Characterization of the effects of the new inotropic agent BDF 9148 in isolated papillary muscles and myocytes of the guinea-pig heart

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1 The cardiotonic agent BDF 9148 (4-[3'-(1"-benzhydryl-azetidine-3"-oxy)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) is structurally related to DPI 201-106 (4-[3'-(4"-benzhydryl-1"-piperaziny)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) which is known to modify cardiac sodium channels. In guinea-pig papillary muscles, both compounds increase force of contraction with similar concentration-response curves. Like DPI 201-106, BDF 9148 prolongs the action potential duration in a tetrodotoxin-sensitive manner. With high concentrations ($>3\text{ }\mu\text{M}$), however, the action potential duration shortens again. In order to elucidate the underlying changes in membrane currents, we have investigated the effects of BDF 9148 in isolated ventricular myocytes of the guinea-pig heart.

2 In isolated cells, a concentration of $1\text{ }\mu\text{M}$ BDF 9148 prolonged the action potential duration and markedly enhanced unloaded cell shortening, indicating that the procedure of cell isolation does not abolish the effect of the drug.

3 Membrane currents were studied with the single electrode voltage clamp technique. With clamp steps from -80 mV to -40 mV , BDF 9148 ($1\text{ }\mu\text{M}$) induced a slowly decaying inward current which was suppressed by tetrodotoxin. Therefore, like DPI 201-106, BDF 9148 slows the inactivation of the sodium channels.

4 In order to quantify the effects of BDF 9148 and DPI 201-106 on sodium current inactivation, we have measured the inward current amplitude still present at 100 ms after a depolarizing clamp step from -80 mV to -30 mV . Both drugs increased this current component in a concentration-dependent manner; however, BDF 9148 had a larger effect in the low concentration range.

5 The calcium current was inhibited by BDF 9148 and DPI 201-106 in a concentration-dependent manner; the pD_2 values were 5.70 and 5.95, respectively.

6 The two compounds are thought to produce similar positive inotropic effects by imposing a sodium load on the muscle cells via modification of the sodium channels. The differences in action potential duration could be due to different contributions of ionic currents other than sodium or calcium currents and of pump and exchange currents. At present, there is not sufficient data to identify clearly distinct current components responsible for the differences in action potential prolongation.

Keywords: Cardiotonic agent, BDF 9148; sodium channel modifying agents; DPI 201-106; papillary muscle; cardiac myocytes; action potential; whole cell patch clamp; calcium current; sodium current

Introduction

The positive inotropic effect of DPI 201-106 (4-[3'-(4"-benzhydryl-1"-piperaziny)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) in isolated heart muscle is accompanied by a marked prolongation of action potential duration (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985). This *in vitro* effect is paralleled *in vivo* by a prolongation of the QT-interval of the electrocardiogram (Rüegg & Nüesch, 1987) which limits its clinical use because of the danger of developing torsades de pointes arrhythmias (for literature, see Roden, 1990). Therefore, a drug with a similar mechanism of action would be considered more favourable if it exhibited less prolongation of action potential duration and hence showed less prolongation of QT time.

DPI 201-106 prolongs the action potential duration due to a delay of the inactivation of the Na^+ channels (Buggisch *et al.*, 1985; Kohlhardt *et al.*, 1986). DPI 201-106 also inhibits the calcium current (I_{Ca}) as shown by its effects on slow action potentials in partially depolarized papillary muscles (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985) and by voltage clamp experiments in guinea-pig cardiomyocytes (Holck & Osterrieder, 1988; Pfeifer *et al.*, 1989). The concentration which blocks calcium channels is one order of magnitude higher than that required for the positive inotropic effect (Scholtysik *et al.*, 1985).

The DPI 201-106 analogue, compound BDF 9148 (4-[3'-(1"-benzhydryl-azetidine-3"-oxy)-2'-hydroxypropoxy]-2-methyl-1H-indole-2-carbonitrile) also has a positive inotropic effect, prolongs the action potential duration, delays inactivation of the sodium current and blocks I_{Ca} (Armah *et al.*, 1990; Honerjäger *et al.*, 1990; Pfeifer & Ravens, 1990).

