledoside F and the non-cumulative tyledoside C. Both drugs originate from the same plant, but they exert different effects when tested in intact animals (Anderson et al., 1983). $K_{0.5}$ values for tyledoside F and C were 3.3×10^{-6} M and 4.2×10^{-6} M, respectively; the slopes were 1.0 and 1.2 for tyledoside F and C, respectively. No statistical difference could be found between both curves. The order of potency for the whole series of drugs tested was: thesiuside > lanceotoxine B > tyledoside F > tyledoside C > ouabain ($K_{0.5} = 6 \times 10^{-6}$ M). When the concentration of Na^+ in the pipette was increased to 100 mM (solution B) the $K_{0.5}$ for lanceotoxin B was significantly lower: 1.6×10^{-7} M (slope 1.19).

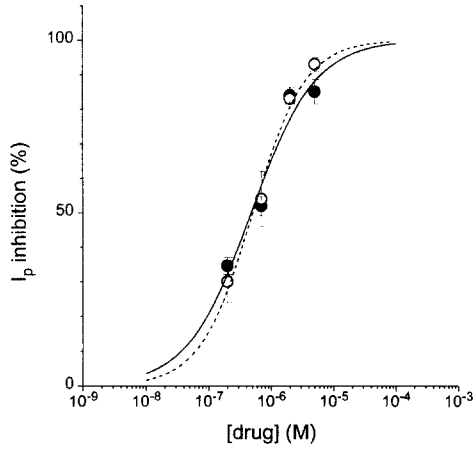

Fig. 3. Concentration–response curves for I_p inhibition by bufadienolides in cardiac cells. Concentration–response curves of I_p inhibition by thesiuside (closed circles), a cardiotoxic, non-cumulative compound and by lanceotoxin B (open circles), a neurotoxic, cumulative compound, in guinea-pig cardiac cells. Total I_p inhibition in K^+-free solution was arbitrarily set to 100%. The sigmoid curves fitted to the data obey the Hill equation: $\%\ \text{inhibition} = (100 \times [\text{drug}]^h)/((K_{0.5})^h + [\text{drug}]^h)$. The curves were obtained by least-square non-linear regression; r^2 for the thesiuside and lanceotoxin B curves were 0.95 and 0.99, respectively. $K_{0.5}$ values for thesiuside and lanceotoxin B were 4.8×10^{-7} M and 5.1×10^{-7} M, respectively, and h values were 0.9 and 1, respectively. The difference between both curves was not statistically significant.

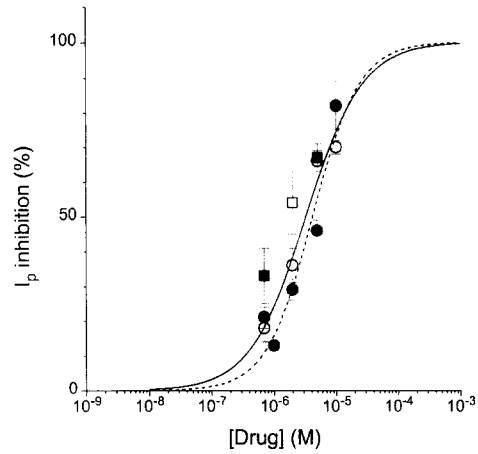

Fig. 4. Comparison of concentration–response curves for I_p inhibition by bufadienolides in cardiac cells and dorsal root ganglion neurones. Concentration–response curves for the inhibition of I_p by the cumulative bufadienolide tyledoside F (open circles, open square) and the non-cumulative tyledoside C (closed circles, closed squares) in cardiac myocytes (circles) and in dorsal root ganglion cells (squares) of the guinea pig. Total I_p inhibition in 0 K_o^+ solution was set to 100%. Curves (full line, tyledoside F; interrupted line, tyledoside C) were fitted as described for Fig. 3. The $K_{0.5}$ values for tyledoside F and C in cardiac myocytes were 3.3×10^{-6} M and 4.2×10^{-6} M, respectively and the h values 1.0 and 1.2, respectively. r^2 values for the tyledoside C and tyledoside F curves were 0.91 and 0.97, respectively. The difference between both $K_{0.5}$ values was not statistically significant.

