British Journal of Pharmacology (1999) 128, 767–773
http://www.stockton-press.co.uk/bjp

Differential effects of bepridil on functional properties of troponin C in slow and fast skeletal muscles

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1 Bepridil (BPD) is a pharmacological compound able to bind to the Ca^{2+} sensor protein troponin C (TnC), which triggers skeletal muscle contraction upon Ca^{2+}-binding. BPD can thereby modulate the Ca^{2+}-affinity of this protein.

2 The Ca^{2+}-sensitizing action of bepridil was investigated on slow and fast isoforms of TnC from skinned slow and fast skeletal muscle fibres, activated by either Ca^{2+} or Sr^{2+} ions.

3 Bepridil did not modify the Ca^{2+} maximal tension of slow and fast fibres, suggesting that binding of the drug to TnC did not induce a change in the number of cross-bridges involved in maximal tension.

4 Sr^{2+} ions induced lower maximal tension than Ca^{2+} ions. However, in fast fibres, these lower Sr^{2+} maximal tensions could be reinforced by bepridil, suggesting an effect of bepridil on the function of site I of fast TnC.

5 Under submaximal tension, bepridil induced an increase in Ca^{2+} affinity of TnC in both slow and fast fibres. However, slow fibres were more drug reactive than fast fibres, and the increase in tension appeared to be modulated by the Ca^{2+} concentration.

6 Thus, bepridil exerted a differential effect on slow and fast fibres. Moreover, the results suggest that bepridil was more effective when activation conditions were unfavourable.

Keywords: Ca^{2+}-sensitizers; bepridil; troponin C; skeletal muscles; skinned fibres

Abbreviations: BPD, bepridil; EGTA, ethylene glycol bis (β-aminoethyl ether) N, N, N′, N′ tetraacetic acid; K Prop, potassium propionate; MgAc, magnesium acetate; MOPS, 3-(N morpholino) propanesulphonic acid

Introduction

Contraction of skeletal and cardiac muscle *in vivo* is elicited by a transient rise in the cytosolic calcium level. This variation is detected by the calcium sensor protein troponin C (TnC). This protein is one of the three subunits of the regulatory troponin complex (with TnI and TnT), regularly spaced along the thin filament composed of two actin and two tropomyosin strands.

Some pathologies, like heart failure, are characterized by a decrease in the contractile response. In this case, to improve muscular contraction, Herzig & Quast (1992) suggested that rather than raising intracellular Ca^{2+} concentration, with attendant danger of calcium overload, increasing Ca^{2+} sensitivity would be a more causative approach. This could be achieved by increasing the calcium affinity of TnC. This dumbbell shaped protein is composed of two heads, each including two Ca^{2+} EF Hand sites, linked by a central α-helix (Grabarek *et al*., 1992; Farah & Reinach, 1995; Tobacman, 1996; for review). The two high affinity carboxy-terminal sites III and IV keep the TnC bound to the thin filament (Zot & Potter, 1982), while the two low affinity amino-terminal sites I and II regulate the muscular switch (Potter & Gergely, 1975). There are two TnC isoforms, with tissue dependent expression (Van Eerd & Takahashi, 1975). The slow isoform is expressed in slow skeletal muscles and in the myocardium (termed in this case the cardiac isoform), and has a defunct site I (Burtnick & Kay, 1977; Leavis & Kraft, 1978). In contrast, the two regulatory sites are functional in the fast isoform, which is expressed in fast skeletal muscles. Since the introduction of the Herzberg-Moult-James model, based on studies of fast TnC, it has been established that calcium fixation on regulatory sites induced conformational changes at the origin of muscular contraction (Herzberg *et al*., 1986).

Among various cardioactive compounds able to improve the Ca^{2+} affinity of TnC (called 'Ca^{2+} sensitizers'), bepridil (BPD) is a polycyclic molecule that can attach hydrophobic parts of TnC buried in close conformation ('apo' state), but accessible to solvent upon Ca^{2+} fixation. As a result, BPD is believed to stabilize the conformational changes, causing an increase in apparent affinity by decreasing the Ca^{2+} off-rate (Mac Lachlan *et al*., 1990). This mechanism is believed to be the same whichever the target isoform, since slow and cardiac isoforms were expected to undergo an opening of the structure upon Ca^{2+} binding similar to that of fast TnC.

