Receptor Subtypes Involved in the $\alpha_1$-Adrenoceptor Mediated Increase in $^{86}$Rb$^+$ Efflux from the Rat Heart

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Received May 6, 1997
Accepted July 18, 1997

ABSTRACT—The aim of this study was to determine the involvement of the different $\alpha_1$-adrenoceptor subtypes in the $\alpha_1$-adrenoceptor mediated increase in $^{86}$Rb$^+$ efflux from rat hearts. Isolated hearts were perfused in the presence of a $\beta$-adrenoceptor antagonist (1 $\mu$M timolol). After loading with $^{86}$Rb$^+$, the efflux was measured during $\alpha_1$-adrenoceptor stimulation by phenylephrine (30 $\mu$M). Phenylephrine increased the $^{86}$Rb$^+$ efflux by about 30%. Pretreatment with the preferentially $\alpha_{1B}$-adrenoceptor inhibitor chloroethylclonidine (CEC), reduced the response to phenylephrine by about 50%. The preferential $\alpha_{1D}$-adrenoceptor inhibitor BMY 7378 inhibited the response to phenylephrine by 35%, with a pKi = 8.4 (95% C.I. 8.2–8.6). The response was sensitive to the preferential $\alpha_{1A}$-adrenoceptor inhibitors (+)niguldipine, 5-methylurapidil (5-MU) and WB-4101 at relatively high concentrations, and 5-MU inhibited the response with a pKi = 7.7 (95% C.I. 7.2–8.0) in CEC pretreated hearts. In conclusion, the phenylephrine stimulated increase in $^{86}$Rb$^+$ efflux in the rat heart is not specifically linked to only one of the $\alpha_1$-adrenoceptor subtypes, but involves the $\alpha_{1B}$- and the $\alpha_{1D}$-adrenoceptor subtypes, and probably the $\alpha_{1A}$-adrenoceptor subtype as well.

Keywords: $\alpha_1$-Adrenoceptor subtype, $^{86}$Rb$^+$ efflux, Rat heart, Chloroethylclonidine, BMY 7378

$\alpha_1$-Adrenoceptors mediate many physiological effects of catecholamines in the mammalian heart (1, 2). Stimulation of myocardial $\alpha_1$-adrenoceptors elicits a positive inotropic response (1, 3), induces cardiac myocyte hypertrophy and gene transcription (4) and stimulates ionic transport (5, 6). Now three $\alpha_1$-adrenoceptor subtypes, showing high sensitivity to prazosin, have been identified by pharmacological methods in contrast to the original subdivision into $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptor subtypes (7). Thus the current classification includes three $\alpha_1$-adrenoceptors named $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$-adrenoceptor subtypes (8). In addition, three $\alpha_1$-adrenoceptor subtypes have been cloned (9–12). We have previously reported that phenylephrine during $\beta$-adrenoceptor blockade causes a concentration-dependent increase in $^{86}$Rb$^+$ efflux in the rat heart (13). The response showed a high sensitivity to prazosin ($K_I=6.1 \times 10^{-11}$ M), implying that it is mediated through $\alpha_1$-adrenoceptors. In order to extend the characterization of the involved $\alpha_1$-adrenoceptors with respect to the subtypes, the effect of phenylephrine stimulation on $^{86}$Rb$^+$ efflux in the presence of subtype preferential antagonists was studied.

MATERIALS AND METHODS

Heart perfusion

Hearts from male Wistar rats, fed ad libitum, with body weight of 200–300 g, were isolated under ether anesthesia. The aorta was cannulated, and the hearts were spontaneously beating and perfused in a non-recirculating system. The flow was kept constant (10 ml/min) by a Bifok FIA 08 roller pump or a HiLoad Pump P-50 reciprocating piston pump (both pumps from Pharmacia Biotech, Uppsala, Sweden). The basic perfusion medium contained: 119.3 mM NaCl, 3.0 mM KCl, 2.4 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2.0 mM CaCl$_2$, 24.9 mM NaHCO$_3$, 10.0 mM glucose, and 2.2 mM mannitol and was equilibrated with 95% $O_2$ / 5% CO$_2$ at 31°C (pH 7.4). When used, $^{86}$Rb$^+$ was added to the perfusion medium to yield approximately 1–2 x 10$^7$ cpm/ml.