3.2. Inhibition of I_p by bufadienolides in dorsal root ganglion neurones of the guinea pig

Although I_p could be measured in dorsal root ganglion cells by the same procedure as used in cardiac myocytes, recordings were less constant and I_p frequently declined during the experiment. The number of good, stable I_p recordings was far below the number of successful registrations in cardiac myocytes. We only used those dorsal root ganglion cells for the calculation of the percentage inhibition by bufadienolides in which a constant I_p for at least 5 min could be registered. The mean steady-state I_p in 5.4 mM K_o^+ was 12 ± 2.4 pA ($n = 51$); in guinea-pig cardiac myocytes it amounted to 71 ± 4 pA ($n = 99$). Fig. 1B shows the effect of the non-cumulative tyledoside C (0.7 and 5×10^{-6} M) in a dorsal root ganglion cell. In dorsal root ganglion cells I_p overshoots after temporary block of the pump in 0 K_o^+ were almost absent or at least much less prominent compared to cardiac myocytes, indicating that $[Na^+]_i$ near the membrane remained more constant during inhibition and activation of the Na^+/K^+ pump. In the presence of 0.7 μM tyledoside C, I_p was only slightly suppressed; at 5 μM tyledoside C, I_p inhibition was prominent. I_p was blocked by bufadienolides in about the same concentration range as in cardiac myocytes. In contrast to cardiac myocytes the holding current in 0 K_o^+ remained constant in the presence of the higher concentration of tyledoside C.

Because of the small amplitude of I_p and the inconstancy of I_p in many cells the range of concentrations tested was limited. To determine $K_{0.5}$ values for I_p inhibition in dorsal root ganglion neurones we applied bufadienolide concentrations which inhibited I_p for 25–75%, and fitted a sigmoidal curve through the experimental point(s) with a similar slope as found in cardiac myocytes. Fig. 4 shows the percentage of inhibition of I_p dorsal root ganglion neurones by the cumulative tyledoside F (filled squares) and the non-cumulative tyledoside C (open square). The inhibition was more pronounced in dorsal root ganglion cells for the same concentration compared to cardiac myocytes, but the difference was not statistically significant. $K_{0.5}$ values for the F derivative and for the C form in dorsal root ganglion cells were 1.7×10^{-6} M and 1.9×10^{-6} M, respectively. $K_{0.5}$ values were calculated for lanceotoxin B, thesiuside and ouabain in a similar way. In Fig. 5 all values estimated in dorsal root ganglion neurones were plotted against values measured for the same drugs in cardiac myocytes. A linear correlation was found between $K_{0.5}$ values in neurones and cardiac cells with a slope of 0.67. This finding indicates that about the same order of potency is present in dorsal root ganglion neurones compared to cardiac myocytes and that neurones bind bufadienolides with a slightly higher affinity than cardiac cells. The $K_{0.5}$ for lanceotoxin B was not affected by increasing the Na^+ concentration in the pipette to 100 mM.