Since the prolongation of action potential duration accompanying a given positive inotropic effect seemed smaller for BDF 9148 than for DPI 201-106 (compare Buggisch *et al.*, 1985 and Armah *et al.*, 1990) the difference in the effects of BDF 9148 and DPI 201-106 on action potential duration can only be explained if either drug has additional effects on other membrane currents. Less prolongation in action potential duration could result if the BDF 9148 concentrations required for modification of I_{Na} and I_{Ca} were closer than those of DPI 201-106 or if the compounds have opposing effects on potassium conductance.

In a first series of experiments, we investigated concentration-response curves for DPI 201-106 and BDF 9148 in guinea-pig papillary muscles in order to quantify the differences in action potential prolongation and positive inotropic effects of these two agents under identical experimental conditions. We then examined the effects of BDF 9148 on membrane currents in isolated myocytes using the single electrode voltage clamp technique. The only difference between both compounds was a larger effect of BDF 9148 on sodium

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current (I_{Na}), particularly in the low concentration range ($<1 \mu\text{M}$); the effects on calcium current (I_{Ca}) were similar. Some of these results have been published in a preliminary form (Pfeifer & Ravens, 1990).

Methods

Experiments with papillary muscles

Right ventricular papillary muscles were carefully dissected from guinea-pig hearts and mounted horizontally in a 5 ml muscle chamber through which oxygenated modified Tyrode solution recirculated from a 100 ml reservoir kept at 35°C . A jet stream of carbogen simultaneously served for circulation and oxygenation of the solution. The preparations were stimulated via platinum electrodes at a frequency of 1 Hz (square wave pulses of 3 ms in duration 20% above threshold voltage). Action potentials were recorded with conventional glass microelectrodes (filled with 3 M KCl) and force of contraction was measured by an isometric force transducer (Statham UC2). Both signals were displayed on the screen of a dual beam oscilloscope, the screen of which was photographed at regular intervals for later analysis of the data. Drugs were added in a cumulative manner to the reservoir of the bathing solution, allowing at least 45 min for equilibration before the next concentration was tested.

Isolated cardiac myocytes

Single ventricular myocytes from guinea-pig hearts were prepared by enzymatic dissociation as described previously (Borchard & Ravens, 1986) and stored at room temperature (20 – 25°C) until use. A small drop of cell suspension was added to the investigation chamber placed on the stage of an inverted microscope (Olympus IMT-2). During the experiments, cells were superfused with modified Tyrode solution at a constant rate of 2 ml min^{-1} . Current recordings were made at room temperature, action potential and contractility recordings at 35 – 36°C . BDF 9148 and DPI 201-106 are poorly soluble in aqueous solutions and strongly adhere to surfaces; thus exact control of drug concentration in the superfusing solution is difficult to obtain. In order to avoid contamination of the chamber with agent from the previous experiment, a freshly cleaned chamber was used in each experiment and new connective tubings were employed. To clean the investigation chambers, they were left over night in detergent (Mucosol, Merz, Frankfurt, Germany) followed by rinsing several times with distilled water.

Action potentials were measured with a LIST EPC-7 patch-clamp amplifier (LIST medical instruments, Darmstadt, Germany) in the current clamp mode. The contractility of the cells was recorded as unloaded shortening via an array of photodiodes placed over the edge of the cell image on a television screen. An electronic device designed by H. Ehrler (Homburg/Saar, Germany) was used to record the light intensity changes due to the contraction of the cells. For current measurements, the single electrode voltage clamp circuit provided by the LIST EPC-7 amplifier was used in the voltage clamp mode with compensation made for series resistance (at least 50%) and membrane capacitance. Membrane currents were monitored and stored on a digital storage oscilloscope (NIC 4094A, Nicolet Instrument Corporation, Madison, Wisconsin, U.S.A.) and registered in parallel on a chart recorder (GOULD Brush 220, Gould Inc., Cleveland, Ohio, U.S.A.) for later evaluation. In some cells, the PClamp software (Axon Instruments, Foster City, CA, U.S.A.) was used for conducting the experiments and analyzing the results.