Experimental design

All hearts were perfused with a basic perfusion medium containing a $\beta$-adrenoceptor antagonist (timolol, 1 $\mu$M) throughout the experiment to suppress the $\beta$-adrenoceptor component of phenylephrine stimulation (14). After a
perfusion period of 10 min, the hearts were loaded with the K⁺ analogue ⁸⁶Rb⁺ for 30 min, followed by a 25-min control period and a 30-min period of agonist stimulation. Perfusate fractions of 3 ml (collection period, 18 sec) were collected consecutively with an LKB 7000 Ultra Rac fraction collector (Pharmacia Biotech) during the washout period. At the end of the experiment, the hearts were freeze-clamped with an aluminium clamp precooled in liquid nitrogen and pulverized in a cooled percussion mortar. The hearts weighed 1.0–1.45 g (wet weight). The tissue powder was stored in plastic vials at −80°C until ⁸⁶Rb⁺ tissue radioactivity was determined. When used, one of the preferential α₁A-adrenoceptor antagonists, WB-4101, (+)-niguldipine and 5-methylurapidil (5-MU), or the preferential α₁D-adrenoceptor antagonist BMY 7378 was added to the perfusion buffer at the start of the washout period. 5-MU was preferred among the α₁A-adrenoceptor antagonists when a concentration-inhibition curve was constructed because it preferentially binds to α₁A-adrenoceptors (15), with a higher degree of selectivity than WB-4101 (16–18), and avoids the problems related to the high lipophilicity of (+)niguldipine (19, 20).

When used, the irreversible preferential α₁B-adrenoceptor antagonist chloroethylethlonidide (CEC) was preincubated for 30 min (the same 30 min period in which the hearts were loaded with ⁸⁶Rb⁺) and was then washed out for 25 min before the hearts were exposed to the α₁-adrenoceptor agonist phenylephrine (30 μM). Corresponding and separate groups were exposed to phenylephrine in the presence or absence of CEC, (+)niguldipine, 5-MU or BMY 7378, respectively. In addition, groups with the combination of CEC and either (+)niguldipine, 5-MU or WB 4101 were included. The maximal response to α₁-adrenoceptor stimulation varied to some degree between the experimental series. Thus the effect of each subtype receptor antagonists were compared with separate groups of hearts exposed to phenylephrine alone.

Determination of ⁸⁶Rb⁺-radioactivity

Radioactivity in the collected perfusate fractions, and in the heart tissue powder was measured with a Packard, model 1900 TR Tri-Carb liquid-scintillation spectrometer (Packard Instrument Company, Downers Grove, IL, USA). The count rates were corrected for radioactivity decay. The radioactivity of the perfusate fractions was determined as Cerenkov radiation directly in the aqueous medium. The heart tissue powder (150 mg) was homogenized in 7.5% trichloroacetic acid at a dilution of 1:10 wt./vol. The radioactivity in 0.1 ml supernatant was determined as Cerenkov radiation after addition of 2.9 ml basic perfusion medium. The contents of the heart radioactivity at time points corresponding to the sampling time of each perfusate fraction were calculated. To the ⁸⁶Rb⁺ contents in the hearts at the end of perfusion, the contents in each previous perfusate fraction were added sequentially and cumulatively.

Calculations of ⁸⁶Rb⁺ washout kinetics and efflux rate

The rate of ⁸⁶Rb⁺ efflux was calculated as radioactivity appearing in the perfusate per min (cpm/min) during washout. The efflux rate index ("fractional efflux" (in min⁻¹)) could be expressed as the ratio between perfusate ⁸⁶Rb⁺ radioactivity and ⁸⁶Rb⁺ contents of the heart at the corresponding time points. A curve fit computer program was used to perform a regression analysis that described the course of the efflux rate index in the control period (25 min) before agonist stimulation. The equation of a hyperbola was used in the computer program (y=(c/x−b)+a, (13)). When the regression line constructed from the data points in the first 15 min period was extrapolated beyond this period, it correlated well with the actual observed course of the efflux rate index in control experiments (r²=0.86, Fig. 1A). Thus the regression lines were calculated individually from the control periods before intervention and were extrapolated into the stimulation period (Fig. 1A). In this way, each heart was used as its own control. The response was expressed as percentage of the extrapolated control index during the corresponding part of the washout period (Fig. 1B). The maximal response in each individual experiment was determined as the maximal increase in percent irrespective of time within the 30-min observation period. The effect of the antagonists was measured as the difference between the maximal response in the presence or absence of antagonists (mean values), respectively.