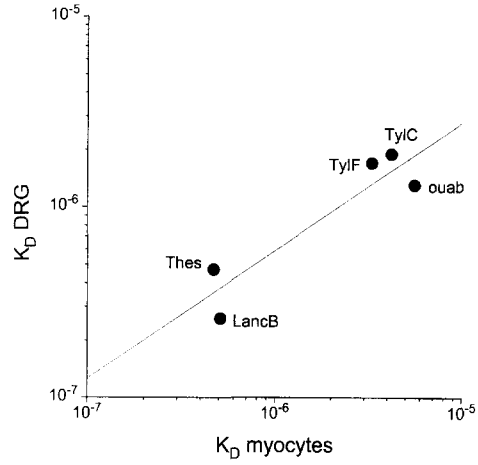

Fig. 5. Correlation between the order of potency for I_p inhibition by bufadienolides in cardiac myocytes and dorsal root ganglion neurones. Relation between the $K_{0.5}$ values (in M) of different bufadienolides (Thes, thesiuside; LancB, lanceotoxin B; TylF, tyledoside F; TylC, tyledoside C) and the cardenolide ouabain (ouab) in cardiac myocytes (abscissa) and dorsal root ganglion cells (ordinate) of the guinea pig. Experimental data were fitted by linear regression ($r = 0.9$). The slope of the curve was 0.67.

3.3. Effect of bufadienolides on I_p, generated by pump molecules with a low and high affinity for cardiac glycosides in rat cardiac cells

The main characteristic of the three different isoforms of the α subunit of the Na^+/K^+ pump is their variable affinity towards cardiac glycosides. Although this characteristic of different isoforms has been extensively studied for ouabain, it is not known whether the order of potency of different cardiac glycosides depends on the type of isoenzyme. To investigate whether the inhibitory action of cumulative and non-cumulative bufadienolides might be determined by the type of functioning isoenzyme we compared the effect of tyledoside F and C on I_p in ventricular myocytes of young rats (110 g; 5 weeks). From concentration–response curves for I_p inhibition we found (unpublished results) that about 15% of total I_p in rat myocytes of this age group is caused by activation of pump molecules with a higher affinity towards dihydroouabain. The experiments investigating the bufadienolide effects were performed with patch pipettes containing solution B (100 mM Na^+) and with 5 mM Ni^{2+} in the superfusion medium.

Fig. 6 illustrates the effect of both drugs on the inhibition of I_p. The interrupted and full lines represent the best fits of the data and they are the sum of two sigmoidal curves calculated by the following equation:

$$\%\ \text{inhibition of } I_p = f_L \frac{100 \times [\text{drug}]^h}{(K_{0.5L})^h + [\text{drug}]^h} + f_H \frac{100 \times [\text{drug}]^h}{(K_{0.5H})^h + [\text{drug}]^h}$$

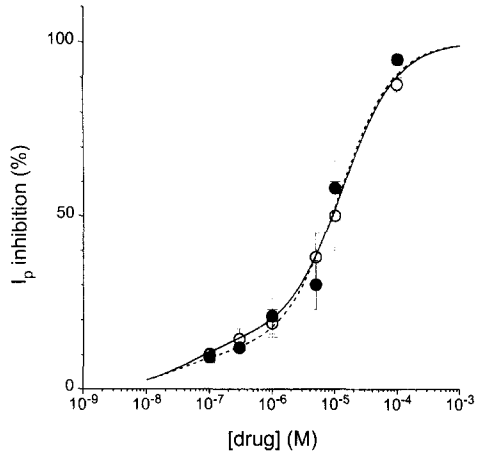

Fig. 6. Both classes of bufadienolides inhibit I_p, generated by high- and low-affinity pump molecules, to the same extent. Concentration–response curves for the inhibition of I_p by the cumulative compound, tyledoside F (open circles) and the non-cumulative drug, tyledoside C (closed circles) in rat cardiac myocytes. Data were fitted as the sum of two sigmoidal curves, following the equation: % inhibition of $I_p = f_L\{(100[\text{drug}]^h)/((K_{0.5L})^h + [\text{drug}]^h)\} + f_H\{(100[\text{drug}]^h)/((K_{0.5H})^h + [\text{drug}]^h)\}$; where f_L and f_H represent the fraction of low- and high-affinity sites; $K_{0.5L}$ and $K_{0.5H}$ are concentrations where 50% of low-, respectively, high-affinity sites are blocked. The slope factor h for binding at both binding sites was taken as 1. r^2 for the tyledoside F curve was 0.98, for the curve of tyledoside C 0.99. The $K_{0.5}$ values for inhibition of I_p generated by low- and high-affinity pump molecules were 1.3×10^{-5} M and 5.0×10^{-8} M, respectively, for tyledoside F, and 1.1×10^{-5} M and 3.5×10^{-8} M, respectively, for tyledoside C. Each data point represents the mean of 4–6 cells. No statistical difference was present between tyledoside F and C; the difference between low and high affinity $K_{0.5}$ values was highly significant for both drugs.