However, it has recently been demonstrated that site I was responsible for the great conformational change that occurred in fast skeletal TnC (Sia *et al*., 1997), and that slow skeletal and cardiac TnC underwent much smaller changes of conformation because of their defunct site I (Spyracopoulos *et al*., 1997). This gave particular significance to a comparative study of the functional effects of bepridil on slow and fast TnC in skinned fibres. Since many papers reported the effect of BPD on the cardiac muscle (Herzig & Quast, 1992; Solaro *et al*., 1986), we decided to compare the functional effects of BPD on slow and fast skeletal muscles to check out the hypothesis of a differential action on the two structurally and functionally distinct TnC isoforms. Moreover, activation of slow and fast fibres was differently modulated by another divalent cation, strontium. Indeed, a much greater affinity of Sr^{2+} for slow fibres compared to fast fibres has previously been demonstrated (Kerrick *et al*., 1980), the TnC isoform being the main

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determinant in establishing such differential Sr^{2+} sensitivity (Babu *et al*., 1987). Therefore, the aim of this study was to examine the effects of BPD as a function of the TnC isoform activated by Ca^{2+} and Sr^{2+} ions.

Methods

Animals and muscle preparation

Experiments were performed on skinned fibres of soleus, gastrocnemius lateralis and tibialis anterior obtained from three adult male rats (IFFA CREDO, I'Arbresle, France) weighing about 310 g. According to myosin ATPase activity, the relative proportion of I, IIa, IId and IIb fibres was, respectively: soleus: 84, 7, 9, 0 ; gastrocnemius lateralis : 0, 0, 8, 92 and tibialis anterior : 2, 18, 34, 46 (Delp & Duan, 1996). Moreover, the soleus and the gastrocnemius should be considered as muscles involved in position bearing. The experiments, as well as the maintenance conditions of the animals received authorizations from the Ministry of Agriculture and the Ministry of Education (veterinary service of health and animal protection, authorization 03805). The animals were anaesthetized with an intraperitoneal (i.p.) injection of pentobarbital sodium (3 mg kg^{-1}), and muscles were immediately removed, before the animals were euthanized with lethal i.p. injection of pentobarbital. After removal, the biopsies were immediately chemically skinned by exposure to a skinning solution (Mounier *et al*., 1989). This procedure permeabilized the sarcolemmic and transverse tubular membranes and allowed the application of activating solutions of various calcium concentrations (pCa, with $pCa = -\log[Ca^{2+}]$) directly to the contractile proteins. The skinned muscles were stored at $-20°C$ in a 50/50 glycerol/skinning solution (storage solution) for up to 2–3 months (Wood *et al*., 1975). Protease inhibitor leupeptin was added to the storage solution (10 μg ml^{-1}) to prevent loss of contractile proteins and preserve the fibre tension (Reiser *et al*., 1988).

Solutions

All reagents were provided by Sigma (St Louis, U.S.A.). The composition of all solutions was calculated by the Fabiato computer program (Fabiato, 1974). The program calculation was used with stability constants listed for Ca^{2+} (Orentlicher *et al*., 1977) and for Sr^{2+} (Moisescu & Thieleczek, 1979), to keep final ionic strength at 200 mM. pH was adjusted to 7.0 and ATP at 2.5 mM was added in each solution. The skinning solution was made up of (mM) MOPS, 10; K Prop, 170; MgAc, 2.5; and K_2 EGTA, 5. The following solutions were used for the experimental procedure: a washing solution (W) composed of (mM) MOPS, 10; K Prop, 185; and MgAc, 2.5; a relaxing solution (R) identical to the skinning solution; pCa or pSr activating solutions consisting of W solution + various concentrations of free Ca^{2+} or Sr^{2+} from $CaCO_3$ or $SrCl_2$, respectively, buffered with EGTA and added in proportions to obtain the different pCa values (7.0 to 4.2) or pSr values (7.0 to 3.0). In order to eliminate a hypothetic influence of the sarcoplasmic reticulum (SR) on the tension developed by the myofilaments, each fibre was bathed for 20 min at the beginning of an experiment in a Brij solution made up of R solution with 2% Brij 58 (polyoxyethylene 20 cetyl ether). The non-ionic Brij 58 detergent irreversibly eliminated the ability of the SR of skinned muscles to sequester and release Ca^{2+}, without altering the actomyosin system (Orentlicher *et al*., 1974).

Experimental protocol

For each experiment, a 2–2.5 mm single fibre segment was isolated from the skinned biopsy. A silk thread was tied at each extremity, allowing the mounting of the fibre in an experimental chamber with constant stirring, initially filled with R (Relaxing) solution. The fibre was held at one end by small fixed forceps and at the other end by a clamp connected to a strain-gauge (force transducer Fort 10 (World Precision Instruments), sensitivity 10 V/g). The mounted fibre was viewed through a high magnifying binocular $\times 80$) with a micrometer, allowing fibre diameter measurements, assuming the cross-section was circular. Fibres with a high degree of ellipticity were discarded. The resting sarcomere length was measured by means of a Helium/Neon laser (Spectra Physics) directed perpendicular to the long axis of the fibre. Then, the fibre was stretched to approximately 120% of resting length to allow maximal isometric tension development upon ionic activation. The resulting sarcomere length (2.6 ± 0.04 μm) was subsequently regularly controlled, and readjusted if necessary. The output of the force transducer was amplified and recorded on a graph recorder (Gould, model 6120) and simultaneously analysed by a computer software.