Construction of concentration-inhibition curves and statistics

The relative response to each concentration of antagonist was plotted as the mean value of the different experiments. The concentration giving 50% reduction (IC₅₀) of phenylephrine-evoked ⁸⁶Rb⁺ efflux was estimated by a curve-fitting computer program (Graph Pad Prism™, Graph Pad Software Inc., San Diego, CA, USA). The estimated IC₅₀ value was used together with the EC₅₀ value for phenylephrine stimulated ⁸⁶Rb⁺ efflux (0.45 μM) obtained from previous work (13) to calculate the Kᵢ (inhibition constant) value according to the Cheng and Prusoff equation (21). Values are given as means with S.E.M., except for the pKᵢ values (−log Kᵢ) which are given with 95% confidence interval (C.I.). Parametric statistical analysis was used (Student’s t-test); P<0.05 was considered to reflect statistically significant differences.
Drugs

Phenylephrine hydrochloride, timolol maleate, mannitol and WB 4101 [2-(2,6-dimethoxyphenoxyethyl)amino-methyl-1,4 benzodioxane] were purchased from Sigma Chemical, St. Louis, MO, USA) and BMY 7378 (8-[(2-(2-methoxyphenyl)-1-piperazinyl)ethyl]-8-azaspiro[4.5] decane-7,9-dione dihydrochloride) and chloroethylclonidine dihydrochloride, from Research Biochemicals Internationals (Natick, MA, USA). (+)Niguldipine and 5-methylurapidil were the kind gifts of Dr. Sanders at Byk Gulden Lomberg (Konstanz, Germany), made available through Pharmacia AS (Oslo, Norway). Stock solutions were prepared in double-distilled water and maintained at -20°C. Further dilutions were made fresh daily and kept cool (0-4°C). Rubidium-86 (specific activity in the range of 37-403 MBq/mg Rb-86) was purchased from Du Pont (Brussel, Belgium) or from Amersham International (Buckinghamshire, UK).

RESULTS

Effect of α₁-adrenoceptor stimulation

The basal 86Rb⁺ efflux rate index decreased with time during the washout period (Fig. 1A). After the 25-min washout (time of agonist intervention), the basal efflux rate index was 3.0±0.05 x 10⁻² min⁻¹ (n=32). Within 5 min after phenylephrine (30 μM) stimulation, the 86Rb⁺ efflux rate index increased and reached a plateau level after 19±0.9 min (Fig. 1A). The new plateau was 0.8±0.04 x 10⁻² min⁻¹ higher than the basal index at the corresponding time point (P<0.0001, n=32). The maximal increase in 86Rb⁺ efflux after α₁-adrenoceptor stimulation by phenylephrine was 28±1.3% (Fig. 2A, P<0.0001, n=32).

Influence of the preferentially α₁B-adrenoceptor subtype antagonist CEC

CEC pretreatment did not affect the basal 86Rb⁺ efflux curve, but the α₁-adrenoceptor mediated increase in 86Rb⁺ efflux was reduced (Fig. 2A). Pretreatment with a maximal concentration of CEC (10 μM, 30 min) reduced the phenylephrine mediated increase in 86Rb⁺ efflux to 51±5.0% of the maximal response (Fig. 3). Time to reach the maximal response was not changed in the CEC pretreated hearts (21±1.2 min vs 23±1.7 min with or without CEC, respectively).