Patch electrodes were pulled from filament borosilicate glass (Hilgenberg, Malsfeld, Germany). Their tip resistances were in the range of 2 – $4 \text{ M}\Omega$ when filled with solution of the following composition (mm): KCl 140, MgCl_2 4, EGTA (ethylene-glycol-bis-(β -aminoethylether-N,N,N',N'-tetraacetic acid) 10, HEPES (hydroxyethyl-piperazinyl-ethane-sulphonic

acid) 10 and Na_2ATP 4. The pH was adjusted to 7.3 with KOH. EGTA was omitted in experiments in which contractility was studied. For recording of sodium and calcium currents, the electrode filling solution contained CsCl instead of KCl and the pH was adjusted with CsOH. Results from cells showing more than 30% decline of I_{Ca} during the initial 5 min of the experiment were discarded. For recording of potassium currents, the electrode filling solution contained (in mm): KCl 120, MgCl_2 0.5, EGTA 10, HEPES 10 and Na_2ATP 4. The pH was adjusted to 7.3 with KOH.

Solutions and drugs

For experiments in guinea-pig papillary muscles, the composition of the modified Tyrode solution used was (in mm): NaCl 137, KCl 5.4, MgCl_2 1.05, CaCl_2 1.8, NaH_2PO_4 0.42, NaHCO_3 22.0 and glucose 5. The solution was gassed with a mixture of 95% oxygen and 5% carbon dioxide, the pH was 7.4.

For experiments with myocytes, the modified Tyrode solution had the following composition (in mm): NaCl 150, KCl 5.4, MgCl_2 2.0, CaCl_2 1.8, HEPES (hydroxyethyl-piperazinyl-ethane-sulphonic acid) 10 and glucose 10. In the experiments for the measurement of sodium current, CsCl was substituted for KCl (Cs-Tyrode) in order to reduce K^+ currents (Isenberg, 1976). The pH was adjusted with NaOH or CsOH to 7.4.

Racemic BDF 9148 was synthesized by Beiersdorf AG, Hamburg, Germany. Racemic DPI 201-106 was kindly provided by Sandoz Co., Basel, Switzerland. Stock solutions of BDF 9148 (1 mm) or DPI 201-106 (1 mm) were prepared in dimethylsulphoxide (DMSO), the final concentration was obtained by direct dilution in Tyrode solution. The final concentration of DMSO in the bathing solution never exceeded 1% (vol/vol) and did not influence the parameters under investigation. Tetrodotoxin was purchased from Boehringer-Mannheim, Mannheim, Germany. All other chemicals were purchased from Merck, Darmstadt, Germany.

Data analysis and statistics

All values are expressed as mean values $\pm$ s.e.mean. The data were analysed by Kruskal-Wallis analysis and by Student's *t* test for paired or unpaired data where appropriate. Differences were considered statistically significant for $P < 0.05$.

Results

Concentration-response curves of DPI 201-106 and BDF 9148 in guinea-pig papillary muscles