where f_L and f_H represent the fractions of low- (L) and high- (H) affinity sites, respectively; $K_{0.5L}$ and $K_{0.5H}$ are the concentrations for half-maximal inhibition of low- and high-affinity pump units, respectively. The slope factor h was assumed to be equal to 1.

$K_{0.5}$ values of tyledoside F and C for binding at the low-affinity sites were 1.3×10^{-5} M and 1.1×10^{-5} M, respectively. The $K_{0.5}$ for the high-affinity sites amounted to 5.1×10^{-8} and 3.5×10^{-8} M for tyledoside F and C, respectively. No statistical difference between both curves and $K_{0.5}$ values in the high- and low-concentration range could be found. The percentage of functioning high-affinity pump units contributing to I_p was 15% in the tyledoside F group, and 11% in the tyledoside C group. Both values were not statistically different.

3.4. Induction of hypercontracture in guinea-pig ventricular myocytes

It is well known that quiescent cardiac cells start to contract spontaneously and undergo an irreversible contracture when they become overloaded with Ca^{2+}. Because of the importance of Na^+/Ca^{2+} exchange for regulating intracellular Ca^{2+} in cardiac cells, an increase in intracellular Na^+ will result in secondary Ca^{2+} overload and, ultimately, in hypercontracture. Counting hypercontracted cells as a function of time is an indirect test of the rate cells will gain Na^+ and secondary Ca^{2+}. To exclude differential effects of cumulative and non-cumulative compounds on Na^+ and Ca^{2+} loading in cells utilizing Na^+/Ca^{2+} exchange for Ca^{2+} extrusion, we studied the effect of tyledoside F and C on hypercontracture development. For comparison ouabain was included in the test. Concentrations for all three substances were 10^{-5} M; at this concentration I_p was inhibited by 63, 70 and 75% for ouabain, tyledoside C and F, respectively. To increase the rate of Na^+ loading, cells were electrically stimulated by field stimulation at 1 Hz. Fig. 7 shows the percentage of surviving, rod-shaped cells as a function of time after the application of tyledoside F (open circles), tyledoside C (filled circles), and ouabain (closed squares). Each count represents the mean of five dishes; in each dish at least 60 regularly contracting, rod-shaped cells were included in the count. For each drug concentration an equal number of drug-free control dishes with cells of the same preparation were counted (open squares). These results indicate that both bufadienolides and ouabain caused a statistically significant increase in the rate of cardiac cell death. After 20 min of drug application no difference in the percentage of hypercontracted cells could be found between the three compounds.

