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Short communication

The new NO donor SPM3672 increases cGMP and improves contraction in rat cardiomyocytes and isolated heart

Georg Kojda [a,*], Klara Brixius [a], Karin Kottenberg [a], Petra Nix [a], Klaus-Dieter Schlüter [b], Hans Michael Piper [b], Eike Noack [a]

[a] *Institut für Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany*
[b] *Institut für Physiologie, Justus-Liebig-Universität, Aulweg 129, 35392 Gießen, Germany*

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Abstract

Recent evidence indicates that organic nitrate esters may directly affect heart muscle. In the present study we investigated the effects of the new organic nitrate ester, *N*-(3-nitratopivaloyl)-l-cysteineethylester (SPM3672), on isolated adult rat ventricular myocytes and on Langendorff preparations of spontaneously beating rat hearts perfused in a volume-constant manner. In cardiomyocytes SPM3672 (100 μM) induced a significant increase in the basal level of cGMP to 232 ± 44% ($n = 8$) indicating its metabolism to nitric oxide. This was associated with an enhanced contractile response to electrical field stimulation (to 174 ± 9%, $n = 108$). In isolated hearts SPM3672 elicited a slight reduction of coronary perfusion pressure (−15 ± 8%) and a significant increase in maximal left ventricular pressure (LVP_{max}), dp/dt_{max} and dp/dt_{min} amounting to 18 ± 7%, 18 ± 6% and 21 ± 7% ($n = 7$), respectively. Oxygen consumption and heart rate remained constant. Thus, SPM3672 improved the contractile response of cardiomyocytes and of isolated heart. This is probably due to the metabolism of SPM3672 to nitric oxide in ventricular cardiomyocytes.

Keywords: SPM3672; Nitric oxide (NO) donor; Nitric oxide (NO); Cardiomyocyte; Heart muscle, contractility

1. Introduction

Pharmacological effects of organic nitrate esters are initiated by enzymatic release of nitric oxide (NO) from the nitrate ester group of these drugs (Feelisch and Noack, 1987; Chung and Fung, 1990). NO activates soluble guanylate cyclase, resulting in the production of cyclic guanosine monophosphate (cGMP), which relaxes vascular smooth muscle by different mechanisms involving reduction of intracellular Ca^{2+} (Ahlner et al., 1991). *N*-(3-Nitratopivaloyl)-1-cysteineethylester (SPM3672) is a sulfhydryl group-containing organic nitrate ester, which was recently developed (Noack et al., 1988). This drug was characterized as a typical nitrovasodilator by demonstration of NO release, activation of soluble guanylate cyclase, increase in arterial cGMP and arterial and venous vasodilation (Kojda and Noack, 1993; Kojda et al., 1994a). In a previous investigation, we found that the organic nitrate esters, glyceryl trinitrate, isosorbide-5-mononitrate and pentaerythrityl-tetranitrate, as well as low concentrations of the spontaneous NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine, increased the basal level of cGMP in isolated rat ventricular cardiomyocytes and enhanced their contractile response to electrical field stimulation (Kojda et al., 1994b, 1995). These results indicate that nitrate esters are metabolized to NO in this cell type. It was therefore very interesting to study whether the new nitrovasodilator, SPM3672, which shows properties differing from those of typical organic nitrates in terms of mechanism of action and tolerance development (Kojda and Noack, 1993), would elicit comparable cardiac effects. Therefore, we investigated the effects of this new drug on heart muscle cells. As an extension to previous investigations, the effects of SPM3672 were also studied in isolated hearts of the same animal species.

* Corresponding author. Tel. +49/221/311-2518, fax +49/221/311-4781.