After 60–90 min of equilibration, the control force of contraction of guinea-pig papillary muscles was $1.54 \pm 0.31 \text{ mN}$ and $1.39 \pm 0.48 \text{ mN}$ in the BDF 9148 and DPI 201-106 group, respectively ($P > 0.2$, not significant); the control values for the action potential duration were $225 \pm 7 \text{ ms}$ and $195 \pm 8 \text{ ms}$. The muscles were then exposed to cumulatively increasing concentrations of BDF 9148 or DPI 201-106 and the changes induced by the compounds were expressed as a factor of the pre-drug control values ($=1$). Under identical experimental conditions, both drugs augmented force of contraction and increased action potential duration in a monotonic time course. However, at the highest concentration of BDF 9148 investigated ($10 \mu\text{M}$), action potential duration declined again. Both drugs had similar concentration-response curves for the positive inotropic effect, but less prolongation of action potential duration was observed with BDF 9148 than with DPI 201-106 (Figure 1). The differences were statistically significant both in relative and absolute terms for concentrations equal to or larger than $1 \mu\text{M}$. Mean action potential amplitude was $117.8 \pm 1.5 \text{ mV}$, resting membrane potential was $-81.6 \pm 0.8 \text{ mV}$, and maximum rate of depolarization (dV/dt_{max}) was $240 \pm 15 \text{ V s}^{-1}$. These values were not significantly influenced by either compound (data not shown).

Figure 1 Concentration-response curves of the effects of BDF 9148 (▲) and DPI 201-106 (□) on force of contraction (a) and action potential duration (b) in guinea-pig papillary muscles. Drug concentration was increased cumulatively in steps of one half log unit. The experimental values were normalized to the control values, measured at the end of the equilibration period. Abscissa scale: molar concentration in log units. Mean values from 5 to 7 experiments; s.e. mean shown by vertical lines. The asterisks indicate statistically significant differences (Kruska-Wallis analysis for group comparison, $*P < 0.05$); NS, not significant.

Effects of BDF 9148 on action potentials and contractility in isolated myocytes

BDF 9148 ($1 \mu\text{M}$) also enhanced the contractility measured as unloaded shortening and prolonged the action potential duration (APD) of electrically stimulated cardiac cells (see Figure 2). After 14 min of washing in drug-free solution, the contraction amplitude declined below the pre-drug control value, whereas the prolongation in action potential duration was only slightly reversed. Neither resting potential nor action potential amplitude were changed; in 5 cells, the mean values of the action potential amplitude were $125.0 \pm 2.1 \text{ mV}$ and $125.5 \pm 1.9 \text{ mV}$ and the mean values of the resting membrane potential were $-74.3 \pm 0.3 \text{ mV}$ and $-75.5 \pm 0.3 \text{ mV}$ for the controls and in the presence of BDF 9148 ($1 \mu\text{M}$), respectively. These results demonstrate that after the isolation procedure, the cells remained responsive to BDF 9148. Therefore, they are a suitable model to study the effects of the compounds on membrane currents.

Effect of BDF 9148 on sodium current

Voltage clamp studies were carried out in order to identify the components of net membrane current responsible for the changes in APD. Because of the large amplitude of the Na^+ current in adult myocytes, adequate voltage control for quantitative analysis is difficult to achieve. At room temperature, however, small cells can be clamped with reasonable quality if low resistance electrodes ($< 2.5 \text{ M}\Omega$) are used. Figure 3 shows tracings from a typical cell: a large, rapidly inactivating

Figure 2 Action potentials and contraction amplitudes measured in a single myocyte (guinea-pig heart) before and after exposure to BDF 9148. After 6 min of exposure to $1 \mu\text{M}$, the cell was washed with drug-free solution for 14 min. Time and voltage scales as indicated, cell shortening in arbitrary units. Stimulation frequency 0.5 s^{-1} , depolarizing current steps of 0.5 nA in amplitude and of 8 ms in duration.

inward current was measured during a potential step from -80 mV to -40 mV . Addition of BDF 9148 ($1 \mu\text{M}$) retarded inactivation of the inward current so markedly that the net membrane current remained inwardly directed for almost 500 ms . The effect was completely abolished by the selective Na^+ channel blocker tetrodotoxin ($30 \mu\text{M}$), suggesting that this current flows through modified Na^+ channels.