Force measurements

All experiments were performed in a thermostatically controlled room ($19 \pm 1°C$). At the beginning of each experiment, a maximal tension (P_0) was induced by applying a pCa 4.2 solution that contained enough calcium to saturate all TnC sites. An experimental sequence was defined as follows: the fibre was bathed in W solution which eliminated EGTA traces from the previously applied R solution. Then, the fibre was activated at a level P in a given pCa (pSr) solution, immediately followed by a maximum contraction P_0 ensured by pCa 4.2 (pSr 3.0) solution. This procedure allowed the calculation of the relative tension P/P_0. Finally, the fibre was relaxed in R solution. For dose response determination, similar sequences were successively performed with 50, 100, 200, 400 and 800 μM BPD added in the pCa 6.0 solution. For the T/pCa (T/pSr) relationships determination, a unique sequence with 100 μM BPD in the previous pCa (pSr) solution was performed. A new set of P/P_0 determination was then defined (same pCa (pSr), with and without BPD), with a step ordinarily equal to 0.2 pCa units. A complete experiment included a series of sequencies as described above from pCa 7.0 or 6.4 to 4.2 (for slow or fast fibres, respectively), and from pSr 7.0 or 5.8 to 3.0 (for slow or fast fibres, respectively). BPD was prepared fresh each day and used as a 20 mM stock solution in absolute ethanol. At concentrations used, ethanol itself had no effect on the developed tension. Fibres were rejected if force declined during a sustained contraction or decreased by more than 20% during the whole experiment, and if T/pCa and T/pSr series (with and without BPD) were not completely achieved. Data from three fibres at least were kept from each muscle biopsy. For dose response determination, fibres were removed from each soleus biopsy ($n = 12$) or tibialis anterior biopsy ($n = 12$).

Identification of fibre type

The criterion for functional fibre identification was based on the difference of Ca^{2+} and Sr^{2+} activation characteristics between fast and slow fibres (Leavis & Kraft, 1978). Indeed, it is generally assumed that fast muscle fibres are less sensitive to Sr^{2+} than slow fibres. T/pCa and T/pSr relationships

provided information about the affinity of the contractile apparatus for Ca^{2+} and Sr^{2+}, respectively. From these curves, pCa_{50} and pSr_{50} values (50% of maximal Ca^{2+} and Sr^{2+} tension responses, respectively) were deduced. The difference between pCa_{50} and pSr_{50} was used to functionally discriminate slow fibres ($pCa_{50}-pSr_{50}<0.3$) and fast fibres ($pCa_{50}-pSr_{50}>1$).

Two other important parameters could be extracted from the T/pCa relationship: the threshold for activation by Ca^{2+} as an indicator of the calcium sensitivity of the contractile system, and the steepness of the T/pCa which reflects the cooperativity between the different regulatory proteins within the thin filament. Fast type fibres could be distinguished from slow type ones by a higher Ca^{2+} threshold (lower pCa value) and a steeper T/pCa curve. The steepness of the T/pCa was determined by the Hill coefficients n_1 and n_2, calculated according to the following equation (Brandt *et al.*, 1982): $P/P_0=(([Ca^{2+}]/K)^n/[1+([Ca^{2+}]/K)^n])$, where P/P_0 is the normalized tension and K is the apparent dissociation constant ($pK=-\log K=pCa_{50}$). n_1 corresponded to $P/P_0>50\%$; and n_2 to $P/P_0<50\%$.

Moreover, to quantify the shift that occurred when bepridil was added to the activating solution, we defined a $\Delta_{X\ n}$, where Δ represented the shift expressed in pCa (or pSr) units, X the nature of the activating ion, and n the percentage of relative tension at which Δ is determined. Thus, in our analyses, we measured $\Delta_{X\ 10}$, $\Delta_{X\ 50}$ and $\Delta_{X\ 90}$, X being Ca^{2+} or Sr^{2+}.

Statistical analysis

All the data were reported as means ± s.e.mean. The statistical significance of the difference between means was determined using the Student's *t*-test or paired *t*-test when data were obtained from the same fibre in different experimental conditions. Difference at or above the 95% confidence level were considered significant.