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2. Materials and methods

2.1. Preparation of rat ventricular myocytes

Ventricular myocytes were isolated from male Wistar rats (200–250 g) as described in detail previously (Piper et al., 1982). The hearts were rapidly excised under deep ether anesthesia, rapidly transferred to ice-cold saline, and mounted on the cannula of a Langendorff perfusion system. Heart perfusion and subsequent steps were all performed at 37°C. First, the hearts were perfused in a non-recirculating manner for 5 min at 10 ml/min (composition of perfusate in mM: 110 NaCl, 1.2 KH_2PO_4, 2.6 KCl, 1.2 $MgSO_4$, 25 Na_2CO_3, 11 glucose, 37°C; gassed with 5% CO_2/95% O_2). Thereafter, perfusion was continued by recirculation of 50 ml of the above perfusate supplemented with 0.06% (w/v) crude collagenase and 25 μM $CaCl_2$ at a flow rate of 5 ml/min. After 30 min, the ventricular tissue was minced and incubated for 20 min in recirculating medium with 1% (w/v) bovine serum albumin under 5% CO_2/95% O_2. Gentle pipetting released the cells from tissue chunks. The resulting cell suspension was filtered through a 200-μm nylon mesh. Twice, the filtered material was washed by centrifugation (3 min, $25 \times g$) and resuspended in the above perfusate, in which the concentration of $CaCl_2$ was increased stepwise to 0.2 and 0.5 mM. After another centrifugation (3 min, $25 \times g$), the cells in the pellet were resuspended at 37°C at a density of 1–2 mg protein/ml in medium 199 supplemented with 4% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary culture of cardiomyocytes was performed by pipetting 1 ml of this cell suspension into 35-mm culture dishes (Falcon 3001) under sterile conditions. After incubation of these dishes for 3–4 h in an atmosphere of 5% CO_2/95% O_2 at 37°C, intact cardiomyocytes had adhered to the culture dish and were separated from broken or dead cells by washing the dishes once with culture medium.

2.2. Determination of cGMP

Freshly prepared rat ventricular myocytes adhered on 30 mm culture dishes (Falcon 3001) were washed once with modified Tyrode buffer of the following composition (in mM): 125 NaCl, 1.2 KH_2PO_4, 2.6 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 10 glucose, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes); pH 7.4, and equilibrated for 15 min in 1 ml of this buffer (37°C). Incubation (37°C) was started by addition of 20 μl of an appropriate stock solution of SPM3672, isoprenaline or vehicle. The medium was then gently mixed (some seconds) and experiments were terminated after 2 min by acidifying the incubate with 250 μl of ice-cold $HClO_4$ (50%). The ice-cold, acidified cell suspension was transferred into 1.5-ml plastic tubes (Eppendorf), sonicated for 10 s (50 W) and centrifuged at 4°C with $10000 \times g$ for 5 min. The pellet was used for protein determination (Bradford, 1976). The supernatant was neutralized (pH 7.4) with K_3PO_4, centrifuged again, and used directly for determination of cGMP by radioimmunoassay using a ^{125}I-labeled antigen. All experiments were performed with cells from at least 3 different cell preparations. Changes of basal cGMP levels are given as percentages related to the basal cGMP level (vehicle treated) observed in parallel experiments.

2.3. Determination of contractile activity

Contractile activity was studied by use of a video microscopic technique as described previously (Piper et al., 1989). Briefly, culture dishes containing attached cardiomyocytes in 2 ml modified Tyrode buffer, which was supplemented with either vehicle, isoprenalin or SPM3672 (addition of 40 μl of an appropriate stock solution to adjust the indicated concentrations) were rapidly placed on a temperature-controlled (37°C) microscope stage. Two AgCl electrodes (diameter 100 μm) fixed on a plastic ring which covered the culture dish were rapidly inserted to a distance of 1 mm in the buffer and positioned laterally to leave the microscopic visual field in their middle free. Application of biphasic electrical stimuli (to avoid hydrolysis) composed of two equal but opposite rectangular 50-V stimuli of 0.5 ms duration at a frequency of 0.5 Hz was started 10 s after addition of the nitrates. The microscopic picture in phase contrast was recorded on a tape using a charge-coupled device video camera and a U-matic video recorder (Sony, model VO-5800PS). The contractions of single cells were determined from frozen consecutive video frames magnifying the cell's picture 500-fold on a video monitor screen. Contractions ocurring 15, 90 and 300 s after addition of the nitrates (5, 80 and 290 s after the onset of cell stimulation) were analysed by measuring the slack length 0.5 s before contraction (A) and the fully contracted cell length (B). Contractile response (cell shortening) is expressed as a percentage: $100 \times (A - B)/A$. Changes in cell shortening induced by isoprenaline or SPM3672 were determined by comparing directly the responses of 30–40 different cells of one preparation to vehicle (all ingredients except the drug) and to the drug. Responses of every single cell in the presence of the drugs are expressed as percentages of the mean control response obtained with cells of the same preparation (100%). Each experiment was repeated with cells from two other cell preparations. The average length of resting cells used for the experiments with SPM3672 was as follows: 91.4 ± 1.4 μm ($n = 114$, vehicle-treated cells) and 92.1 ± 1.9 μm ($n = 108$, drug-treated cells). The average

shortening of cells treated with vehicle was 9.8 ± 0.4% (n = 114).

