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Pharmacological Characterization of a New Ca^{2+} Sensitizer[1]

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ABSTRACT

The benzimidazole molecule was modified to synthesize a Ca^{2+} sensitizer devoid of additional effects associated with Ca^{2+} overload. Newly synthesized compounds, termed **1**, **2**, **3**, **4**, and **5,** were evaluated in spontaneously beating and electrically driven atria from reserpine-treated guinea pigs. Compound **3** resulted as the most effective positive inotropic agent, and experiments were performed to study its mechanism of action. In spontaneously beating atria, the inotropic effect of **3** was concentration-dependent (3.0 μM–0.3 mM). Compound **3** was more potent and more active than the structurally related Ca^{2+} sensitizers sulmazole and caffeine, but unlike them it did not increase the heart rate. In electrically driven atria, the inotropic activity of **3** was well preserved and it was not inhibited by propranolol, prazosin, ranitidine, pyrilamine, carbachol, adenosine deaminase, or ruthenium red. At high concentrations (0.1–1.0 mM) **3** inhibited phosphodiesterase-III, whereas it did not affect Na^+/K^+-ATPase, sarcolemmal Ca^{2+}-ATPase, Na^+/Ca^{2+} exchange carrier, or sarcoplasmic reticulum Ca^{2+} pump activities of guinea pig heart. In skinned fibers obtained from guinea pig papillary muscle and skeletal soleus muscle, compound **3** (0.1 mM, 1 mM) shifted the pCa/tension relation curve to the left, with no effect on maximal tension and no signs of toxicity. Compound **3** did not influence the basal or raised tone of guinea pig isolated aorta rings, whose cells do not contain the contractile protein troponin. The present results indicate that the inotropic effect of compound **3** seems to be primarily sustained by sensitization of the contractile proteins to Ca^{2+}.

Although over the past 20 to 30 years the mortality rates due to coronary artery disease have declined steadily in Western countries (Sharpe and Doughty, 1998), congestive heart failure, which is in most cases secondary to coronary disease, remains an important and increasing public health problem (Eriksson, 1995). The management of heart failure has improved but there is no clear evidence that therapeutic advances have made any impact on the overall burden of disease in the community (Sharpe and Doughty, 1998) and the prognosis remains poor (SOLVD Investigators, 1991). Thus, greater efforts are required to identify new and effective treatments that decrease mortality, lessen disease progression, and improve the overall quality of life of patients. Lessons from 15 years of heart failure trials show that the reduction of progressive deterioration of myocardial function is the therapeutic target and provide guidance for future drug development (Massie, 1998).

The fundamental mechanism underlying progressive myocardial dysfunction has been termed remodeling (for review, see Sharpe, 1994). This process, particularly active in severely dilated ventricle (Katz, 1998), consists of a complex of molecular and cellular events that lead to important changes in the structure and function of the myocardium (Givertz and Colucci, 1998). Among factors contributing to myocardial remodeling, inflammatory cytokines, nitric oxide, and reactive oxygen species all exert negative inotropic effects, which may be related with worsening of the disease (Givertz and Colucci, 1998). Inflammatory cytokines reduce cardiac contractility, both directly and indirectly, the latter being mediated by enhancement of nitric oxide production (for review, see Ceconi et al., 1998). Nitric oxide impairs mechanical myocardial function by elevating intracellular cGMP contents, leading to a reduced Ca^{2+} current in cardiomyocytes (Wahler and Dollinger, 1995) and to myofilament desensitization to Ca^{2+} (Shah et al., 1994). Free radicals decrease contractility by reducing Ca^{2+} sensitivity of contracting proteins (Perez et al., 1998), and also reduce Ca^{2+} accumulation by the sarcoplasmic reticulum (Okabe et al., 1991). Thus, although data concerning changes in Ca^{2+} sensitivity in

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ABBREVIATIONS: PDE, phosphodiesterase; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; IR, infrared; MS, mass spectrometry; RYR, ryanodine receptor.

chronically failing heart are still controversial (Zakhary et al., 1999), blunted myofibrillary Ca^{2+} sensitization may be expected when the concentration of remodeling factors increases or when hypoxia or ischemia induce cellular acidosis (Solaro et al., 1988). Therefore, restoring cardiac contractility by increasing myofilament sensitivity to Ca^{2+} is an attractive and logical adjunct to drug treatment of heart failure. Unfortunately, up to now, all pharmacological agents described as having noteworthy Ca^{2+}-sensitizing properties also exert marked phosphodiesterase (PDE) inhibitory activity (for review, see Endoh, 1998). Both PDE inhibitors and all the positive inotropic agents that are widely used in clinical practice, such as digitalis and catecholamines, increase the likelihood of harmful cardiac arrhythmias by increasing intracellular Ca^{2+} content and have no beneficial effect on prognosis (Massie, 1998).

In the present study the benzimidazole molecule, structurally related to other Ca^{2+} sensitizers, such as sulmazole, caffeine, and pimobendan, was modified to obtain a Ca^{2+} sensitizer devoid of significant influence on PDE III activity and cardiac rhythm. The molecule of the chromophore, termed compound **1**, previously synthesized (Caroti et al., 1987), was modified to obtain different analogs termed compounds **2**, **3**, **4**, and **5**.

All these compounds were initially tested on the contractility of spontaneously beating or electrically driven guinea pig atria. The mechanism of action of the most effective positive inotropic agent was further evaluated in chemically skinned fibers of guinea pig right papillary muscle and skeletal soleus muscle and in isolated aorta rings. Lastly, the influence of these compounds on PDE and other enzymes involved in cardiac contractility was investigated.

Experimental Procedures

Isolated Atria Preparation

Reserpine-treated Dunkin-Hartley male guinea pigs (300–500 g) were killed by a blow to the head followed by exsanguination. Atria were separated from ventricles and suspended vertically in a bath containing 30 ml of physiological salt solution of the following composition: 120 mM NaCl, 2.7 mM KCl, 0.09 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.37 mM $CaCl_2$, 11.9 mM $NaHCO_3$, and 5.5 mM *d*-glucose. The solution was maintained at 29°C and bubbled vigorously with a mixture of 95% O_2 and 5% CO_2 to produce a pH of 7.5 ± 0.02. Where indicated, acidosis was induced by switching to superfusion with an identical solution bubbled with 15% CO_2 and 85% O_2, which gave a pH of 6.67 ± 0.03 (Lee et al., 1993).

Resting tension was adjusted to about 5 mN and developed tension was recorded isometrically by means of high-sensitivity transducers (type DYO for isolated auricles; Basile, Comerio, Varese, Italy) and recorded on a writing oscillograph (Basile Unirecord System, model 7050; Basile). The basic developed tension ranged from 4.8 to 5.3 mN. Where indicated, left atria were mounted on punctate electrodes with a load of 0.5 g and were stimulated by square-wave electrical pulses of 3-ms duration and voltage 10 to 20% greater than the threshold value, at a frequency of 1.5 Hz, by a Grass stimulator (model 24KR; Grass Instruments Corporation, Quincy, MA). Developed tension was 3.28 ± 0.51 mN. Electrical stimulation was performed to eliminate any influence on contractile function due to variation in frequency.

Inotropic Activity

Experiments were performed on spontaneously beating atria or on electrically driven left atria obtained from reserpine-treated guinea pigs. Reserpine (2 mg kg^{-1} i.p.) was given 48 and 24 h before the animals were killed, to eliminate the influence of noradrenaline, which may be released from sympathetic nerve terminals (Temma et al., 1977). Noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (2 μg ml^{-1}) before starting the experiments. Experiments were performed only in preparations that did not respond to tyramine. Unless otherwise indicated, the inotropic agents were added cumulatively to the perfusion fluid after 90 min of equilibration. Inotropic effects were recorded for 5 min before adding a higher concentration. The effect of the compounds was defined as the difference between the force of contraction before and after its addition to the bathing fluid and was expressed as a percentage of the response induced by 1 μM noradrenaline in the same preparation. The EC_{50} value was graphically determined as the concentration that produced half the maximum effect obtainable with the drug. Benzimidazole derivatives were dissolved in dimethyl sulfoxide (DMSO), the final concentration of which in the medium did not exceed 0.3% and did not itself influence the basal activity of the atrial preparations.

Isolated Aorta Preparation

The thoracic aorta was removed from reserpine-treated guinea pigs as reported above, dissected free from connective tissue, and cut into rings that were denuded from the endothelium by gently rubbing the intimal surface with polyether string. The denuded vessel preparations did not respond to acetylcholine, thus excluding the involvement of the endothelium in the vascular response to the agents studied. The denuded aorta rings were mounted vertically by means of stainless steel hooks in 10-ml organ baths containing the same physiological solution used for atria preparation, aerated as described above, and maintained at 37 ± 0.3°C. Changes in tension were recorded by means of an isotonic transducer (E.C.T.A. Linearcorder Mark III, Watanabe, Japan). An initial tension of 0.8 g was applied to the rings, which were then allowed to equilibrate for 2 h. Initially, contractions were evoked by exposure to 1 μM noradrenaline (5-min contact time) at 30-min intervals, until three responses of equal amplitude were obtained (corresponding to 83 ± 55% of the maximal contraction induced by 120 mM KCl, n = 10). The effect of acetylcholine (10 nM–10 μM) was tested on the contraction induced by the last addition of noradrenaline to evaluate the lack of functional integrity of the endothelium. Mean tension generated by noradrenaline was 10.03 ± 0.99 mN (n = 9).