In order to quantify the drug effects on Na^+ current inactivation, we have established concentration-response curves (Figure 4a). Sodium currents were activated by clamp steps from -80 mV to -30 mV (clamp step duration 250 ms , frequency 0.2 s^{-1}). Under control conditions, the Na^+ current was completely inactivated 100 ms after the beginning of the depolarizing step. In the presence of various concentrations of BDF 9148 or DPI 201-106, however, an appreciable inward

Figure 3 Effects of BDF 9148 ($1 \mu\text{M}$) and subsequent addition of tetrodotoxin (TTX, $30 \mu\text{M}$) on net membrane current in response to 2.5 s long clamp pulses from -80 mV to -40 mV at a frequency of 0.1 s^{-1} . Calibrations as indicated by the bars. The pre-drug control tracing consists of a large, rapid downward deflection (inward current). After addition of BDF 9148, the inward current decays slowly and persists for almost 500 ms . It is blocked by TTX.

Figure 4 Concentration-response curves for the effects of BDF 9148 ($\blacktriangle$) and DPI 201-106 ($\square$) on membrane currents in cardiac myocytes. (a) Persisting I_{Na} after 6 min of drug exposure measured 100 ms after a step depolarization from -80 mV to -30 mV (arrow in inset), duration of test pulse 250 ms, frequency 0.2 s^{-1} . Only one drug concentration was studied in each cell ($1\text{ }\mu\text{M}$ in inset). Mean values of at least 3 experiments in each group; vertical lines show s.e.mean. The asterisks indicate statistically significant differences (Kruskal-Wallis analysis for group comparison, $P < 0.05$); NS, not significant. (b) Decrease in I_{Ca} expressed as percentage inhibition of the control value measured 8 min after the beginning of the experiment. The effect was measured after 7 min of drug exposure (one concentration only in each cell, $10\text{ }\mu\text{M}$ in inset). Clamp pulses were applied at a frequency of 0.2 s^{-1} , holding potential -40 mV, clamp potential 0 mV, duration of depolarization 300 ms. The control value (C) was time-matched and presents the spontaneous decline of I_{Ca} ('run-down') in the control cells. Mean values from 4–8 experiments; vertical lines show s.e.mean. pD_2 values of 5.70 and 5.96 for BDF 9148 and DPI 201-106, respectively, were estimated from the fit of a 4 parameter logistic equation. Insets: original tracings of membrane currents in response to the depicted clamp steps before (C) and after drug application ($\blacktriangle$).

current was detectable at this time (see inset in Figure 4a), the amplitudes of which were taken as a measurement of the effects of the drugs on Na^+ current inactivation. Figure 4a clearly shows that BDF 9148 affected this parameter at lower concentrations than DPI 201-106. There was no statistically significant difference between the maximum effects.

Effect of BDF 9148 on calcium current

For the study of Ca^{2+} currents, cells were kept at a holding potential (V_h) of -40 mV to inactivate the Na^+ current and were depolarized by clamp pulses of 300 ms duration to 0 mV at a frequency of 0.2 Hz . BDF 9148 was added 8 min after the start of the experiment. All experiments were terminated after 15 min. Taking the value at the end of the 8 min control period as 100% I_{Ca} of control cells decreased to $87 \pm 6\%$ during the subsequent 7 min ($n = 6$; spontaneous 'run-down'), but BDF 9148 ($10\text{ }\mu\text{M}$) reduced I_{Ca} to $13 \pm 2\%$ ($n = 4$). The

concentration-response curves for BDF 9148 and DPI 201-106 are depicted in Figure 4b. Both drugs inhibited I_{Ca} in a similar concentration range, the pD_2 values were 5.70 and 5.96, for BDF 9148 and DPI 201-106, respectively. For both drugs, inhibition of I_{Ca} was almost complete at the highest concentrations of $10\text{ }\mu\text{M}$. Neither BDF 9148 nor DPI 201-106 shifted the current voltage relation of I_{Ca} along the voltage axis (data not shown).