2.4. Preparation of isolated hearts

Male Wistar rats (350–400 g) were anesthetized by an i.m. injection of 5 mg/kg xylazine (Rompun) and 25 mg/kg ketamine. An i.m. injection of 600 U/kg heparin (Liquemin) prevented blood coagulation during further preparation. After tracheotomy and under artificial ventilation (frequency: 80/min, pressure: 200 mm H_2O), the thorax was opened and the aorta was cannulated. The hearts were rapidly excised and perfused by the technique of Langendorff at a constant pressure of 110 cm H_2O with an oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (pH 7.4, 37°C) of the following composition (in mM): Na^+ 143.07, K^+ 5.87, Ca^{2+} 1.6, Mg^{2+} 1.18, Cl^- 125.96, HCO_3^- 25.00, $H_2PO_4^-$ 1.18, SO_4^{2-} 1.18 and glucose 5.05. The pulmonary artery was cannulated for recording of coronary flow and oxygen content. A tip manometer (Millar Instruments, Houston, TX, USA) connected to a small water-filled balloon was inserted into the left ventricle via the mitral valve in order to measure frequency, maximal left ventricular pressure (LVP_{max}), dp/dt_{min} and dp/dt_{max}. With another tip manometer we measured coronary perfusion pressure. Oxygen content of the buffer was recorded before and after heart passage by means of oxygen electrodes (Radiometer Deutschland, Willich, Germany). Tip manometers and electrodes were connected to a computer to provide simultaneous on-line recording. After cannulation of the left atrium, coronary flow was measured under constant pressure Langendorff conditions. The constant pressure Langendorff perfusion was then switched into a constant flow mode. The flow rate was adapted to the coronary flow measured under constant pressure Langendorff conditions, which amounted to 9.0 ± 0.6 ml/min (n = 7). Drugs were applied with a perfusor pump (Gerhard Heinemann, Schwäbisch Gemünd, Germany) using a cannula which was placed in the aorta proximal to the aortic valve. The maximal flow of the buffer containing drugs was 5.0% of the coronary flow rate. Oxygen consumption was calculated as follows: coronary flow $\times (O_{2,before} - O_{2,after\ heart})$/heart wet weight. After an equilibration period of half an hour, cumulative concentration-response-curves of SPM3672 (10–100 μM) were obtained.

2.5. Substances and solutions

SPM3672 was generously provided by Schwarz Pharma (Monheim, Germany); isoprenaline and crude collagenase were obtained from Serva Feinbiochemika (Heidelberg, Germany); fetal calf serum and antibiotics were obtained from Gibco (Eggenstein, Germany), medium 199 was obtained from Life Technologies (Paisley, Scotland, UK), ^{125}I-labeled cGMP was obtained from DuPont NEN (Bad Homburg, Germany) and all other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany).

Stock solutions (10 mM) of isoprenaline in double-distilled water and of SPM3672 in dimethyl sulfoxide were prepared daily and kept, protected from daylight, on ice until use. All concentrations given are expressed as final bath concentrations. Because of the low solubility of SPM3672, organ bath concentrations higher than 100 μM could not be used, since this would result in organ bath concentrations of dimethyl sulfoxide exceeding 1% (v/v), which is unacceptable owing to direct interference with the cells and hearts under investigation.

2.6. Statistics

All data were analysed by one-way analysis of variance (ANOVA) with subsequent Student-Newman-Keuls test (SAS PC Software 6.04, PROC ANOVA, also used to calculate the plots) and are expressed as mean values and standard error of the mean (S.E.M.). The significance of differences was evaluated by using Student's t-test, and a P value below 0.05 was considered as significant.

3. Results

3.1. Isolated cardiomyocytes

Incubation of isolated rat cardiomyocytes with 100 μM SPM3672 for 2 min resulted in a significant increase in basal cGMP levels. Expressed as percentage of the respective basal level of cGMP (average value: 1.7 ± 0.5 pmol/mg protein, n = 8) measured with the same preparations of cells, this increase amounted to 232 ± 44% (n = 8). A lower concentration of SPM3672 (10 μM) did not significantly increase cGMP levels. SPM3672 also elicited a pronounced effect on the contractile response of rat cardiomyocytes. As shown in Fig. 1, the drug induced a significant improvement of the contractile response to electrical field stimulation. This effect started rapidly, within 15 s. The maximal improvement of the control contractile response, observed after 5 min, was 173.9 ± 9.2%. Experiments with isoprenaline, conducted for comparison, revealed a significant ($P < 0.01$) dose-dependent increase in contractile response to 141.4 ± 6.4% (10 nM, n = 126) and 191.8 ± 6.3% (30 nM, n = 122).