Assay of Soluble Type III PDE Activity from Guinea Pig and Rat Heart

Type III PDE was isolated from guinea pig heart using the procedure described by Weishaar et al. (1986). Type III PDE from guinea pig heart had an apparent K_m for cAMP of 1.33 ± 0.15 μM and V_{max} of 4.54 ± 0.29 nmol/mg of protein/min (n = 8). When assayed at 0.4 μM cAMP, the activities of guinea pig cardiac type III PDE were inhibited by about 80 ± 4% by 4 μM cGMP (n = 8). The fraction was insensitive to calmodulin and only slightly inhibited by 100 μM rolipram, the specific inhibitor of type IV PDE. PDE activity was measured by the two-step procedure of Thompson et al. (1974).

Assay of ATP-Dependent $^{45}Ca^{2+}$ Uptake by Cardiac Sarcoplasmic Reticulum Vesicles

A crude cardiac membrane vesicle preparation enriched in sarcoplasmic reticulum was obtained by the method of Jones et al. (1977) from guinea pig ventricular tissue. Ca^{2+} uptake was determined as previously described (Floreani et al., 1996). Benzimidazole derivatives were dissolved in DMSO; the same amount of DMSO was always added to the controls.

Assay of Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Na^+/Ca^{2+} Exchange Carrier Activities in Cardiac Sarcolemmal Vesicles

Cardiac sarcolemmal vesicles were prepared from guinea pig ventricular tissue by the method of Slaughter et al. (1983). Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Na^+/Ca^{2+} exchange carrier activities were measured as previously described (Floreani et al., 1996).

Protein Assay

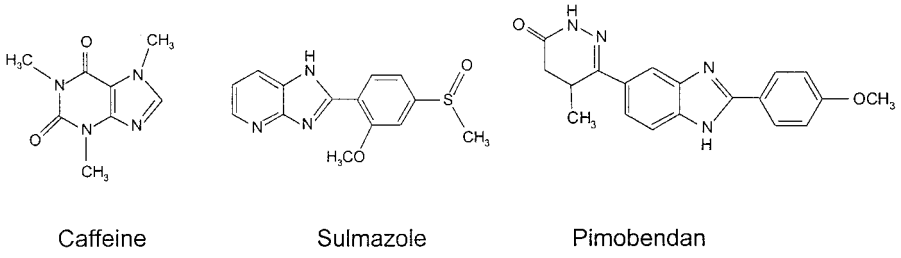
Protein content was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Skinned Fiber Preparation and Analysis

Soleus and right ventricular papillary muscles from guinea pigs (300–500 g) were used. The muscles were chemically skinned as previously described (Danieli-Betto et al., 1990). Immediately after dissection, muscle specimens were exposed at 0–4°C to a skinning solution containing 170 mM potassium propionate, 2.5 mM magnesium propionate, 5 mM K_2-EGTA, 2.5 mM Na_2K_2ATP, and 10 mM imidazole, pH 7.0. At the 1st, 2nd, 4th, and 23rd h, the solution was replaced with fresh solution. After 24 h, skinned muscles were transferred to a skinning solution containing 50% (v/v) glycerol and stored at −20°C.

Single chemically skinned skeletal muscle fibers or small papillary bundles (4–5 mm in length and 1–2 mm in width) were isolated under a dissecting microscope and transferred to a chamber containing 0.8 ml of a relaxing solution containing 170 mM potassium propionate, 2.5 mM magnesium propionate, 5 mM K_2-EGTA, 5 mM Na_2K_2ATP, and 10 mM imidazole, pH 7.0. The fiber segments or single bundles were inserted between two clamps, one of which was connected to a tension transducer (AK; Sensonor, Horten, Norway). The fibers were stretched to 130% of their resting length. All experiments were performed at room temperature.

The Ca^{2+} sensitivity of tension generation was determined as previously described (Danieli-Betto et al., 1990). Preliminarily, fibers were briefly incubated with 0.2% (w/v) BRIJ-58 to eliminate Ca^{2+} control by the sarcoplasmic reticulum (Salviati et al., 1982). pCa/tension curves were obtained by exposing the fibers sequentially to solutions with increasing free Ca^{2+} concentrations (from pCa 7.0 to 5.0). The various pCa solutions used throughout the experiments



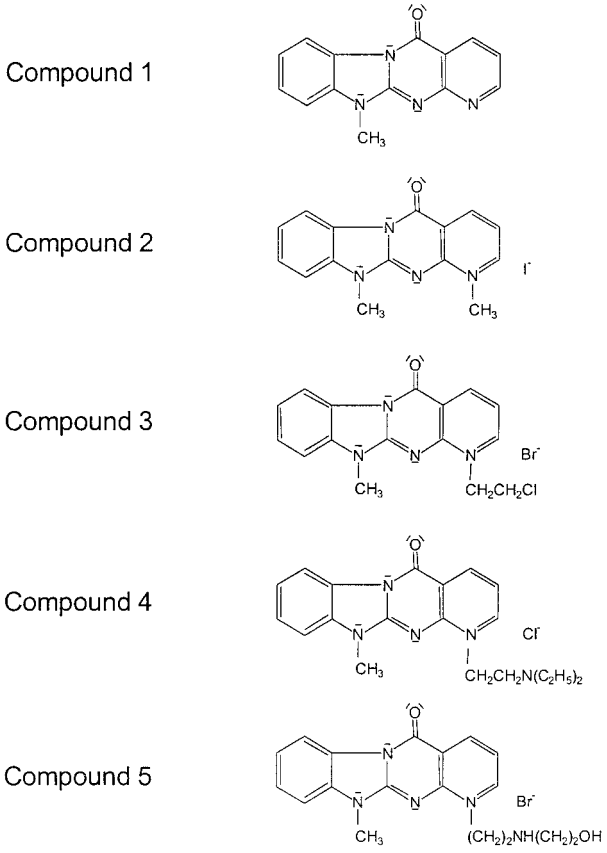


Fig. 1. Chemical structure of caffeine, sulmazole, pimobendan, and benzimidazole derivatives. Compound **1**: 5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*α*]benzimidazole; compound **2**: 1-methyl-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5] pyrimido[1,2-*α*]benzimidazol-1-ium iodine; compound **3**: 1-(*β*-chloroethyl)-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*α*]benzimidazol-1-ium bromide; compound **4**: 1-(*β*-diethylaminoethyl)-5,11-dihydro-11 methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*α*]benzimidazol-1-ium chloride; compound **5**: 1-(*β*-ethanolaminoethyl)-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*α*]benzimidazol-1-ium bromide.

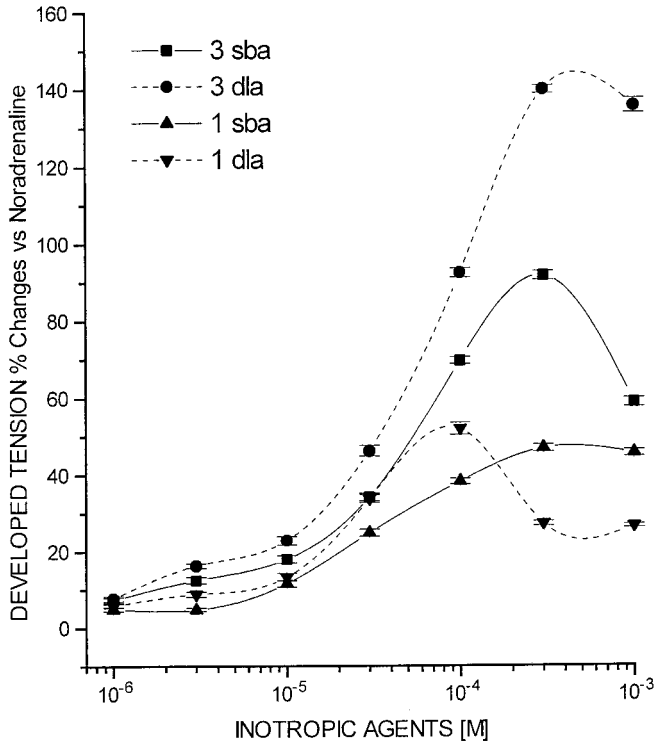


Fig. 2. Cumulative concentration-effect curves for inotropic effect of compounds **1** and **3** in spontaneously beating and electrically driven atria from reserpine-treated guinea pigs. The effect of each compound was defined as the difference between the force of contraction before and after its addition to the bathing fluid and was expressed as a percentage of the response induced by 1 μM noradrenaline in the same preparation. All data are means ± S.E.M. of 6 to 10 assays from 10 different experiments. 1, compound **1**; 3, compound **3**; sba, spontaneously beating atria; dla, driven left atrium.

were divided into two equal parts, one being used for the control and the other, with the addition of the selected concentration of compound **3**, for the treated fibers. At each pCa tension measurement, the fibers or the bundles were exposed first to the control solution, then to the solution containing the drug and, finally, rinsed in the relaxing solution.