Influence of preferentially α₁A-adrenoceptor subtype antagonists in CEC pretreated hearts

In order to increase the experimental selectivity, the effect of three different preferentially α₁A-adrenoceptor antagonists was tested against the CEC insensitive part of the response to phenylephrine. 5-MU inhibited the

![Fig. 1](image-url)
response concentration-dependently (Fig. 4) with a $-\log IC_{50}$ value of 5.6±0.14, corresponding to a calculated p$K_i$ = 7.7 ($-\log K_i$, 95% C.I. 7.2–8.0). The combination of 5-MU and CEC were tolerated well by the spontaneously beating heart, and high concentrations (10 μM) of both antagonists almost eliminated the $\alpha_1$-adrenoceptor mediated increase in $^{86}$Rb$^+$ efflux (Figs. 2B and 3). Also WB 4101 and (+)niguldipine inhibited the response in a concentration-dependent manner (Fig. 3). Both antagonists gave a statistically significant reduction compared to

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Effect of the preferential $\alpha_{1B}$-adrenoceptor subtype antagonist chloroethylclonidine (CEC pretreatment, 10 μM, 30 min, 31°C), the preferential $\alpha_{1A}$-adrenoceptor subtype antagonist 5-methylurapidil (5-MU) and the combination of 5-MU and CEC pretreatment on the phenylephrine (PHE) stimulated increase in $^{86}$Rb$^+$ efflux rate index (in the presence of 1 nM timolol). Each data point represent the mean value of 5–26 separate hearts. All groups were stimulated by PHE (arrow). A: Open circles: Efflux rate index in PHE-stimulated hearts in the absence of $\alpha_1$-receptor antagonists (n=7). Closed circles: Efflux rate index in PHE-stimulated hearts (closed circles from panel A included for comparison). Open triangles: Efflux rate index in non-CEC-pretreated hearts in the presence of 5-MU (10 μM, n=8). Open squares: Efflux rate index in CEC-pretreated hearts in the presence of 5-MU (10 μM, n=5). Ordinate: Increase in $^{86}$Rb$^+$ efflux rate index in percent of basal efflux rate. Abscissa: Time in min after addition of agonist.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Effect of chloroethylclonidine (CEC) pretreatment and three different preferential $\alpha_{1A}$-adrenoceptor subtype antagonists on the $\alpha_1$-adrenoceptor mediated increase in $^{86}$Rb$^+$ efflux rate index (in the presence of 1 μM timolol). Groups of hearts were subjected to stimulation by phenylephrine (PHE) alone or after CEC pretreatment without or with either 5-methylurapidil (MU), niguldipine (NIG) or WB 4101 (WB). The response was expressed as increase of $^{86}$Rb$^+$ efflux rate index in percent of the maximal response to PHE stimulation in the absence of receptor antagonists. Values are given as mean values ± S.E.M. **P<0.001, vs PHE alone; $^{11}P<0.001, vs PHE + CEC.**
CEC alone (P<0.001, Fig. 3). The occurrence of heart arrhythmias prevented the use of higher concentrations of (+)niguldipine.

**Influence of preferentially α1A-adrenoceptor subtype antagonists in the absence of CEC pretreatment**

5-MU also inhibited the response to phenylephrine in the absence of CEC pretreatment (Fig. 2B), reducing the response to 27±5.1% of the maximal response at 10 μM (Fig. 5). Time to reach the maximal response was also reduced from 18±1.7 with phenylephrine alone (n=8) to 10±1.7 min in the presence of 5-MU (n=8, P=0.006, Fig. 2B). The phenylephrine mediated increase in 86Rb+ efflux was reduced to 71±7.8% and 57±6.5% of the maximal response by the presence of 0.1 and 1 μM (+)niguldipine, respectively (Fig. 5). Again, the occurrence of heart arrhythmias prevented the use of higher concentrations of (+)niguldipine.

**Influence of the preferential α1D-adrenoceptor subtype antagonist BMY 7378**

To investigate the possible involvement of the α1D-adrenoceptor subtype, phenylephrine stimulation was performed in the presence of BMY 7378 (Fig. 6). The effect of BMY 7378 was studied without CEC pretreatment of the hearts because the α1D-adrenoceptor subtype shows a relatively high sensitivity to CEC inactivation (22, 23). The α1-adrenoceptor mediated increase in 86Rb+ efflux rate index was inhibited by BMY 7378 with a -log IC50 value of 6.6±0.2, corresponding to a calculated pKi = 8.4 (95% C.I. 8.2-8.6, Fig. 7). The response was reduced to 69±5.6% of maximal response (Fig. 5) by 1 μM BMY 7378, without changing the time to reach the maximal response (19±3.1 min, n=5). A higher concentration of BMY 7378 (10 μM) gave no further statistically significant inhibition of the response (66±6.8% of maximal response, n=11).