Results

Dose response

Dose response curves were determined for slow and fast fibres from the soleus and tibialis anterior, respectively (Figure 1). They were established at pCa 6.0. This concentration was chosen for two reasons. First, it caused the development of a tension the amplitude of which was similar in slow and fast fibres (around pCa_{50}), and secondly, it permitted a clear estimation of the tension reinforcement as it occurred in the presence of BPD. Figure 1A shows typical records of submaximal tensions obtained for the soleus muscle at pCa 6.0 followed by the maximal tension elicited in pCa 4.2 solution. Three records were presented and corresponded to tensions without (No BPD) and with two different BPD concentrations (100 and 800 μM). The amplitude of the submaximal tension was found to be increased in the presence of BPD and dependent on its concentration. In contrast, the maximal tension was similar without and with BPD. The curves in Figure 1B reported the dose response with BPD concentrations in the range 25–800 μM of fibres from slow (soleus) and fast (tibialis anterior) muscles. BPD induced larger reinforcement in fast fibres than in slow ones. From these curves, we chose a concentration of BPD equal to 100 μM for all our other experiments since this concentration corresponded for both fibre types to approximately half the maximal effect obtained with 800 μM.

Calcium activation

The maximal isometric tension P_0 was recorded in the saturating pCa 4.2 solution (Table 1). Fast fibres from the tibialis anterior exhibited significant higher P_0 absolute values (in μN) than slow fibres from the soleus. When expressed per cross sectional area, fibres from both fast muscles showed identical normalized P_0, which were significantly higher than those obtained for the slow soleus fibres. This was observed also in control conditions as well as in the presence of BPD. Moreover, for the three muscles, the P_0 values were not

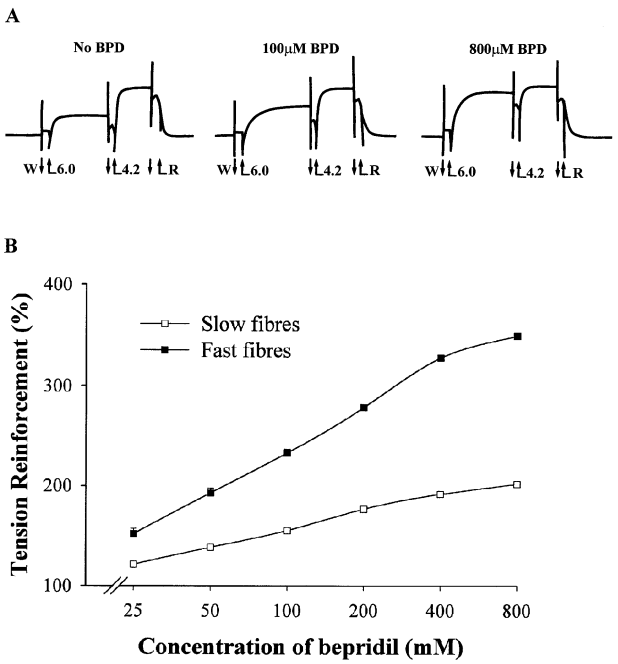

Figure 1 Effects of bepridil on Ca^{2+} activated force in skinned muscle fibres. (A) Submaximal contraction records obtained for the soleus muscle at pCa 6.0 followed by maximal tension elicited by pCa 4.2 solution. The records corresponded to tensions without (No BPD) and with 100 μM or 800 μM BPD. Fast transient peaks on the records and the transient shift of the base line were produced by the changes of the solutions. Upward arrows represented the application of a new solution, while downward arrows indicated withdrawal of the previously applied solution. (B) Tension reinforcement at pCa 6.0 as a function of drug concentration (logarithmic scale). The reinforcement was expressed relative to the tension at pCa 6.0 without BPD. Slow fibres ($n=12$, open squares) and fast fibres ($n=12$, filled squares) were obtained from the soleus and tibialis anterior, respectively. s.e.m. were not reported when they merged with the mean points.

Table 1 Effect of 100 μM bepridil on the P_0 characteristics of soleus, gastrocnemius and tibialis anterior fibres

	Soleus (n = 18)	*Gastrocnemius* (n = 18)	*Tibialis* (n = 18)
$pCa_{50}-pSr_{50}$	0.25 ± 0.02	1.21 ± 0.01*	1.22 ± 0.01*
Diameter (μm)	85.0 ± 3.2	78.75 ± 2.7	87.5 ± 2.4
No P_0 (μN)	650.5 ± 58.3	757 ± 50.1	938.6 ± 101*
BPD P_0 ($kN.m^{-2}$)	115 ± 10.4	156.8 ± 9.9*	152.2 ± 9.9*
With P_0 (μN)	650 ± 58.3	716.5 ± 39	874.5 ± 77*
BPD P_0 ($kN.m^{-2}$)	115.1 ± 10.5	148.7 ± 7.9*	142.8 ± 6.9*

n represented the number of fibres. *indicated significant difference between slow soleus fibres and fast fibres from either gastrocnemius or tibialis anterior. The type of each fibre was controlled by the value of the difference pCa_{50}-pSr_{50}.

modified by BPD, neither for the slow soleus muscle fibres nor for the fast gastrocnemius and tibialis anterior muscle fibres (Table 1).