Sarcoplasmic reticulum sensitivity to compound **3** was determined according to the procedure previously described (Salviati and Volpe, 1988; Danieli-Betto et al., 1995). Ca^{2+} was accumulated into the sarcoplasmic reticulum by exposing single skeletal muscle fibers to a pCa 6.8 solution for 30 s, whereas papillary bundles were exposed for 1 min. Fibers were then rinsed twice in a wash solution containing 170 mM potassium propionate, 2.5 mM magnesium propionate, 1.25 mM Na_2K_2ATP, and 10 mM imidazole, pH 7.0. Ca^{2+} release from the sarcoplasmic reticulum was monitored indirectly by following tension development. Type 1 soleus muscle fibers were sequentially exposed to solutions containing various concentrations of compound **3**, emptying the sarcoplasmic reticulum after each dose with 20 mM caffeine. The threshold of compound **3** was defined as the lowest concentration capable of inducing a detectable tension. The ratio between the threshold of compound **3** and the 20 mM caffeine contracture was measured. Papillary bundles were exposed only to two concentrations of compound **3** (0.3 and 1 mM).

SDS-PAGE

The skinned fibers used for the analysis described above were subjected to 6% SDS-PAGE to determine myosin heavy chain composition (Danieli-Betto et al., 1990). Experimental data from type 1 fibers, i.e., fibers containing the myosin heavy chain 1 only, were taken into consideration.

TABLE 1

EC_{50} and E_{max} for inotropic activity of compounds **3** and **1** in spontaneously beating and in electrically driven left atria from reserpine-treated guinea pigs

All data are means ± S.E.M. of values from results of six experiments. Statistical significance was calculated by Student's t test.

Atrium	Compound **1**		Compound **3**	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	μM	%	μM	%
Spontaneous	36.0 ± 6.6	46.6 ± 0.9	42.2 ± 9.9	91.5 ± 1.1
Driven	22.5 ± 6.9	51.7 ± 1.58[a]	53.3 ± 8.8[b]	137.6 ± 1.0[c]

%, percentage of contractile response to 1 μM noradrenaline.
[a] Marginally significant difference ($P = .05$) vs. E_{max} of the same compound in spontaneously beating atria.
[b] Significantly different vs. EC_{50} of compound **1** in electrically driven atria.
[c] Significantly different vs. E_{max} of the same compound in spontaneously beating atria.

Statistical Analysis

pCa/tension data were fitted according to the following equation: $Y = Po \cdot P^N/(P^N + K^N)$, where Po is the maximum tension normalized to 1, K the pCa at 50% of maximum tension, and N the Hill coefficient. Data are shown as mean ± standard error of the mean. The statistical significance of the differences between means was calculated by Student's t test for paired data. Values were considered statistically different at $P < .05$.

Chemistry

Synthesis of Compound 1, 5,11-Dihydro-11-methyl-5-oxopyrido [2′,3′:4,5] pyrimido[1,2-*a*]benzimidazole, and Compound 2, 1-Methyl-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*a*]benzimidazol-1-ium Iodide. Compounds **1** and **2** were synthesized by previously reported methods (Caroti et al., 1987).

Synthesis of Compound 3, 1-(β-Chloroethyl)-5,11-dihydro-11-methyl-5-oxopyrido [2′,3′:4,5] pyrimido[1,2-*a*]benzimidazol-1-ium Bromide. To a suspension of the heterocycle derivative **1** (0.600 g, 2.4 mmol) in 50 ml of ethanol, 0.2 ml (0.02 mol) of 1-bromo-2-chloroethane was added dropwise and the mixture was refluxed for 40 h. During this time an additional amount of 0.2 ml of 1-bromo-2-chloroethane was slowly added. The resulting solution was evaporated to dryness under reduced pressure to give crude compound **3**, which was purified by recrystallization from methanol, yielding 0.180 g (24% yield) of pure derivative **3** (melting point >300°C). Infrared (IR) spectroscopy 1700, 1620, 1580, 1480, 1390, 1200, 1100, 700, 620 cm^{-1}; ^1H NMR (proton magnetic resonance) (DMSO-d_6 as solvent), chemical shifts from tetramethylsilane δ: 3.98 [singlet, 3H, NCH_3]; 4.22 [triplet, 2H, NCH_2CH_2Cl]; 5.22 [triplet, 2H, NCH_2CH_2Cl]; 7.61–9.27 [multiplet, 7H, Ar-H]; mass spectroscopy (MS), m/z 250 (base, M^+). Elemental analysis ($C_{16}H_{14}N_4OClBr$) C, H, N.

Synthesis of Compound 4, 1-(β-Diethylaminoethyl)-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*a*]benzimidazol-1-ium Chloride. To a suspension of compound **1** (1.00 g, 4 mmol) and of 1-chloro-2-diethylaminoethane hydrochloride (0.76 g, 4.4 mmol) in 50 ml ethanol, 0.2 ml of triethylamine was added dropwise. The mixture was allowed to stir at room temperature for 2 days. During this time two additional amounts of 1-chloro-2-diethylaminoethane hydrochloride (0.76 g) and triethylamine (0.2 ml) were slowly added. The solution was evaporated to dryness under reduced pressure and the resulting crude residue was purified by recrystallization from methanol. The insoluble initial compound **1** was recovered by filtration; the methanolic mother liquors were evaporated to dryness and the residue was washed with water and extracted with chloroform. The solid was collected by evaporation of the organic layer and was recrystallized from methanol/ether to give 0.200 g (20% yield) of pure compound **4** (melting point 232–235°C). IR 3350, 2600, 2400, 1680, 1600, 1560, 1480, 1400, 1300, 1200, 1120,

TABLE 2

EC_{50} and E_{max} for inotropic activity of compounds **3** and **1** in electrically driven left atria from reserpine-treated guinea pigs preincubated in the presence of various inhibitors

All data are means ± S.E.M. of values from results of five different experiments. Statistical significance was calculated by Student's t test.

Inhibitors (M)	Compound **1**		Compound **3**	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	μM	%	μM	%
Control	22.5 ± 6.9	51.8 ± 1.6	55.3 ± 8.8	137.6 ± 1.0
Prazosin (5×10^{-9})	20.2 ± 2.1	53.1 ± 1.2	55.2 ± 2.3	136.4 ± 3.0
Propranolol (1×10^{-7})	20.3 ± 1.8	58.2 ± 2.3	49.2 ± 4.4	142.9 ± 4.2
Pyrilamine (1×10^{-7})	22.1 ± 0.9	50.6 ± 1.3	52.1 ± 5.1	141.4 ± 2.2
Ranitidine (1×10^{-5})	25.1 ± 1.2	54.5 ± 1.2	48.8 ± 3.6	136.7 ± 3.2
ADA (2 U ml^{-1})	26.1 ± 2.1	55.6 ± 3.2	49.9 ± 6.1	136.2 ± 4.3
Carbachol (5×10^{-4})	20.4 ± 9.9	131.0 ± 4.4[a]	55.4 ± 8.8	260.3 ± 9.9[a]

%, percentage of the contractile response to 1 μM noradrenaline; ADA, adenosine deaminase.
[a] Significantly different vs. control.

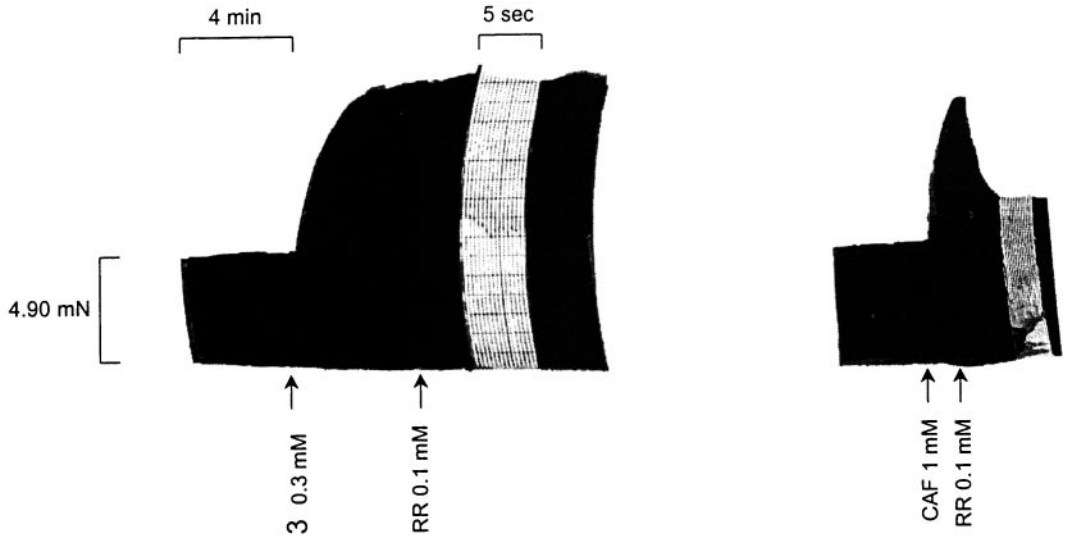


Fig. 3. Typical tracings showing influence of ruthenium red on inotropic effect exerted by compound **3** or caffeine on electrically driven left atria from reserpine-treated guinea pigs. Tracings represent results obtained from five different experiments. Similar results were obtained in the presence of compound **1**. 3, compound **3**; CAF, caffeine; RR, ruthenium red.