**DISCUSSION**

We have studied the phenylephrine stimulated increase in 86Rb+ efflux in the rat heart in the presence of subtype preferential antagonists against the α1A+, α1B+ and α1D- adrenoceptor subtypes. The increased efflux was partially inhibited by antagonists against all three subtypes. The
Evidence for involvement of the \(\alpha_{1B}\)-adrenoceptor subtype

The 50\% reduction of the \(\alpha_1\)-adrenoceptor-stimulated increase in \(86\text{Rb}^+\) efflux after pretreatment with CEC in the present study shows the involvement of the \(\alpha_{1B}\)-adrenoceptor subtype. This was supported by the effects of 5-MU without or with CEC pretreatment, showing a part of the response (about 30\%) which was 5-MU insensitive, but was eliminated by CEC pretreatment. The 5-MU insensitive part of the response must be mediated mainly by the \(\alpha_{1B}\)-adrenoceptor subtype since 5-MU has higher affinity for the \(\alpha_{1D}\)-subtype than for the \(\alpha_{1B}\)-adrenoceptor subtype (8). The alkylating substance CEC irreversibly and preferentially inactivates \(\alpha_{1B}\)-adrenoceptors (24, 25), and the degree of inactivation depends on concentration, incubation time and temperature (26). The experimental conditions (10 \(\mu\)M, CEC, 30 min, 31°C) were chosen in order to obtain inactivation of the major part of the \(\alpha_{1B}\)-adrenoceptor population (27) with a minimum effect on the \(\alpha_{1A}\)-adrenoceptors. CEC inactivates the \(\alpha_{1D}\)-adrenoceptor subtype with an affinity that is intermediate between the affinity for \(\alpha_{1B}\) and \(\alpha_{1A}\)-adrenoceptors (23), and about 50\% of the \(\alpha_{1D}\)-adrenoceptor population can be inactivated by CEC treatment (12, 28). A 25-min washout of CEC before phenylephrine stimulation prevented the possibility of remaining CEC acting as a reversible antagonist at the other \(\alpha_1\)-adrenoceptors (18, 26). As pretreatment with 10 \(\mu\)M CEC in the rat heart reduced the maximal number of high affinity sites for prazosin by 86\% (29), the present CEC treatment probably inactivated a major part of the \(\alpha_{1B}\)-adrenoceptors. The results thus indicate that the \(\alpha_{1B}\)-subtype apparently contributes to the \(\alpha_1\)-adrenoceptor stimulated \(86\text{Rb}^+\) efflux.

Evidence for involvement of the \(\alpha_{1D}\)-adrenoceptor subtype

We found that BMY 7378 inhibited the phenylephrine evoked response in a concentration-dependent manner with a calculated \(pK_1\) value = 8.4, corresponding to the value reported for the rat \(\alpha_{1D}\)-adrenoceptor (\(pK_1=8.2\), ref. 30). Thus most of the inhibitory effect of BMY 7378 is probably due to inhibition of the \(\alpha_{1D}\)-adrenoceptors. As CEC is able to inactivate a part of the \(\alpha_{1D}\)-adrenoceptor population (see above), the effect of BMY 7378 was thus...
studied without CEC pretreatment of the hearts. BMY 7378 was recently found to be a selective (approximately 100-fold compared to the \( \alpha_{1A} \)- and the \( \alpha_{1B} \)-subtype) antagonist against the \( \alpha_{1D} \)-adrenoceptor subtype (30–32). Most binding studies have shown that the rat heart contains \( \alpha_{1A} \)- and \( \alpha_{1B} \)-adrenoceptors in a 20 : 80010 ratio (16, 33). Han and Minneman (20), however, found in the CEC pretreated rat heart a significant proportion of low affinity sites for 5-MU which could at least partly represent the \( \alpha_{1D} \)-adrenoceptor subtype. Recent studies have found 5-MU to have affinity for the \( \alpha_{1D} \)-adrenoceptor that is intermediate between the \( \alpha_{1A} \)- and \( \alpha_{1B} \)-adrenoceptor subtypes (12, 34). Thus the \( \alpha_{1D} \)-subtype may escape detection when only CEC and 5-MU are used as antagonists. Very recently, BMY 7378 was shown to inhibit the prazosin binding and the inotropic response and phospholipase C activation by phenylephrine in rat myocardium (28). The contribution of \( \alpha_{1D} \)-adrenoceptors to the inotropic response seems, however, to be small (35). The present study indicates that the \( \alpha_{1D} \)-adrenoceptor subtype apparently contributes to the phenylephrine stimulated \( ^{86}\text{Rb}^+ \) efflux in the rat heart.