The T/pCa relationships of the soleus, gastrocnemius and tibialis anterior muscle fibres were illustrated in Figure 2A, C and D, respectively. For the three muscles in control conditions (without BPD), the curves exhibited classic profiles. Thus, pCa values for Ca activation threshold (Figure 2 and Table 2) were significantly higher for the slow soleus fibres (pCa 6.69) than for the fast gastrocnemius (pCa 6.41) or tibialis anterior fibres (pCa 6.36). The pCa_{50} values were not significantly different for the soleus and gastrocnemius fibres

Table 2 Calcium and strontium activation characteristics of 18 fibres from the three muscles, without (No BPD) and with 100 μM BPD

		Soleus		*Gastrocnemius*		*Tibialis Anterior*	
		Ca^{2+}	Sr^{2+}	Ca^{2+}	Sr^{2+}	Ca^{2+}	Sr^{2+}
No BPD	$pCa_{threshold}$	6.69±0.03	6.62±0.07	6.41±0.05*†	5.36±0.06*	6.36±0.02*†	5.40±0.03*
	$pCa\ (Sr)_{50}$	5.89±0.02†	5.64±0.03	5.92±0.03†	4.70±0.04*	5.82±0.03†	4.61±0.04*
	n_1	1.81±0.10†	1.53±0.06	2.53±0.21*†	1.85±0.13*	2.18±0.07*†	1.76±0.07*
	n_2	2.39±0.13†	2.04±0.09	4.93±0.42*†	3.51±0.18*	4.01±0.22*†	2.63±0.10*
With BPD	$pCa_{threshold}$	7.20±0.05§	7.15±0.08§	6.64±0.04*†§	5.67±0.05*§	6.59±0.02*†§	5.73±0.06*§
	$pCa\ (Sr)_{50}$	6.09±0.03†§	5.89±0.03§	6.07±0.03†§	4.96±0.03*§	5.96±0.03*†	4.88±0.03*§
	n_1	1.44±0.07§	1.29±0.05	2.19±0.15*†	1.67±0.12*	2.14±0.07*†	1.56±0.05*
	n_2	1.79±0.10†§	1.62±0.09	4.03±0.22*†	2.96±0.11*	3.55±0.20*†	2.55±0.06*
Δ	$\Delta_{x\ 10}$	0.31±0.01†	0.39±0.01	0.19±0.02*†	0.31±0.01*	0.18±0.01*†	0.29±0.01*
	$\Delta_{x\ 50}$	0.20±0.01†	0.25±0.02	0.14±0.01*†	0.27±0.01	0.14±0.01*†	0.27±0.01
	$\Delta_{x\ 90}$	0.05±0.02	0.13±0.03	0.08±0.02†	0.24±0.02*	0.09±0.01†	0.23±0.02*
	$\Delta_{x\ 90\text{-}10}$	−0.26±0.02	−0.26±0.03	−0.11±0.02*	−0.07±0.03*	−0.09±0.02*	−0.06±0.02*

Δ represents the difference between two curves (with and without BPD) for a specific ion (X being Ca^{2+} of Sr^{2+}) at the relative tension shown in indice. * Indicates significant difference between slow soleus fibres and fast fibres from either gastrocnemius or tibialis anterior. † Indicates difference between Ca^{2+} and Sr^{2+} activation of same muscle fibres (symbols reported on Ca^{2+} values). § Indicates significant difference between No BPD and With BPD conditions (symbols reported on 'With BPD' values).