1020, 840, 740, 700 cm^{-1}; ^1H NMR (DMSO-d_6 as solvent), chemical shifts from tetramethylsilane δ: 1.34 [triplet, 6H, $N(CH_2CH_3)_2$], 2.93–3.5 [quartet, 4H, $N(CH_2CH_3)_2$], 3.83 [triplet, 2H, $CH_2CH_2N(CH_2CH_3)_2$], 4.03 [singlet, 3H, NCH_3], 5.36 [triplet, 2H, NCH_2CH_2], 7.37–9.4 [multiplet, 7H, Ar-H]; MS, m/z 351 (1.2, M^+), 86 (base). Elemental analysis ($C_{20}H_{24}N_5OCl$) C, H, N.

Synthesis of Compound 5, 1-(β-Ethanolaminoethyl)-5,11-dihydro-11-methyl-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-*a*]benzimidazol-1-ium Bromide. To a suspension of compound **3** (0.100 g, 0.25 mmol) in 10 ml of ethanol with a little amount of triethylamine, 0.1 ml (1.67 mmol) of ethanolamine was added dropwise. The reaction mixture was refluxed for 4 h. The resulting suspension was filtered and the crude product obtained was purified by recrystallization from ethanol (0.045 g, 43% yield, melting point >300°C). IR 3250, 1680, 1600, 1520, 1300, 1140, 740 cm^{-1}; ^1H NMR was not determined because of insolubility in the usual deuterated solvents; MS, m/z 338 (2, M^+), 290 (base). Elemental analysis ($C_{18}H_{20}N_5O_2Br$) C, H, N.

Materials

Tyramine hydrochloride, noradrenaline bitartrate, acetylcholine chloride, pyrilamine maleate, prazosin hydrochloride, propranolol hydrochloride, ranitidine hydrochloride, adenosine deaminase, carbachol, ouabain, sulmazole, reserpine, verapamil hydrochloride, caffeine anhydrous, ruthenium red, ATP, and BRIJ-58 were from Sigma Chemical Co. (St. Louis, MO). 8-[^{3}H]cAMP was from Amersham Italia (Milan, Italy). $^{45}CaCl_2$ was from New England Nuclear (Florence, Italy).

Results

All the benzimidazole derivatives, **1**, **2**, **3**, **4**, and **5**, are closely related, as shown in Fig. 1, a tetracyclic basic structure being present in all of them. Compound **1** is a lipophilic molecule, whereas **2** is charged and is slightly more hydrophilic. Compound **3** was prepared to enhance the lipophilicity of **2**, by means of the chloroethyl chain in N_1. Finally, compounds **4** and **5** were synthesized in the attempt to modify the bulkiness and basicity of the molecule. A long-chain diethylaminoethyl and etanolaminoethyl increases the bulkiness in compound **4** and **5**, respectively. With respect to compound **2**, lipophilicity was unchanged, as in **4**, or slightly decreased, as in **5**.

Effect of Benzimidazole Derivatives on Guinea Pig Atria Contractility

We first determined the effects of the new compounds on the contractility and frequency of spontaneously beating atria, and then tested the contractile effect of more active inotropic agents on electrically driven left atrium. Com-

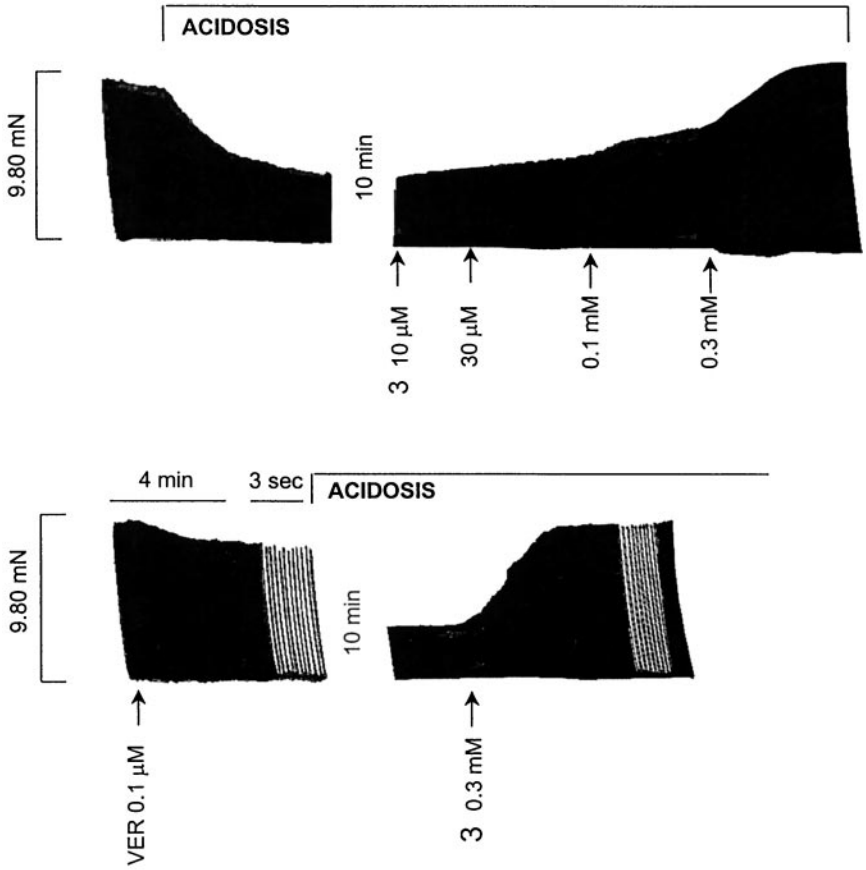


Fig. 4. Typical tracings showing effect of compound **3** on contractility of electrically driven left atria exposed to acidosis. Atria were obtained from reserpine-treated guinea pigs. Tracings represent results obtained from six different experiments. Similar results were evoked by compound **1**. 3, compound **3**; VER, verapamil.

pounds **2**, **4**, and **5** were devoid of any influence on cardiac contractility and frequency of spontaneously beating atria (data not shown).

In spontaneously beating atria, compounds **1** and **3** caused a concentration-dependent increase in the force of contraction (Fig. 2). The increase was very rapid in onset and reached its peak within 4 min. The inotropic effect was already present at micromolar concentrations of both compounds and reached its peak at a concentration of 0.3 mM. This contractile effect was particularly interesting, inasmuch as it did not correlate with unwanted increases in heart rate. At inotropic effective concentrations, both compounds **1** and **3** exerted a slight and not statistically significant negative chronotropic effect ($-8.50 \pm 0.59\%$, $n = 8$). Millimolar concentrations of the compounds evoked signs of toxicity, such as a reduction of contractile force, together with an increase in frequency and sometimes the appearance of moderate arrhythmias. These toxic effects were completely reversed by washing the heart preparation.

In electrically driven left atria, the contractile influence of compound **1** and, even more, that of compound **3** was not only well preserved but even potentiated (Fig. 2). Comparing the EC_{50} and E_{max} values of the compounds in electrically driven atria (Table 1), compound **1** is slightly more potent than compound **3**, whereas **3** is more active. Furthermore, in electrically driven heart preparations, in which inotropism is not influenced by spontaneous oscillations of frequency, compound **3** was more effective than in spontaneously beating atria, thus excluding any correlation between contractile and chronotropic effects.

As shown in Table 2, in electrically driven left atria, the contractile activity of compounds **1** and **3** does not involve receptor activation. It was not influenced by prazosin (5 nM), thus excluding direct interaction of the new compounds with α_1- and/or α_2-adrenergic receptors (Skomedal et al., 1980), by propranolol at a concentration (0.1 μM) that abolished maximal contractile response to isoprenaline in the same heart preparation (Dorigo et al., 1993), by 0.1 μM pyrilamine, or by 10 μM ranitidine, excluding an interaction with H_1- and H_2-histamine receptors. The contractile activity of compounds **1** and **3** does not involve antagonism toward endogenous adenosine because it was not modified by preincubation of left atria with adenosine deaminase (2 U ml^{-1}), the enzyme that inactivates adenosine by metabolizing it to inosine. Lastly, the positive inotropic activity of compounds **1** and **3** was not inhibited by carbachol at a concentration of 50 μM, which inhibits the inotropic responses induced by increases in intracellular cAMP in the same preparation (Dorigo et al., 1993). On the contrary, in the presence of carbachol, the inotropism evoked by compounds **1** and **3** was amplified. An explanation for this amplification is not readily evident, although it may have at least in part resulted from the depression of baseline contractility induced by the muscarinic agent. In any case, these results show that an elevation in cAMP levels does not seem to mediate the contractile effect of the benzimidazole derivatives under study.