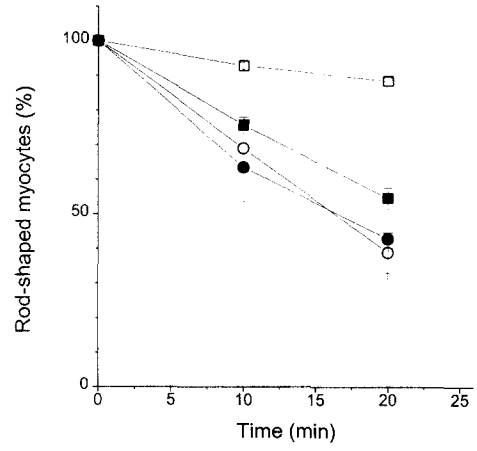

Fig. 7. Both types of bufadienolides have similar effects on hypercontracture of guinea-pig myocytes. Ordinate: the percentage of rod-shaped myocytes; abscissa: time in the presence or absence (control, open squares) of cardiac glycosides. Cells were stimulated at 1 Hz by field stimulation. Closed squares: in the presence of ouabain (10^{-5} M); open circles: tyledoside F (10^{-5} M); closed circles: tyledoside C (10^{-5} M). Each data point represents the mean count of cells in 4–9 dishes; at 0 time at least 60 rod-shaped myocytes were defined in each dish. A control dish of the same cell preparation was included for each dish to which cardiac glycosides were applied. After 20 min the difference in the number of rod-shaped cells between the control and the drug-treated cells was highly significant ($P < 0.001$). The difference between cell counts in the presence of drugs was not significant.

3.5. Bufadienolides and Ca^{2+} currents in guinea-pig ventricular myocytes

Although it is generally accepted that cardiac glycosides are specific blockers of the Na^+/K^+ ATPase, other mechanisms contributing to the positive inotropic effect have been mentioned in the literature (see Le Grand et al., 1990). A stimulatory as well as an inhibitory effect on the L-type Ca^{2+} current, and a stimulatory effect on the T-type Ca^{2+} current have been reported for ouabain (Le Grand et al., 1990; Tseng and Boyden, 1991). Because no results have been published so far on the effects of bufadienolides on Ca^{2+} currents, we investigated the effect of the cumulative drug, tyledoside F (10^{-6} M), and the non-cumulative compound, tyledoside C (10^{-6} M), on both types of Ca^{2+} currents in guinea-pig ventricular myocytes. The effects of both tyledosides were estimated 4 min after addition of the drugs. The L-type Ca^{2+} current was not influenced by any of these drugs. Both substances, however, increased the T-type Ca^{2+} current. Fig. 8 illustrates the effect of tyledoside F (D) and tyledoside C (B, C) on the current recorded at −30 mV and elicited alternatively from a holding potential of −90 mV (record 2 in A and B) and −50 mV (record 1 in A and B). Fig. 8A shows the control records; in B, the effect of tyledoside C on both currents of the same cell as in A is illustrated. Fig. 8C shows the difference currents from A (C) and B (tyl C). The difference current was clearly increased. The mean increase of the T-type Ca^{2+} current was 63 ± 13% ($n = 4$) after application of tyledoside C, and 40 ± 15% ($n = 5$) after addition of tyledoside F. Although both drugs increased the peak current, the extent of increase was variable and no statistical difference could be found. For comparison, ouabain was supplementary tested in two cells (10^{-6} M); in both myocytes the T-type current was equally increased (60% on the average). As for both tyledosides, ouabain had no effect on the L-type Ca^{2+} current.

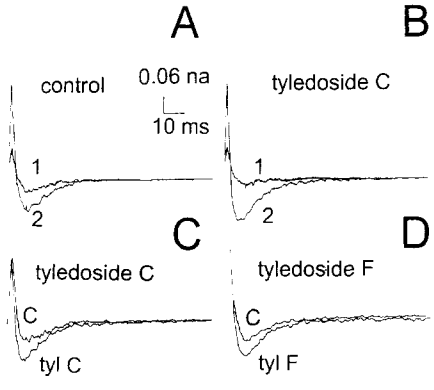

Fig. 8. Bufadienolides enhance T-type Ca^{2+} currents. Effect of bufadienolides on the low-threshold, T-type Ca^{2+} current in guinea-pig cardiac myocytes. A: Membrane currents recorded during test pulses to −30 mV, elicited from a holding potential of −50 (trace 1) or −90 mV (trace 2); duration of test pulses: 100 ms; frequency of pulses: 0.1 Hz. B: Membrane currents of the same cell as in A, 5 min after application of tyledoside C (10^{-6} M). C: Difference currents calculated from the records in A (control, C) and in B (tylC). D: Difference currents estimated in another cell in control conditions (C) and in the presence of tyledoside F (tylF; 10^{-6} M).