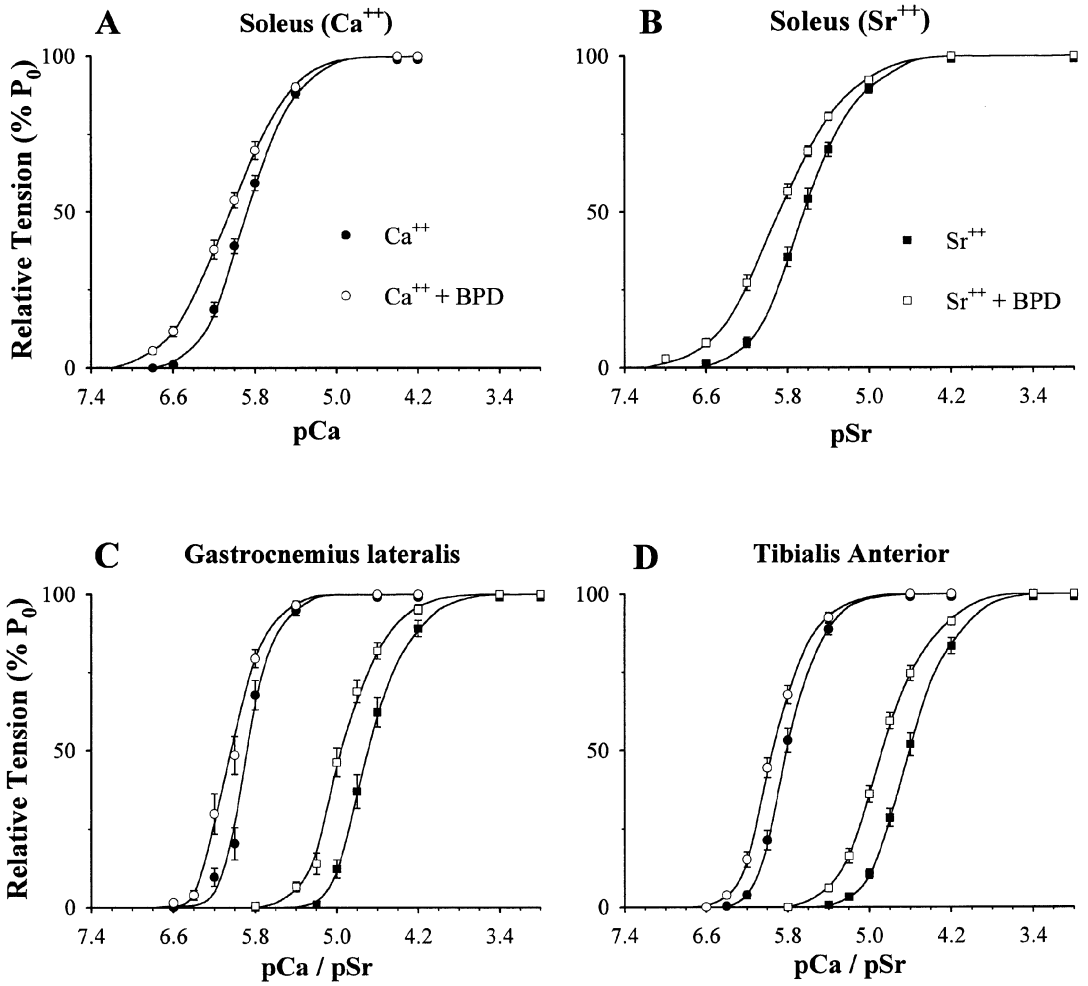

Figure 2 T/pCa and T/pSr relationships T/pCa and T/pSr relationships of single skinned fibres from soleus (A and B), gastrocnemius (C) and tibialis anterior (D). BPD was used at a concentration of 100 μM. Curves were fitted with the Hill parameters (n_1 for $P/P_0 > 50\%$ and n_2 for $P/P_0 < 50\%$).

and slightly lower for the tibialis anterior. The slopes of the curves (n_1 and n_2 parameters) were significantly higher for fast gastrocnemius or tibialis anterior than for slow fibres.

In the presence of BPD, the T/pCa relationships were shifted towards higher pCa values for the three muscles. However, the shift was significantly larger for the slow soleus fibres (Table 1: $\Delta_{Ca50} = 0.20$) than for the fast gastrocnemius or tibialis anterior fibres ($\Delta_{Ca50} = 0.14$ for both muscles).

Another difference between the sensitivity of slow and fast fibres to BPD was evidenced by the Δ_{Ca10} and the Δ_{Ca90} analysis (Table 1). In the slow fibres, there was a large difference between Δ_{Ca90} and Δ_{Ca10} values (-0.26 ± 0.02), which reflected a flattening of the T/pCa curve in the presence of BPD, as shown by the significantly lower n_1 and n_2 parameters, when compared with control values (No BPD). In the fast fibres, the difference between Δ_{Ca90} and Δ_{Ca10} values was lower and equal to -0.11 ± 0.02 and -0.09 ± 0.02 for the gastrocnemius and tibialis anterior, respectively, and the two T/pCa relationships (with and without BPD) appeared almost parallel. No significant differences were found for the n_1 and n_2 parameters of the curves established in the absence or in the presence of BPD.