The above-mentioned data indicate that in guinea pig atria both compounds **1** and **3** may work through the same cellular influence. Therefore, in the subsequent experiments designed to ascertain the mechanism of action of the new molecules, only the more active compound **3** was further investigated.

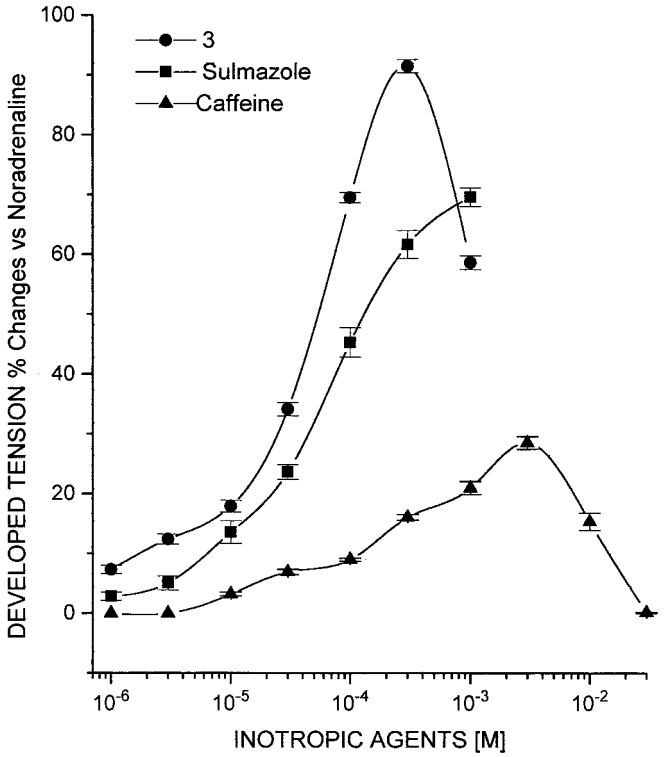


Fig. 5. Comparison of cumulative concentration-effect curves for inotropic effect of compound **3**, sulmazole, and caffeine in spontaneously beating atria from reserpine-treated guinea pigs. The effect of each compound was defined as the difference between the force of contraction before and after its addition to the bathing fluid and was expressed as a percentage of the response induced by 1 μM noradrenaline in the same preparation. All data are means ± S.E.M. of 8 to 10 assays from 10 different experiments. **3**, compound **3**.

In electrically driven left atria, ruthenium red (0.1 mM), a potent although nonselective blocker of the ryanodine receptor (Ma, 1993), inhibited the inotropic effect of caffeine (1 mM), whereas at the same concentration, it did not influence the contractile effect evoked by compound **3** (Fig. 3). This may suggest that Ca^{2+} release from the sarcoplasmic reticulum is not involved in the coupling of excitation and contraction in response to compound **3**, although other explanations may be possible because ruthenium is not highly selective as an inhibitor of ryanodine receptors.

The cardiac activity of compound **3** was then studied in electrically driven left atria perfused by an acid solution (pH 6.69 ± 0.03). Figure 4 shows that acidosis reduced the amplitude of basal contractile activity (−67.45 ± 2.56%, $n = 6$), whereas the cumulative addition of increasing concentrations of compound **3** gradually restored it. The ability of **3** to restore the initial contractile activity after exposure of the atrial preparation to acidosis was also evident in the presence of 0.1 μM verapamil (Fig. 4), thus excluding an involvement of Ca^{2+} uptake through voltage-operated channels in the inotropic action of the compound.

Comparison of Cardiac Effects Evoked by Compound 3, Sulmazole, and Caffeine in Spontaneously Beating Guinea Pig Atria

The inotropic and chronotropic effects of compound **3** were compared with those induced in the same cardiac preparation by the structurally related Ca^{2+} sensitizers, sulmazole and caffeine, which are endowed with the ability to inhibit PDE and release Ca^{2+} from the sarcoplasmic reticulum (for review, see Endoh, 1998). As shown in Fig. 5, compound **3** is the most potent (EC_{50} 4.22 ± 0.9 × 10^{-5} M for compound **3**; 8.32 ± 0.7 × 10^{-5} M for sulmazole; 2.45 ± 0.8 × 10^{-4} M for caffeine) and the most active (E_{max}, expressed as percentage of the contractile effect induced by 1 μM noradrenaline, 91.54 ± 1.12% for compound **3**; 69.57 ± 21.55% for sulmazole; 28.49 ± 6.01% for caffeine). Furthermore, whereas compound **3** did not alter the frequency rate at inotropic concentrations, both sulmazole and caffeine significantly increased it (Table 3).

Effect of Compound 3 on Enzyme Activities and Transport Systems Involved in Cardiac Contractility

When tested on PDE III, the proposed target of several cardiotonic drugs, compound **3** significantly inhibited the enzyme activity only at the highest concentrations tested (0.1–1.0 mM) (Table 4). More specifically, at 0.1 mM, a concentration that evoked an inotropic effect quantitatively equal to that induced by 1 μM noradrenaline, compound **3** inhibited PDE III by only 19 ± 2% ($n = 4$). At the same concentration (0.1 mM) compound **3** did not significantly affect the activity of Na^+/K^+-ATPase (191 ± 2.3 nmol of ATP hydrolyzed/mg/min in the presence of compound **3** versus 226 ± 2.5 nmol of ATP hydrolyzed/mg/min in the absence of compound **3**, $n = 4$), sarcolemmal Ca^+-ATPase (61.52 ± 6.01 nmol of ATP hydrolyzed/mg/min in the presence of compound **3** versus 65.02 ± 2.03 nmol of ATP hydrolyzed/mg/min in the absence of compound **3**, $n = 4$), Na^+/Ca^{2+} exchange carrier (14.87 nmol of $^{45}Ca^{2+}$taken up/mg/min in the presence of compound **3** versus 13.80 ± 0.61 nmol of $^{45}Ca^{2+}$taken up/mg/min in the absence of compound **3**, $n = 4$) and sarcoplasmic reticulum Ca^{2+} pump (11.58 ± 0.40 nmol of $^{45}Ca^{2+}$taken up/mg/min in the presence of compound **3** versus 12.19 ± 0.18 nmol of $^{45}Ca^{2+}$taken up/mg/min in the absence of compound **3**, $n = 4$) of guinea pig cardiac tissue.

Effect of Compound 3 on Myofibrillary Protein Sensitivity to Ca^{2+}

Papillary Muscle. To determine whether compound **3** produces a positive inotropic effect on cardiac muscle by increasing its myofibrillary protein sensitivity to Ca^{2+}, the pCa/tension relationship of guinea pig right papillary muscle was analyzed. The test was performed on small bundles of papillary muscle that showed only the presence of type 1 myosin heavy chain when analyzed by SDS-PAGE (data not shown). The specific tension was not affected by the presence of compound **3**. In particular, in the chemically skinned papillary muscle, **3** did not cause signs of toxicity as observed in isolated myocardial preparation. As shown in Fig. 6B, the pCa/tension curves of muscle treated with 0.1 or 1 mM compound **3** were significantly shifted to the left compared with those of controls. There were appreciable differences between the two groups treated with different concentrations of the compound. However, both the pCa threshold and the Hill coefficient were not significantly different from controls, whereas the pCa_{50} was significantly increased by compound **3**. Lower concentrations of compound **3** were ineffective (data not shown). To verify whether the positive inotropic effect of **3** on cardiac muscle was related to the ability to induce Ca^{2+} release from sarcoplasmic reticulum, the minimum concen-

TABLE 3

Comparison of the influence of compound **3**, sulmazole, and caffeine on the frequency of spontaneous contraction of guinea pig atria

The effect is expressed as percentage change from control (atrial frequency in the absence of drug) and reported as mean ± S.E.M. of 8 to 10 assays from 10 different experiments. Negative changes indicate negative chronotropic effect. Statistical significance was calculated by Student's *t* test.

Compound **3**							
Concentration (M)	10^{-6}	3×10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}
Effect	0.00 ± 0.00	0.35 ± 0.06	0.33 ± 0.03	−2.47 ± 0.59	−7.67 ± 0.82	−8.49 ± 0.59	−4.16 ± 0.40
Sulmazole							
Concentration (M)	10^{-6}	3×10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}
Effect	−9.34 ± 1.07	−6.22 ± 5.05	−0.21 ± 0.83	5.33 ± 0.67	7.84 ± 1.64	20.99 ± 1.06[a]	32.01 ± 0.43[a]
Caffeine							
Concentration (M)	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}
Effect	0.00 ± 0.00	5.21 ± 0.32	7.31 ± 1.17	10.49 ± 1.23[a]	14.12 ± 1.35[a]	31.49 ± 2.72[a]	20.65 ± 2.48[a]

[a] Significantly different vs. control.