3.2. Intact heart

The effects of SPM3672 on an isolated, spontaneously beating rat heart perfused in a constant vol-

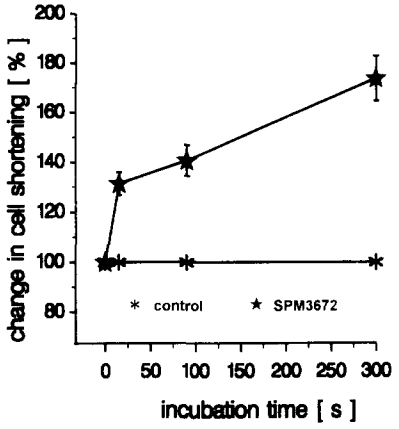

Fig. 1. Effects of the new organic nitrate ester *N*-(3-nitratopivaloyl)-1-cysteineethylester (SPM3672) on isolated, adult rat ventricular myocytes. Cell shortening induced by electrical field stimulation under control conditions, as measured with 114 cells, was 9.8 ± 0.4% (related to the maximal cell length). Percent changes in cell shortening induced by SPM3672 are related to control values observed at the same time point and in the same cell preparation ($n = 108$). Plot shows mean values (± S.E.M). All values obtained in the presence of SPM3672 are significantly different from the controls ($P < 0.05$).

ume manner were restricted to the contractile function of the ventricle. Average control values for seven hearts, measured after equilibration and before starting the infusion of SPM3672 proximal to the aortic valve, were as follows ($n = 7$): coronary perfusion pressure: 58 ± 9 mm Hg; oxygen consumption: 106 ± 6 μl O_2/min/g; LVP_{max}: 74 ± 9 mm Hg; dp/dt_{max}: 1737 ± 202 mm Hg/s; dp/dt_{min}: 1195 ± 164 mm Hg/s and frequency 167 ± 3 beats/min. In a concentration of 100 μM, SPM3672 showed no influence on coronary vasomotor tone (coronary perfusion pressure: −15 ± 8%, n.s.). In parallel, all parameters measured to determine left ventricular function were moderately, but significantly increased (Fig. 2). This effect was not associated with an increase in oxygen consumption. Spontaneous beating rate (frequency) was also not affected. As shown in Fig. 2, the effects on LVP_{max}, dp/dt_{min} and dp/dt_{max} were significant at a dose of 100 μM SPM3672.

4. Discussion

In this study we investigated the effects of SPM3672, a new sulfhydryl-containing nitrovasodilator, on the basal level of cGMP and on contractile function of freshly prepared rat ventricular myocytes and intact heart. The major finding is that this drug induces an increase in basal level of cGMP associated with an improvement of contractile response of isolated cells and of left ventricular myocardial function.

In vascular smooth muscle an increase in basal level of cGMP induced by organic nitrate esters like glyceryl trinitrate is most likely mediated by their enzymatic conversion to nitric oxide and subsequent activation of soluble guanylate cyclase (Feelisch and Noack, 1987; Chung and Fung, 1990; Ahlner et al., 1991). Earlier investigations of the new nitrate ester, SPM3672, revealed an increase in arterial cGMP comparable to that induced by glyceryl trinitrate, but a different mechanism of liberation of nitric oxide in a cell-free system and in coronary arteries (Kojda and Noack, 1993). As shown in the present study, liberation of nitric oxide from SPM3672 most likely also occurs in rat ventricular cardiomyocytes, because exposure of