Strontium activation

Maximal tensions P_0 were elicited using Sr^{2+} ions (pSr 3.4 or 3.0 solutions) instead of Ca^{2+} ions (pCa 4.6 or 4.2 solutions). However, these P_0 (Sr) tensions appeared significantly lower than the P_0 (Ca) ones, the decrease in force being equal to 7, 20 and 20% for the soleus, gastrocnemius and tibialis anterior, respectively (Figure 3). Addition of BPD did not modify the maximal P_0 (Sr) tension of the soleus muscle fibres, but limited the decrease in P_0 (Sr) to 13% for both gastrocnemius and tibialis anterior. In the three muscles, the T/pSr relationships were also established. In the control (No BPD) soleus muscle (Figure 2B and Table 2), the T/pSr relationship was shifted towards higher ionic concentrations (by 0.25 units) and exhibited lower steepness than the T/pCa curve, as shown by significantly decreased n_1 and n_2 parameters. In the two fast muscles, when compared to the T/pCa relationships, the T/pSr curves were clearly shifted towards lower pSr values (by 1.21 units) and the Hill coefficients (n_1 and n_2) were more decreased

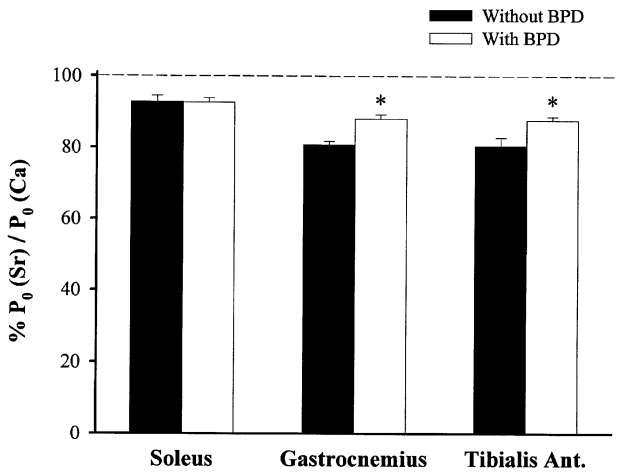

Figure 3 Amplitude of P_0 (Sr) relative to P_0 (Ca). The dashed line indicated P_0 (Ca) of each muscle taken as 100%. Amplitudes of tension elicited by Sr^{2+} ions were expressed relative to P_0 (Ca) in the absence (black bars) and presence (white bars) of 100 μM BPD. * Indicated significant difference between Sr and Sr + BPD activations.

than in the slow soleus fibres. As observed in the presence of Ca^{2+}, BPD induced a leftward shift of the T/pSr of both fibre types. However, when compared to Δ_{Ca50}, the difference Δ_{Sr50} between the two curves (with and without drug) was significantly increased, by a factor 1.25 for the slow soleus fibres ($\Delta_{Sr50} = 0.25$), and by a factor 1.9 for the gastrocnemius and the tibialis anterior ($\Delta_{Sr50} = 0.27$). For the two fast muscles, the slopes of the T/pSr curves (n_1 and n_2 parameters) were not significantly different in the presence or not of BPD, and the two curves were almost parallel. On the contrary, for the slow fibres, BPD diminished the Hill coefficients n_1 and n_2 of T/pSr relationships, as shown above with Ca^{2+} activation ($\Delta_{Ca90\text{-}10} = \Delta_{Sr90\text{-}10} = -0.26$). Thus, BPD induced a modification in the slopes of slow fibres but not of fast ones.

Discussion

This study reported a comparison of the effects of bepridil on skinned fibres from slow and fast skeletal muscles. Moreover, we have attempted to clarify the possible mechanism of action of BPD on the two TnC isoforms using Ca^{2+} and Sr^{2+} as contractile activating ions.

Effects of BPD on Ca^{2+} and Sr^{2+} maximal tensions

As usually described, fast fibres (from gastrocnemius and tibialis anterior) developed higher maximal tensions P_0 than did slow soleus muscle fibres. Addition of the drug BPD, when Ca^{2+} was the activating ion, did not modify P_0, whatever the fibre type. Thus, we believe that the effect of BPD did not imply variations in the maximal number of loaded Ca^{2+} sites. Therefore, the maximal number of attached myosin heads as well as the force developed for each cross-bridge were not modified in the presence of BPD.