TABLE 4

Effect of compound **3** on soluble PDE III isolated from guinea pig heart

Experimental conditions are reported in the experimental section. Data are mean ± S.E.M. from four duplicate experiments. The statistical significance of the changes induced by compound **3** was calculated by Student's *t* test. *P* values were calculated vs. respective control (enzyme activity in the absence of inotropic agent).

Control	Compound **3** (M)						
	10^{-6}	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}
	nmol/mg protein/min						
1.08 ± 0.05	1.09 ± 0.03	1.11 ± 0.05	1.05 ± 0.06	0.94 ± 0.07 (−13%)	0.87 ± 0.02 (−19%)[a]	0.57 ± 0.04 (−47%)[a]	0.42 ± 0.03 (−61%)[a]

%, percentage changes vs. control.
[a] Significantly different vs. control.

tration able to produce detectable tension in chemically skinned guinea pig papillary muscle was measured. Only 57.1% of the 28 papillary muscle bundles analyzed were sensitive to compound **3**, the majority responding to 0.1 mM (data not shown). Tension developed by compound **3** was 17.33 ± 5.9% (n = 6) of the tension developed by 20 mM caffeine, a concentration that is considered to empty the sarcoplasmic reticulum almost completely (Salviati and Volpe, 1988).

Skeletal Muscle. Because the similarity of the contractile machinery both in cardiac and in skelatal muscles is well known, to confirm the Ca^{2+}-sensitizing action of compound **3**, the pCa/tension relationship was analyzed also on type 1 soleus skinned skeletal muscle fibers. Besides one control group of seven untreated fibers, a group of nine fibers was treated with 0.1 mM compound **3** and another group of seven fibers with 1 mM compound **3**. All fibers used in the experiments were electrophoretically identified and only type 1 fibers were considered. The specific tension produced by single skinned fibers, as calculated by normalizing the maximum tension to cross-sectional area, was not affected by the drug.

Compound **3** caused a significant leftward shift of the pCa/tension relationship in a concentration-dependent manner (Fig. 6A). The pCa threshold, i.e., the minimum Ca^{2+} concentration able to produce detectable tension, was significantly increased by 0.1 mM compound **3** but not by 1 mM. In contrast, the Hill coefficient N (calculated from the equation described under *Statistical Analysis*) was significantly reduced only by 0.1 mM compound **3**. The pCa_{50} value, i.e., the pCa causing the development of 50% of the maximum tension, was significantly increased by both concentrations of the drug, although the effect was higher at 0.1 mM (Fig. 6).

Effect of Compound 3 on Sarcoplasmic Reticulum Ca^{2+} Release in Type 1 Soleus Muscle Fibers

Almost all (87%) type 1 soleus muscle fibers (n = 24) were responsive to compound **3**, about 58% of the fibers showing a threshold for Ca^{2+} release of 0.5 to 1 mM (data not shown). Tension developed by the drug at threshold concentrations was 32.7 ± 5.5% (n = 8) of that developed by 20 mM caffeine. Ca^{2+} release caused by 1 mM compound **3** was abolished or markedly reduced by 1 mM ruthenium red, a potent antagonist of the sarcoplasmic reticulum Ca^{2+} release channel (Salviati and Volpe, 1988).

Lack of Influence of Compound 3 on Basal or Raised Tone of Endothelium-Denuded Guinea Pig Aorta Rings

Taken together, the above-mentioned results strongly indicate the sensitization of contractile myofibrils to Ca^{2+} as the main mechanism responsible for the positive inotropic action of compound **3**. To confirm this hypothesis, some experiments were performed on a muscular tissue, such as the guinea pig aorta, which lacks the contractile protein troponin. As expected, in denuded vessel preparations, the cumulative addition of compound **3** (10 μM–1 mM) did not modify the basal tone or the muscular contractile response to 1 μM noradrenaline (Fig. 7).

Discussion

Although several compounds possess remarkable Ca^{2+} sensitizing activity, most of them also possess additional effects that lead to increased intracellular Ca^{2+} concentrations, particularly PDE-inhibiting activity (for review, see Endoh, 1998). Inasmuch as the positive inotropic effect of PDE inhibitors is greatly diminished in end-stage human heart failure (Feldman et al., 1987), we tried to develop an

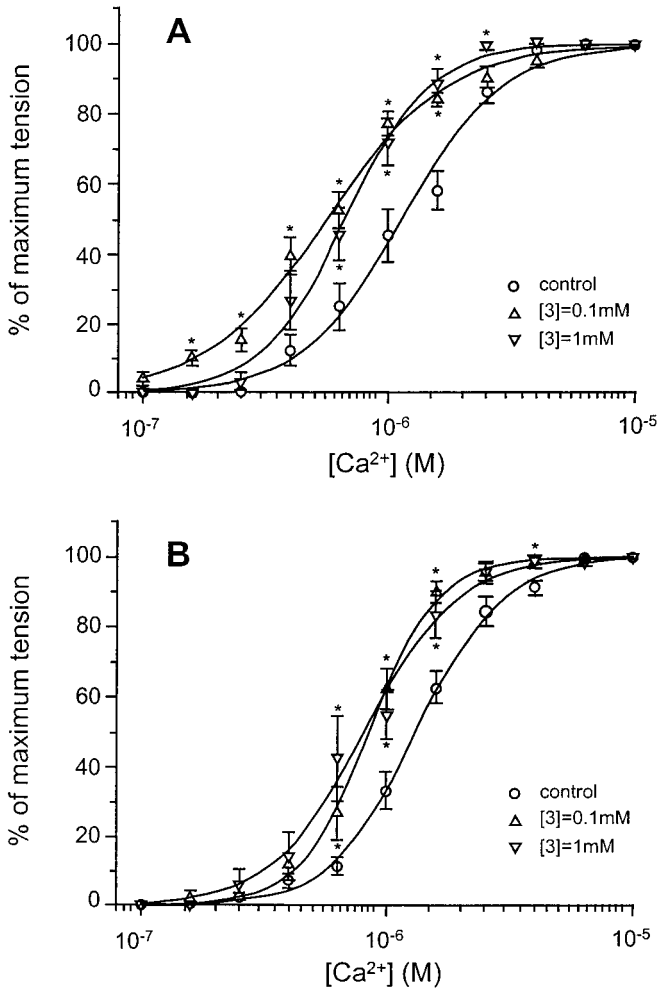


Fig. 6. pCa/tension relationships in control and treated soleus muscle fibers (A) and in control and treated papillary bundles (B). In guinea pig soleus muscle, control values for pCa threshold (pCa threshold for tension development), pCa_{50} (pCa for half-maximal tension) and N (Hill coefficient calculated by fitting the pCa/tension data to the Hill equation as indicated under *Experimental Procedures*) were 6.43 ± 0.07, 5.94 ± 0.05, and 2.41 ± 0.14, respectively (n = 7). In soleus muscle fibers, compound **3** induced 1) a significant increase in the pCa threshold at 0.1mM (6.87 ± 0.07, n = 9), being almost almost ineffective at 1 mM (6.43 ± 0.06, n = 7), 2) a significant increase in pCa_{50} at both 0.1 and 1 mM (6.25 ± 0.05 and 6.17 ± 0.05, respectively), and 3) a decrease of N at 0.1 mM (1.87 ± 0.07), with no effect at 1 mM (2.80 ± 0.20), and with a significant difference of 0.1 mM versus both control and 1 mM compound **3**. In guinea pig papillary muscle bundles, control values for pCa threshold, pCa_{50} and N were 6.54 ± 0.09, 5.88 ± 0.03, and 2.96 ± 0.27, respectively (n = 13). In papillary muscles, compound **3** induced 1) no effects on pCa threshold at both 0.1 and 1 mM (6.60 ± 0.06, n = 8, and 6.60 ± 0.09, n = 5, respectively), 2) a significant increase in pCa_{50} values at both 0.1 and 1 mM (6.08 ± 0.03 and 6.11 ± 0.08, respectively), and 3) no effects on N at both 0.1 and 1 mM (3.58 ± 0.50 and 2.92 ± 0.17, respectively). *, significantly diffferent versus the respective control. 3, compound **3**.