4. Discussion

The results of this study show that all phyto-bufadienolides tested are potent blockers of the Na^+/K^+ pump in cardiac myocytes as well as in dorsal root ganglion cells. In this respect phyto-bufadienolides share characteristics with bufadienolides from animal origin (Cruz and Matsuda, 1993) and with synthetic bufenolides (Glitsch et al., 1990), substances which bind to the pump with the highest affinity described until now. Most of the bufadienolides tested in our experiments dissociated extremely slowly from the Na^+/K^+ pump molecules, a property which favours accumulation when chronically ingested by animals and which may account for the high toxicity of these drugs. Although exact kinetics of association and dissociation could not be determined therefore, no trend seemed to be present for non-cumulative drugs to dissociate systematically faster than the cumulative ones. Affinities of neurotoxic drug were not systematically higher than those of cardiotoxic compounds. The sequence of potency for I_p inhibition of all bufadienolides tested was similar in dorsal root ganglion cells and cardiac cells. $K_{0.5}$ values of different bufadienolides were slightly smaller in dorsal root ganglion neurones, but no difference could be detected between cumulative and non-cumulative drugs. This finding might be surprising since neurones are generally expected to have a higher sensitivity towards cardiac glycosides, because α isoforms with a higher affinity towards cardiac glycosides are preferentially expressed in nervous tissue (Sweadner, 1989; Levenson, 1994). Although low- and high-affinity isoforms have been described in the peripheral nervous system (Sweadner, 1989) based on ouabain and specific antibody binding, no data are available on the quantitative distribution of different functionally active isoforms. The results described here are in line with findings on I_p suppression by dihydroouabain in dorsal root ganglion neurones and cardiomyocytes from the guinea pig (Hermans et al., 1994), showing that the major isoform generating more than 80% of total I_p is similar in both cell types. Although variable expression of different pump isoforms is of major interest for understanding selective toxicity of cardiac glycosides, studies on the contribution of different isoforms to total I_p have been limited to relatively large cells as cardiomyocytes. Even in these cells, controversy exists between studies on isoform expression based on functional measurements of I_p (Mogul et al., 1989; Gao et al., 1995) or on binding of [^{3}H]ouabain and monoclonal antibodies to different pump molecules (McDonough and Schmitt, 1985; McDonough et al., 1992, 1996; Sweadner et al., 1994). From binding studies it has been concluded that only the low-affinity α_1 isoform is

expressed in the guinea-pig heart. These findings agree with our results (not shown) on the inhibition of I_p by low concentrations of dihydroouabain in guinea-pig myocytes, but are in disagreement with findings of Mogul et al. (1989) and Gao et al. (1995). In contrast to our results in guinea-pig myocytes, the data in rat cells are consistent with the presence of two pump isoforms. The affinities of both isoforms for the bufadienolides tyledoside F and C (Fig. 6) differed by a factor of about 300. The $K_{0.5}$ values for I_p inhibition by both tyledosides in guinea-pig myocytes were nearly the same as the $K_{0.5}$ of ouabain. In the rat, α_1 has a low affinity for ouabain ($K_{0.5} = 5 \times 10^{-5}$ M), whereas the high-affinity isoforms α_2 and α_3 have affinities between 10^{-7} and 10^{-8} M (Sweadner, 1989). The $K_{0.5}$ values of both tyledosides for the inhibition of low- (about 10^{-5} M) and high- (about 5×10^{-8} M) affinity pump units fitted very well with the values expected from the ouabain data in guinea pig. The percentage of current generated by the high-affinity pumps in the rat cardiomyocytes ranged between 10 and 15%. This finding is consistent with results previously described for I_p inhibition by dihydroouabain (Hermans et al., 1995). The number is also in agreement with estimations obtained from active site phosphorylation (Sweadner and Farshi, 1987) and ouabain-binding (Noel and Godfraind, 1984), suggesting a ratio for α_1 to α_2 sodium pumps of 10 to 1, and from Na^+/K^+-ATPase inhibition suggesting a 4 to 1 ratio (Lucchesi and Sweadner, 1991). Although data on the ratio of low- to high-affinity pump units obtained by selective binding and electrophysiological methods are very consistent, the ratio between both α_2 and α_3 high-affinity isoforms could only be determined by isoform-specific antibodies. The type of high-affinity form expressed in the rat heart highly depends on the stage of maturation, with a developmental switch from α_3 to α_2 between 14 and 21 days after birth (Lucchesi and Sweadner, 1991). At all stages both isoforms should make equivalent contributions to ion pump capacity. The rat cardiac cells we have used in this study originated from 3-week-old rats. Therefore, the high-affinity form contributing to I_p in our study was most probably the α_2 isoform. In rat the overall distribution between low- and high-affinity isoforms is similar in the heart and the brain (Lichtstein et al., 1986), so that rat cardiomyocytes are well suited for performing electrophysiological studies on the interaction of both kinds of isoforms with cardiac glycosides. Because of the small amplitude of I_p in dorsal root ganglion cells and, presumably also in other neurones, assessment of the contribution of different isoforms to I_p by electrophysiological techniques will be difficult to perform. Our results in this study show that the affinity of the low-affinity isoform in cardiomyocytes and dorsal root ganglion neurones of the guinea-pig ('ouabain-sensitive' species), and the affinity of low- as well as high-affinity isoforms of rat cardiomyocytes ('ouabain-resistant' species) are similar for both classes of bufadienolides.