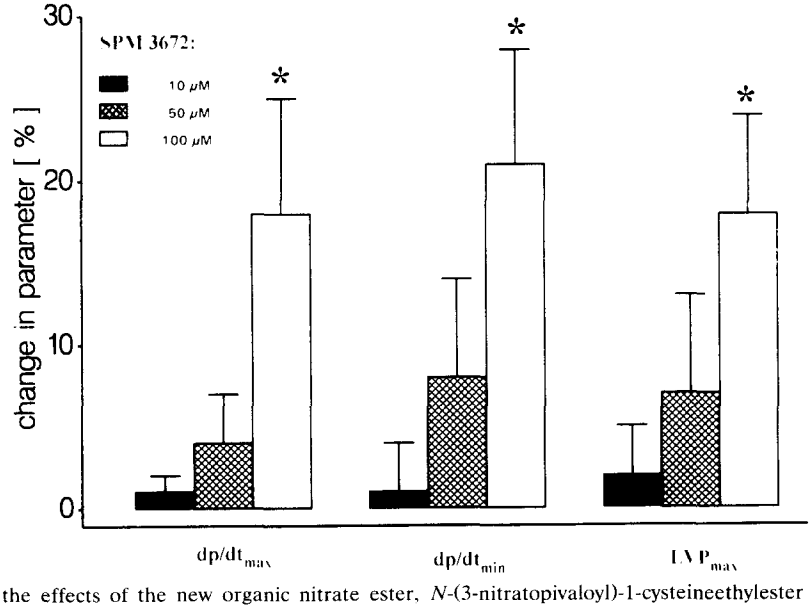

Fig. 2. Bar graph showing the effects of the new organic nitrate ester, *N*-(3-nitratopivaloyl)-1-cysteineethylester (SPM3672), on Langendorff preparations of spontaneously beating rat hearts perfused in a constant volume manner. Concentration-dependent changes in parameters are related to the values measured directly before infusion of SPM3672, which amounted on average to 1737 ± 202 mm Hg/s (dp/dt_{max}), 1195 ± 164 mm Hg/s (dp/dt_{min}) and 74 ± 9 mm Hg (maximal left ventricular pressure, LVP_{max}). Plot shows mean values (± S.E.M, $n = 7$) and significant differences are indicated ($^*P < 0.05$).

these cells to SPM3672 resulted in an increase in basal level of cGMP (see Results). In the same cell type similar results were obtained with glyceryl trinitrate, isosorbide-5-mononitrate and pentaerythrityl tetranitrate (Kojda et al., 1994b, 1995). Thus, the action of SPM3672 on soluble guanylate cyclase resembles the effects of typical organic nitrate esters not only in vascular tissue, but also in isolated rat cardiomyocytes.

As shown in Fig. 1, exposure of isolated rat cardiomyocytes to SPM3672 resulted in a marked improvement of their contractile response to electrical field stimulation. This result is consistent with the action of other typical nitrovasodilators like glyceryl trinitrate, isosorbide-5-mononitrate and pentaerythrityl tetranitrate as well as low concentrations (1 μM) of the spontaneous NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine in the same cell type (Kojda et al., 1994b, 1995). Other investigators observed positive inotropic responses induced by the NO donor, sodium nitroprusside (Sys et al., 1993; Diamond et al., 1977). In contrast, Brady et al. (1993) observed a dose-dependent depressive action of sodium nitroprusside and NO itself, an effect that also occurred with high concentrations (100 μM) of *S*-nitroso-*N*-acetyl-D,L-penicillamine (Kojda et al., 1994b, 1995). At present, the reason for the diversity of these results is not known, but it seems possible that NO exerts stimulatory as well as inhibitory effects on cardiac contraction. The underlying mechanism is not yet clear, but recent results suggest an involvement of both cAMP- and cGMP-dependent protein kinase (Kojda et al., 1994b, 1995).

We were also interested in whether the enhanced contractile response of isolated cardiomyocytes induced by SPM3672 also occurs in isolated heart of the same species. To exclude effects of increased coronary flow, which might occur due to the vasodilating action of SPM3672, we used a constant flow Langendorff preparation. As shown in Fig. 2, SPM3672 elicited a moderate but significant increase in LVP_{max}, dp/dt_{max} and dp/dt_{min} and these effects developed in a dose-dependent manner. Thus, SPM3672 moderately improves the contractile function of isolated rat heart. According to the suggestions discussed above, it seems likely that this improvement is due to a direct action of SPM3672 on ventricular myocytes. Since this drug is a potent vasodilator (Kojda and Noack, 1993), it is interesting to note that SPM3672 did not induce a significant reduction of coronary perfusion pressure, despite the high concentrations used (see Results). In rat aorta, the concentration producing half-maximal relaxation was found to be 1.2 ± 0.09 μM (Kojda and Noack, 1993) and, thus, two orders of magnitude lower than the concentration used here. It might be speculated that this obvious difference is due to a low activity of the drug in coronary resistance vessels as described previously for glyceryl trinitrate (Sellke et al., 1991). However, the low efficacy of SPM3672 in the coronary circulation is an advantage, because it prevents vasodilator-induced angina related to redistribution of myocardial perfusion from ischemic regions; the so-called coronary steal phenomenon (Harrison and Bates, 1993).

In summary, our investigation on cardiac effects of the new organic nitrate ester, SPM3672, revealed that this drug is metabolized to nitric oxide in ventricular cardiomyocytes as evidenced by a significant increase in cGMP. This is associated with an improvement of the contractile reponse of isolated rat cardiomyocytes as well as left ventricle of isolated rat heart.

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