Discussion

In the present study, we confirm that the new inotropic agent BDF 9148 enhances force of contraction in papillary muscles to a similar maximum as its parent compound DPI 201-106, but that this effect is accompanied by less prolongation in action potential duration (Armah *et al.*, 1990). Since the positive inotropic effect of the two compounds is abolished by the sodium channel blocking agent tetrodotoxin, it is concluded that it is intimately linked to their inhibitory effect on I_{Na} inactivation (Ravens, 1976; Honerjäger, 1982; Isenberg & Ravens, 1984; Buggisch *et al.*, 1985). BDF 9148- or DPI 201-106-modified sodium channels allow Na^+ influx to persist during membrane depolarization and the resulting increase in intracellular sodium concentration causes a rise in the cellular calcium content due to decreased calcium efflux via the $\text{Na}^+/\text{Ca}^{2+}$ exchange (Ravens & Wettwer, 1989). This shift in intracellular ionic distribution is considered to be responsible for the positive inotropic effect (Honerjäger, 1982).

Changes in the shapes of the action potentials are more difficult to interpret because the action potential duration is determined by a delicate balance of ionic, pump and carrier currents directed both outward- and inwardly. The persisting Na inward current induced by both DPI 201-106 and BDF 9148 probably contributes directly to the APD prolonging effect, although it cannot be the only cause since we have found that BDF 9148 causes *less* APD increase in spite of the fact that it appears to have a greater influence on I_{Na} . Differential effects in I_{Ca} can be ruled out, because BDF 9148 and DPI 201-106 concentration-dependently reduced I_{Ca} without significant differences between the two compounds. The effect of BDF on APD could be explained if the concentration-response curves for modification of I_{Na} and I_{Ca} lay close together so that the APD-prolonging effect of persisting I_{Na} is counteracted by the APD-shortening effect of inhibition of I_{Ca} . This is, however, not the case; on the contrary, BDF 9148 is more potent in modifying I_{Na} than I_{Ca} whereas DPI 201-106 influences both currents with similar potencies. Therefore, either compound must have an additional effect on other membrane currents.

The inward rectifier, I_{K1} , or the delayed rectifier, I_K , could also be targets for BDF 9148 and DPI 201-106-induced changes in APD. In preliminary experiments, we were unable to observe clear effects on these currents, however, the changes may have been too small for detection yet still relevant for plateau prolongation (Trautwein, 1973). This point needs to be addressed in a further carefully designed study, because there is some evidence from the literature, that DPI 201-106 does indeed decrease I_{K1} (Holck & Osterrieder, 1988).

The concentration-response curves for the positive inotropic effects of BDF 9148 and DPI 201-106 were found to be superimposed. If we assume that the increase in force is mainly due to sodium load, both drugs should induce a similar load (estimated as sodium current integral corrected for the action potential duration). This corresponds well with the larger effect of BDF 9148 on I_{Na} combined with a shorter action potential, whereas DPI 201-106 increases I_{Na} to a lesser extent yet causes more AP prolongation.

In addition, the increased intracellular Na^+ concentration may modify physiological pump and exchanger currents which also affect the action potential duration. With BDF 9148, the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ could contribute more outwardly directed pump current and therefore shorten the APD. In

the potential range of the plateau phase, the $\text{Na}^+/\text{Ca}^{2+}$ exchange is also outwardly directed and an increase in exchange current caused by Na^+ load should also shorten the action potential duration (Hilgemann, 1988; Ravens & Wettwer, 1989; Wettwer & Ravens, 1991).

Each of the compounds used was the racemic mixture of a pair of enantiomers with opposing actions on I_{Na} (Scholtysik *et al.*, 1986; Romey *et al.*, 1987; Wang *et al.*, 1990) and similar effects on I_{Ca} (see Ravens *et al.*, 1991 for DPI 201-106 enantiomers; no data available for BDF 9148 enantiomers). Therefore, different contributions of the enantiomers in each racemic mixture to the various effects on membrane currents could also explain our observations. This problem must be solved in future experiments with the enantiomers.

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