In the narrow therapeutical range of cardenolide concentrations clinically used for treatment of cardiac failure, neurological side effects are mostly absent, indicating that at concentrations influencing cardiac contractile force, binding to neuronal high-affinity isoforms is negligible or, at least, has no major side-effects on nervous function. The only peripheral neurological effect which becomes evident at these concentrations is the higher parasympathetic tone to the heart. These clinical observations are in agreement with the assumption that cardenolides do not preferentially bind to and influence function of neurones compared to the heart. Toad bufadienolides stimulate the release of noradrenaline from orthosympathetic nerve endings (Cress et al., 1991) at concentrations which have a positive inotropic effect on the heart too, indicating that high-affinity isoforms present in the heart and the peripheral nervous system are almost equally sensitive towards toad bufadienolides. From these indirect observations and our results discussed above, it is most likely that neurotoxicity of some phyto-bufadienolides does not originate from a preferential effect on high-affinity α pump subunits present in neurones. The hypercontracture study is in agreement with the fact that, for equal inhibition of I_p, the rate of gain of intracellular Ca^{2+} and irreversible cell damage in guinea-pig cardiomyocytes is quantitatively the same for both classes of bufadienolides, underlining again the similarity of action.

Besides differences in the density of functioning pump isoforms as a cause for tissue- and cell-specific toxicity by cardiac glycosides, sensitivity is also determined by the conformational state of the pump molecule. Intracellular Na^+ is known to enhance binding of cardiac glycosides (Stimers et al., 1990, 1991; Bielen et al., 1992). Binding only occurs when the pump molecule is in the E_2-P conformation of the pump cycle. A rise in intracellular Na^+ will favour association of cardiac glycosides with a pump molecule by increasing the cycling rate (Bielen et al., 1992). Previous experiments (Bielen et al., 1991), and the results presented in this paper, demonstrate that the Na^+ concentration at the binding sites is not well controlled during activation and inhibition of the Na^+/K^+ pump in cardiac myocytes. These changes are responsible for the time-dependent changes of I_p. The Ni^{2+}-sensitive shifts in background current during variation of pump activity are in line with the expected changes of the subsarcolemmal Na^+ concentration. I_p in the steady state may fall to one-third of its maximal value after a long period of pump inhibition (Bielen et al., 1991; Fig. 2 in this paper). In cultured chick cardiac myocytes a 10-fold change in $K_{0.5}$ was found for a threefold increase in pump current (Stimers et al., 1990). In dorsal root ganglion neurones I_p overshoots after temporary inhibition of the pump did not occur as in cardiomyocytes, indicating that the Na^+ concentration at the internal cation binding sites seems to be better controlled. The absence of a rise in background current in 0 K_o^+ was also in favour of a more