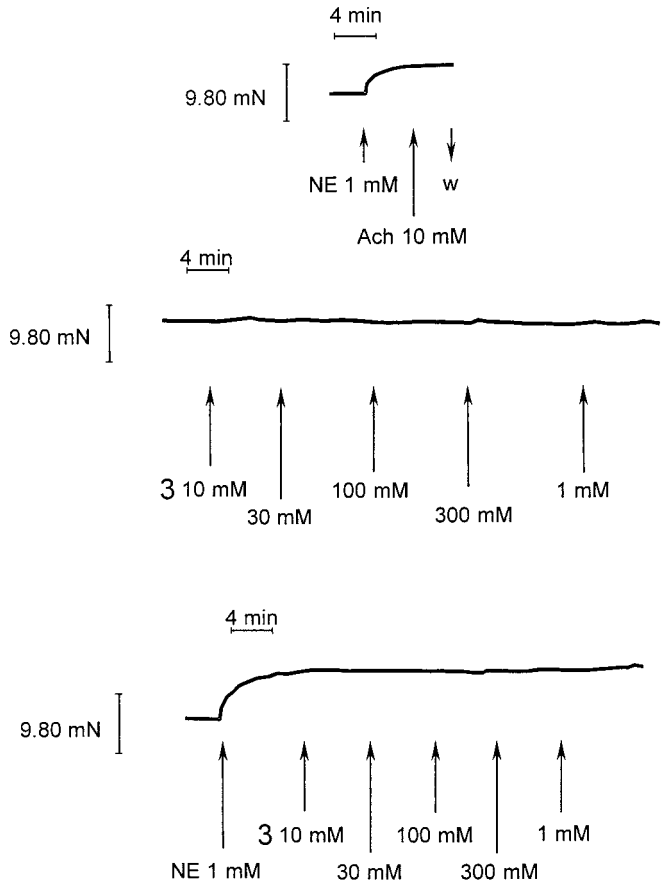


Fig. 7. Typical tracing showing lack of influence of compound **3** on basal and raised tone of aorta rings from reserpine-treated guinea pigs. The tracings represent the results from five different experiments. Similar results were obtained in the presence of compound **1**. 3, compound **3**; NE, noradrenaline; Ach, acetylcholine.

agent showing Ca^{2+}-sensitizing properties at concentrations devoid of untoward additional effects.

In this report we investigated the cardiac activity of a new series of derivatives of benzimidazole structurally related to sulmazole, pimobendan, and the xanthines. In addition to their Ca^{2+}-sensitizing activity, sulmazole and pimobendan inhibit PDE III activity, and sulmazole also inhibits the sarcolemmal Na^+/K^+-ATPase as well as the binding to A_1 adenosine receptors (for review, see Endoh et al., 1998). Xanthines are well known PDE inhibitors, adenosine antagonists, Ca^{2+} sensitizers, and promoters of Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine channels (Sitsapesan and Williams, 1990). Because all cardiac effects linked to Ca^{2+} overload may ultimately damage the myocardial cells, our aim was to obtain a molecule with selective mechanism of action, i.e., the increase in myofilament responsiveness to intracellular Ca^{2+}.

Among the newly synthesized molecules, only compounds **1** and **3**, at concentrations ranging between 1 μM and 0.3 mM, increased the contractile force in spontaneously beating guinea pig atria. Unlike other well known Ca^{2+} sensitizers, such as sulmazole or caffeine, the inotropic effect of **1** and **3** was not accompanied by an increase in heart rate; on the contrary, heart rate appeared slightly reduced. At concentrations higher than 1 mM, contractility declined while frequency increased and, in some atrial preparations, moderate arrhythmias appeared. Toxicity was completely reversed by washing the heart preparation.

In electrically driven left atria, the potency of the two compounds remained unmodified but the maximum contractile effect was increased. Comparing EC_{50} and E_{max} parameters, compound **1** was the most potent and compound **3** was the most effective inotropic agent. The cardiac activity of **1** and **3** does not involve catecholamine release because the compounds were tested in atria isolated from reserpine-treated guinea pigs not responding to the tyramine test.

Adrenergic receptors were not directly activated because both propranolol and prazosine were inactive. Neither H_1- nor H_2-histamine receptors were involved in the contractile activity of **1** and **3**, which remained insensitive to pyrilamine and ranitidine. The new benzimidazole derivatives do not share with caffeine and sulmazole the ability to displace endogenous adenosine from its cardiac receptors because their contractile effect was still present in atria pretreated with adenosine deaminase. Moreover, increases in intracellular cAMP content seems not to be involved, inasmuch as the contractile effect was not inhibited by carbachol, an agent that selectively abolishes the elevation of heart contractility sustained by increases in cAMP levels induced either by adenylyl cyclase stimulation or PDE inhibition in different preparations (Karth et al., 1987).

These evidences suggest that the receptor-operated events generally involved in cardiac contractility do not sustain the inotropic activity of compounds **1** and **3**, which probably exert similar cellular effects. Consequently, in the following experiments only the mechanism of action of the most effective compound **3** was studied.

The contractile effect exerted by compound **3** in electrically driven left atria does not seem to require Ca^{2+} release from the sarcoplasmic reticulum because it is not reduced by ruthenium red at concentrations that inhibit the positive inotropic response to caffeine in the same preparation. Compound **3** did not influence Na^+/K^+-ATPase, Ca^{2+}-ATPase, Na^+/Ca^{2+} exchange carrier, or sarcoplasmic reticulum Ca^{2+} pump activities. In contrast, at the highest concentrations tested (0.1 to 1 mM), it inhibited PDE III of guinea pig heart. However, because atrial contractility was significantly increased by micromolar concentration of compound **3**, no relationship seems to exist between PDE III and the positive inotropic action. PDE III inhibition might be involved in the toxic effects exerted by **3**, such as the increase in frequency and the occurrence of cardiac arrhythmias.

On the contrary, some experimental evidence suggests that a Ca^{2+}-sensitizing effect on myofilaments could be involved in the inotropic activity of compound **3**. First, compound **3** is still active in acidosis conditions. In electrically driven left atria, a slight reduction in the pH, to reach values observed in conditions of myocardial ischemia (Garlick et al., 1979), significantly reduced (−67%) basal cardiac contractility. This reduction is generally related to reduced myofibrillary responsiveness due to decreased Ca^{2+} sensitivity in response to decreased Ca^{2+} binding to troponin C on the myofilaments and/or decreased maximum force, possibly by a direct action on cross bridges (Orchard and Kentish, 1990). In these experimental conditions, ouabain remained inactive (data not shown), being devoid of influence on myofibrillary sensitization, probably because Na^+/K^+-ATPase is inhibited by reduced pH (Orchard and Kentish, 1990). Compound **3** on the contrary, was able to reverse completely the effect of acidosis and to restore the contractile activity to its previous maximum value, as previously observed with Ca^{2+} sensitizers, such as EMD 57033 and Org 30029 (for review, see Endoh, 1998). The effect of **3** was also evident in the presence of the Ca^{2+} channel blocker verapamil at concentrations inhibiting Ca^{2+} uptake from the external compartment. Second, compound **3** directly altered the Ca^{2+} responsiveness of myofilaments when assayed in skinned fibers isolated from both guinea pig cardiac papillary muscle of the right ventricle and skeletal soleus muscle. In these preparations sarcolemma and sarcoplasmic reticulum have been destroyed by detergents to make the relation between $[Ca^{2+}]$ and developed tension directly accessible. In these experimental conditions, compound **3** at 0.1 mM (its nearly maximum inotropic effective concentration) and at 1 mM (its minimum toxic concentration on the isolated atria preparation) significantly shifted to the left the pCa/tension relation curves without signs of toxicity. Like pimobendan (for review, see Endoh, 1998), compound **3** shifted the pCa/tension curves to the left, with no effect on the maximal tension. Furthermore, in the presence of 0.1 and 1 mM compound **3**, a concentration-effect relationship was not observed; lower concentrations of compound **3** were ineffective. The significance of these data is emphasized by the observation that, in electrophoretic assay, both cardiac and skeletal fibers were shown to contain the same isoform of the myosin heavy chain, i.e., type 1 or slow isoform. Furthermore, in the atrial wall of failing heart the rapid isoform turns into slow isoform (Dubus et al., 1993).

The influence of compound **3** on Ca^{2+} release from the sarcoplasmic reticulum was also evaluated, and the results stress the substantial difference between soleus and papillary muscle. In the soleus, but not in the cardiac preparation, compound **3** clearly released Ca^{2+} from the sarcoplasmic reticulum. The mechanism responsible for this release may be similar to that evoked by caffeine because it could be inhibited by ruthenium red. At present, no data are available to explain the apparent different sensitivity of skeletal and cardiac sarcoplasmic reticulum to compound **3**. One possible explanation may be related to the fact that skeletal muscle contains two distinct isoforms of the sarcoplasmic reticulum Ca^{2+} release channel, i.e., RYR1 and RYR2 (Nakai et al., 1997). Furthermore, skeletal and cardiac Ca^{2+} release channels have distinct functional properties related to the different mechanism of activation, that is, the depolarization-induced Ca^{2+} release versus the Ca^{2+}-induced Ca^{2+} release, respectively (Copello et al., 1997). In this respect, compound **3** may be useful to distinguish the two mechanisms of activation. In any case, data obtained in soleus preparations emphasize the therapeutic potential of this compound in heart failure, a syndrome characterized by decreased exercise capacity, with early appearance of fatigue and dyspnea. Although the origin of these symptoms is not clear, muscle myopathy has been demonstrated, accompanied by reduced intracellular pH and reduced numbers of type 1, slow, aerobic, fatigue-resistant fibers (Vescovo et al., 1996). Thus, the increases in both Ca^{2+} release from the sarcoplasmic reticulum and myofibrillary responsiveness to Ca^{2+} in skeletal muscle may provide a cardiac inotropic agent with an additional beneficial effect.