Is the \( \alpha_{1A} \)-adrenoceptor subtype involved?

The phenylephrine evoked response was extensively reduced by three different preferentially \( \alpha_{1A} \)-adrenoceptor subtype antagonists. This could indicate the involvement of the \( \alpha_{1A} \)-subtype in the response to phenylephrine. The reduction, however, appeared at concentrations that may influence also the \( \alpha_{1B} \)- and the \( \alpha_{1D} \)-adrenoceptor subtypes. The estimated pK1 value = 7.7 for 5-MU in CEC treated hearts was lower than the values usually found for the \( \alpha_{1A} \)-subtype (mean 8.8, range 7.7–9.3; ref. 26). This indicates that 5-MU may have acted on the \( \alpha_{1B} \)- (mean 7.1, range 6.0–7.8) or \( \alpha_{1D} \)-adrenoceptor (intermediate affinity) subtypes (26), and makes a contribution from the \( \alpha_{1A} \)-adrenoceptors somewhat uncertain.

There are, however, additional arguments in favor of an involvement of the \( \alpha_{1A} \)-subtype in addition to the \( \alpha_{1B} \)- and the \( \alpha_{1D} \)-adrenoceptor subtypes. The relative reduction (in percent) of the response to phenylephrine by 5-MU was higher in CEC treated hearts than in untreated ones. 5-MU influenced the time course of the response (Fig. 2B) to phenylephrine differently compared to CEC and BMY 7378. While CEC treatment or the presence of BMY 7378 reduced the response to phenylephrine without affecting the time to the maximal response, 5-MU reduced the time to the maximal response significantly. A possible explanation for these differences may be that the different \( \alpha_{1} \)-adrenoceptor subtypes are coupled to different \( ^{86}\text{Rb}^+ \) translocation mechanisms. The order of potency of (+)-niguldipine and 5-MU with respect to the inhibitory effect (in CEC pretreated hearts) may also suggest a contribution from the \( \alpha_{1A} \)-adrenoceptors to the phenylephrine stimulated \( ^{86}\text{Rb}^+ \) efflux. Thus the results as a whole can apparently best be accounted for by assuming that the \( \alpha_{1A} \)-adrenoceptor subtype is also involved in the stimulated \( ^{86}\text{Rb}^+ \) efflux.

Concluding remarks

The results of this study revealed that the increased \( ^{86}\text{Rb}^+ \) efflux involves the \( \alpha_{1D} \)- and the \( \alpha_{1B} \)-adrenoceptors. Also the \( \alpha_{1A} \)-adrenoceptor subtype seems to be involved in the response to phenylephrine, although these results are less clear. It is, however, difficult to quantify the contribution of the different receptor subtypes, because of the lack of complete selectivity of the antagonists available. The cross inhibition of the antagonists is obvious from the finding that the sum of inhibition (at maximal concentrations of antagonists) exceeded 100%. It is likely that several mechanisms are involved in the phenylephrine-induced increase of total \( ^{86}\text{Rb}^+ \) efflux. This may be related to a contribution from all three \( \alpha_{1} \)-adrenoceptor subtypes, as they may at least partly be coupled to different translocation mechanisms. In conclusion, the demonstrated inhibition obtained by subtype selective antagonists indicates that the phenylephrine stimulated increase in \( ^{86}\text{Rb}^+ \) efflux in the rat heart is not specifically linked to only one of the \( \alpha_{1} \)-adrenoceptor subtypes, but involves the \( \alpha_{1B} \)- and the \( \alpha_{1D} \)-adrenoceptor subtypes, and probably the \( \alpha_{1A} \)-adrenoceptor subtype as well.

Acknowledgments

Iwona Gutowska Schaider is gratefully acknowledged for technical assistance. This work was supported by The Norwegian Council on Cardiovascular Diseases, the Norwegian Research Council, and the Norwegian Medical Depot.

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