Compared with Ca^{2+}, Sr^{2+} activation produced lower P_0 on fast as well as on slow fibres. This suggested that it was more difficult for Sr^{2+} to bind to the TnC amino-terminal low affinity sites. It has been previously demonstrated (Fuchs, 1974) that the affinity of TnC was related to the ionic radius of divalent activating cations, and that optimal radii were close to that of Ca^{2+} (about 1 Å). Thus, the radius of Sr^{2+} (1.12 Å) and chemical properties of Sr^{2+} such as hydration (Fuchs, 1971) should be limiting factors for TnC binding. Therefore, even high Sr^{2+} concentrations might not activate all the TnC proteins, and P_0 (Sr) did not reach P_0 (Ca). This phenomenon was more pronounced on fast fibres (P_0 (Sr) = 80% of P_0 (Ca)) than on slow fibres (P_0 (Sr) = 92% P_0 (Ca)), and was most likely due to the number of regulatory binding sites of TnC. Indeed, unlike its slow counterpart, the fast skeletal isoform has a functional site I, which is necessary for protein function (Sheng *et al*., 1990). Furthermore, this site was demonstrated to have a lower affinity than site II (Li *et al*., 1997). Consequently, it was probably more difficult for Sr^{2+} to bind to the site I than to the site II, and therefore to activate simultaneously both available sites I and II than site II alone.

As described above for Ca^{2+} maximal activation, BPD did not induce any modification of the P_0 (Sr) values in slow fibres. On the contrary, in fast fibres, BPD increased P_0 (Sr), suggesting that more fast TnC molecules were activated. Since BPD was ineffective in tension reinforcement on slow fibres (with slow TnC lacking site I), this differential effect on slow and fast fibres might be attributed to the presence of site I and its lower binding capacity for Sr^{2+} than for Ca^{2+} ions. Thus, binding of BPD would decrease Sr^{2+} off-rate of site I, as was the case with Ca^{2+} (Mac Lachlan *et al*., 1990). As a result, a

greater number of fast TnC were activated, and the decrease in P_0 in fast fibres was limited in the presence of BPD.

Effects of BPD on submaximal tensions

BPD reinforced the submaximal tensions and induced a leftward shift of the T/pCa relationships, on fast and on slow fibres. This increase in apparent affinity suggested that BPD had targeted both fast and slow TnC isoforms. Interestingly, the amplitude of affinity reinforcement (Δ_{50}) was greater on slow fibres expressing slow TnC (Van Eerd & Takahashi, 1975), as already described for another Ca^{2+} sensitizer, EMD 53998 (Barth *et al*., 1995). This demonstrated that site I function was not required for optimal drug effects. Moreover, on these slow fibres only, the leftward shift induced by BPD was higher at low Ca^{2+} concentration ($\Delta_{10} = 0.31$) than at high Ca^{2+} concentration ($\Delta_{90} = 0.05$). This result would suggest that BPD had a greater effect under less favourable activation conditions. This opinion was further confirmed by the comparison of the position of the T/pSr and T/pCa relationships of both types of fibres: in the case of slow fibres, half maximal activation required 1.75 fold more Sr^{2+} than Ca^{2+} and the Δ_{Sr50} shift was 25% greater than Δ_{Ca50}. In the case of fast fibres, which needed 16 fold more Sr^{2+} than Ca^{2+} for half maximal activation, a 2 fold increase in Δ_{Sr50} vs Δ_{Ca50} was evidenced.

If we now consider the slopes of the T/pCa (T/pSr) with BPD, an additional differential effect of BPD between fast and slow fibres was noted. In fast fibres, the slopes of T/pCa (T/pSr) were not significatively different in the absence or presence of the drug. This suggested that BPD increased the apparent affinity without disturbing cooperativity. On the contrary, in slow fibres, a large alteration of the slopes of T/pCa (T/pSr) was shown in the presence of the drug. This suggested a decrease in the cooperativity between the different regulatory proteins of the thin filament. If the target of BPD, TnC, is responsible for Ca^{2+} switch and cooperativity propagation (Gulati *et al*., 1988), this protein represents only one link in the chain of the regulatory proteins. Thus, it could be proposed that the modifications induced by BPD probably resulted from integrated effects which are particularly pronounced within the slow myofibrillar lattice, (i) either from the signalling cascade downstream of the TnC, involving especially TnT, which is known to influence cooperativity (Nassar *et al*., 1991), or (ii) from a feedback of cycling cross-bridges, which in turn have been demonstrated to increase TnC affinity (Zot & Potter, 1989). Further research will be necessary to determine the influence of nearby subunit isoforms involved in the regulatory process and the intrinsic role of TnC, in order to provide new insights into the pharmacological modulation of muscular contraction.

To summarize, these findings indicate that bepridil induced an increase in the affinity of the contractile system, which depended first, on the fibre type, and second, on the activating cation. The BPD effect appeared all the more important as the activation of TnC was achieved with difficulty, justifying its pharmacological use as well in cardiac as in other muscle insufficiencies.

This work was supported by the Centre National d'Etudes Spatiales (CNES, grant n°982711), and by the Nord-Pas de Calais Regional Council.

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(Received April 8, 1999
Revised June 30, 1999
Accepted July 5, 1999)