In conclusion, our results indicate that compound **3** may evoke sustained inotropic effect at concentrations suitable to induce Ca^{2+} sensitization without affecting PDE III activity and heart frequency. The lack of influence of compound **3** on guinea pig aorta, a muscular tissue devoid of troponin, locates the action of the compound at the level of this contractile protein, but the specific step involved in the sensitization process remains to be elucidated. In any case, the site of action sensitive to new benzimidazole derivatives may be more easily reached or activated by the most lipophilic compound **3**, in which a chloroethyl chain increases the bulkiness of the structure. Thus, compound **3** may be a useful tool to

counteract negative influences exerted by remodeling factors on cardiac contractility and to reverse the myocardial dysfunction encountered in pathological conditions, such as ischemia, hypoxia, and acidosis, when Ca^{2+}-mobiliting agents fail to increase the contractile force.

References

Caroti P, Ceccotti C, Da Settimo A, Palla F and Primofiore G (1987) Synthesis of 5,11-dihydro-5-oxopyrido[2′,3′:4,5]pyrimido[1,2-a]benzimidazoles as potential antitumor agents. *Gaz Chim Ital* **117:**263–266.

Ceconi C, Curello S, Bachetti T, Corti A and Ferrari R (1998) Tumor necrosis factor in congestive heart failure: A mechanism of disease for the new millennium? *Prog Cardiovasc Dis* **41:**25–30.

Copello JA, Barg S, Onoue H and Flischer S (1997) Heterogeneity of Ca^{2+} gating of skeletal muscle and cardiac ryanodine receptors. *Biophys J* **73:**141–156.

Danieli-Betto D, Betto R, Megighian A, Midrio M, Salviati G and Larsson L (1995) Effects of age on sarcoplasmic reticulum properties and histochemical composition of fast- and slow-twitch rat muscles. *Acta Physiol Scand* **154:**59–64.

Danieli-Betto D, Betto R and Midrio M (1990) Calcium sensitivity and myofibrillar protein isoforms of rat skinned skeletal muscle fibres. *Pfluegers Arch* **417:**303–308.

Dorigo P, Gaion RM, Belluco P, Fraccarollo D, Maragno I, Bombieri G, Benetollo F, Mosti L and Orsini F (1993) A pharmacological, crystallographic, and quantum chemical study of new inotropic agents. *J Med Chem* **36:**2475–2484.

Dubus I, Samuel JL and Swynghedauw B (1993) Origin and mechanism of heart failure in hypertensive patients: Left ventricular remodeling in hypertensive heart disease. *Eur Heart J* **14 (Suppl J):**76–81.

Endoh M (1998) Changes in intracellular Ca^{2+} mobilization and Ca^{2+} sensitization as mechanisms of action of physiological interventions and inotropic agents in intact myocardial cells. *Jpn Heart J* **39:**1–43.

Eriksson H (1995) Heart failure: A growing public health problem. *J Intern Med* **237:**135–141.

Feldman AM, Copelas L, Gwathmey JK, Phillips P, Warren SE, Schoen FJ, Grossman W and Morgan JP (1987) Deficient production of cyclic AMP: Pharmacologic evidence of an important cause of contractile dysfunction in patients with end-stage heart failure. *Circulation* **75:**331–339.

Floreani M, Forlin A, Pandolfo L, Petrone M and Bellin S (1996) Mechanisms of plumbagin action on guinea pig isolated atria. *J Pharmacol Exp Ther* **278:**763–770.

Garlick PB, Radda GK and Seely PJ (1979) Studies of acidosis in the ischaemic heart by phosphorus nuclear magnetic resonance. *Biochem J* **184:**547–554.

Givertz MM and Colucci WS (1998) New targets for heart-failure therapy: Endothelin, inflammatory cytokines, and oxidative stress. *Lancet* **352:**St34–St38.

Jones LR, Besch HR Jr and Watanabe AM (1977). Monovalent cation stimulation of Ca^{2+} uptake by cardiac membrane vesicles. *J Biol Chem* **252:**3315–3323.

Karth M, Engels J and Shafer Korting M (1987) Influence of phosphodiesterase inhibition and carbachol on inotropic effects of P-substituted AMPc analogues. *Naunyn-Schmiedeberg's Arch Pharmacol* **335:**166–175.

Katz AM (1998) Evolving concepts of heart failure: Cooling furnace, malfunctioning pump, enlarging muscle: II: Hypertrophy and dilatation of the failing heart. *J Card Fail* **4:**67–81.

Lee JE, Shah N, White J and Orchard CH (1993) A novel thiadiazinone derivative fully reverses acidosis-induced depression of force in cardiac muscle by a calcium-sensitizing effect. *Clin Sci* **84:**141–144.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Ma J (1993) Block by ruthenium red of the ryanodine-activated calcium release channel of skeletal muscle. *J Gen Physiol* **102:**1031–1056.

Massie BM (1998) 15 Years of heart-failure trials—What have we learned. *Lancet* **352:**St29–St33.

Nakai J, Ogura T, Protasi F, Franzini-Armstrong C, Allen PD and Beam KG (1997) Functional nonequality of the cardiac and skeletal ryanodine receptors. *Proc Natl Acad Sci USA* **94:**1019–1022.

Okabe E, Kuse K, Sekishita T, Suyama N, Tanaka K and Ito H (1991) The effect of ryanodine on oxygen free radical-induced dysfunction of cardiac sarcoplasmic reticulum. *J Pharmacol Exp Ther* **256:**868–875.

Orchard CH and Kentish JC (1990) Effects of changes of pH on the contractile function of cardiac muscle. *Am J Physiol* **258:**C967–C981.

Perez NG, Gao WD and Marban E (1998) Novel myofilament Ca^{2+}-sensitizing property of xanthine oxidase inhibitors. *Circ Res* **83:**423–430.

Salviati G, Sorenson MM and Eastwood AB (1982) Calcium accumulation by sarcoplasmic reticulum in two populations of chemically skinned human muscle fibers. *J Gen Physiol* **79:**603–632.

Salviati G and Volpe P (1988) Ca^{2+} release from sarcoplasmic reticulum of skinned fast- and slow-twitch muscle fibers. *Am J Physiol* **23:**C459–C465.

Shah AM, Spurgeon HA, Sollott SJ, Talo A and Lakatta EG (1994) 8-Bromo-cGMP reduces the myofilaments response to Ca^{2+} in heart cardiac myocytes. *Circ Res* **634:**779–787.

Sharpe N (1994) Cardiac remodeling in congestive heart failure, in *Congestive Heart Failure* (Hosenpud JD and Greenberg DH eds) pp 83–94, Springer Verlag, New York.

Sharpe N and Doughty R (1998) Epidemiology of heart failure and ventricular dysfunction. *Lancet* **352:**S13–S17.

Sitsapesan R and Williams AJ (1990) Mechanisms of caffeine activation of single calcium-release channel of sheep cardiac sarcoplasmic reticulum. *J Physiol (Lond)* **423:**425–439.

Skomedal T, Osnes JB and Oye J (1980) Competitive blockade of α-adrenergic receptors in rat heart by prazosin. *Acta Pharmacol Toxicol* **47:**217–222.

Slaughter RS, Sutko JL and Reeves JP (1983) Equilibrium calcium-calcium exchange in cardiac sarcolemmal vesicles. *J Biol Chem* **258:**3183–3190.

Solaro RJ, Lee JA, Kentish JH and Allen DG (1988) The effects of acidosis on ventricular muscle from adult and neonatal rats. *Circ Res* **634:**779–787.

Studies Of Left Ventricular Dysfunction (SOLVD) Investigators (1991) Effects of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med* **325:**293–302.

Temma K, Akera T, Brody TM and Mannian AA (1977) Hydroxylated chloropromazine metabolites: Positive inotropic action and the release of catecholamines. *Mol Pharmacol* **13:**1076–1085.

Thompson WJ, Brooker G and Appleman MM (1974) Assay of cyclic nucleotide phosphodiesterase with radioactive substrates. *Methods Enzymol* **38:**205–214.

Vescovo G, Serafini F, Facchin L, Tenderini L, Carraro U, Dalla Libera, L, Catani C and Ambrosio GB (1996) Specific changes in skeletal muscle myosin heavy chain composition in cardiac failure: Differences with disuse atrophy as assessed on microbiopsies by a new electrophoretic micromethod. *Heart* **76:**337–343.

Wahler GM and Dollinger SJ (1995) Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am J Physiol* **268:**C45–C54.

Weishaar RE, Burrows SD, Kobylarz DC, Quade MM and Evans DB (1986) Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. *Biochem Pharmacol* **35:**787–800.

Zakhary DR, Moravec CS, Stewart RW and Bond M (1999) Protein kinase A (PKA)-dependent troponin I phosphorylation and PKA regulatory subunits are decreased in human dilated cardiomyopathy. *Circulation* **99:**505–510.

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