constant Na^+ concentration. These findings are in agreement with the observation that the $K_{0.5}$ value for I_p inhibition by lanceotoxin B in dorsal root ganglion neurones was not influenced by increasing the pipette Na^+ concentration to 100 mM. In guinea-pig cardiomyocytes a similar increase resulted in a threefold decrease in $K_{0.5}$. Therefore, it is reasonable to assume that in the steady state and at a pipette concentration of 30 mM Na^+, the Na^+ concentration near the pumping sites is higher in dorsal root ganglion cells compared to cardiac cells, favouring binding of cardiac glycosides.

Although the effect of intracellular Na^+ on steady-state binding and binding kinetics of cardenolides has only been measured for low-affinity isoforms (Stimers et al., 1990, 1991; Bielen et al., 1992), it most probably applies for high-affinity forms too. The higher affinity for intracellular Na^+ at the intracellular cation binding site of high-affinity isoforms (Levenson, 1994) may favour cardiac glycoside binding when these cells are electrically active and the pumps are maximally activated. Furthermore, it may be expected that an increase in exposure time of the cardiac glycoside binding site will influence the probability of binding of compounds with a slow association rate constant more. Toxicity of cardiac glycosides in different cell types will depend therefore on the type of pump isoforms expressed, the dependency of individual isoforms on internal Na^+, the Na^+ concentration at the internal cation binding sites, and the type of cardiac glycoside.

In addition to similarity in suppression of I_p, both types of bufadienolides influenced hypercontracture and Ca^{2+} currents in cardiac myocytes to the same extent. We confirmed the observations of Le Grand et al. (1990) that ouabain can increase Ca^{2+} currents in ventricular myocytes. We found that ouabain and bufadienolides of either group increased the T-type Ca^{2+} current. In contrast to the findings of Le Grand et al. (1990), the L-type Ca^{2+} current was not influenced by ouabain and bufadienolides in our experimental conditions. However, these authors could only find an increase if the Na^+/K^+ pump was inhibited previously by superfusing the cell with 0 K_o^+. In our conditions K^+ was absent in the external medium, but another activator cation of the pump, Cs^+, was present in sufficient amounts (20 mM) to activate the pump substantially. If total Na^+/K^+ pump inhibition was the necessary requisite for the effect on the L-type Ca^{2+} current, then our findings agree very well with the published results. Although alternative hypotheses have been presented for these effects (Le Grand et al., 1990), the observed phenomena might be related to the gain in intracellular Ca^{2+}, secondary to the rise in internal Na^+. The T-type Ca^{2+} current is augmented by an increase in internal Ca^{2+} (Tseng and Boyden, 1991). The L-type Ca^{2+} current may be facilitated in a similar way, but the increase in peak current may be counteracted by the simultaneous rise in the rate of inactivation. The net effect, either a suppression, a rise or no effect on the L-type current may depend therefore on the elevation of intracellular Ca^{2+} during pump inhibition. These effects explain why the quantitative effects of bufadienolides and ouabain on both currents were almost the same for all three drugs, because the Na^+/K^+ pump was inhibited to the same extent in all three conditions.

In conclusion, these results show that phyto-bufadienolides isolated are potent inhibitors of the Na^+/K^+ pump in cardiac myocytes as well as in dorsal root ganglion neurones. The preferential effect of some of these bufadienolides on the nervous system could not directly be related to different effects on the Na^+/K^+ pump current generated by different pump isoforms in cardiac cells and dorsal root ganglion neurones. Neurospecificity of some bufadienolides may be caused indirectly by differences in intracellular Na^+ and, ultimately, by differences in distribution and accumulation in the nervous system.

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