Pflügers Arch (1993) 425:462–468

Pflügers Archiv
European Journal of Physiology

Effects of 2,3-butanedione monoxime on smooth-muscle contraction of guinea-pig portal vein

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Received February 9, 1993/Received after revision and accepted July 20, 1993

Abstract. Effects of 2,3-butanedione-2-monoxime (BDM) on the contraction of intact and skinned smooth muscles from guinea-pig portal vein were examined. In intact preparations loaded with fura-2, 5–10 mM BDM markedly suppressed Ca^{2+} transients and force developments induced by 154 mM potassium and by phenylephrine (0.1 mM). On the other hand, in Ca^{2+}-free depolarizing solution, BDM did not suppress phenylephrine (0.1 mM)-induced Ca^{2+} transient and force development. In skinned preparations obtained with *Staphylococcus aureus* α-toxin treatment, BDM did not markedly affect active force development. The above results indicate that BDM suppresses contraction of the portal vein mainly by the inhibition of voltage-dependent cytosolic Ca^{2+} transients. An additional result suggests that BDM suppresses the force-enhancing effect of α_1-adrenergic agents on the contractile elements.

Key words: Portal vein – 2,3-Butanedione monoxime – Fura-2 – Ca^{2+} transient – α-Toxin – Skinned smooth muscles – Force-enhancing effect of α_1-adrenergic agent

Introduction

The agent 2,3-butanedione-2-monoxime (BDM; $CH_3C:NOHCOCH_3$) is well known to suppress contraction of skeletal [5, 7, 10, 20], cardiac [1, 14, 15, 23] and smooth muscles [13, 17, 20] reversibly. In skeletal and cardiac muscles, BDM has been shown to affect not only contractile apparatus [7, 10, 14, 15] but also excitation/contraction coupling [1, 5, 10] near the threshold dose for the suppression of contraction. In other words, BDM does not necessarily have a specific site of action in skeletal and cardiac muscles. In smooth muscles, on the other hand, little work has been done on the suppressive mechanisms. Recently Lang and Paul [13] reported that 2–10 mM BDM inhibited voltage-dependent Ca^{2+} channel currents in single cells of guinea-pig taenia caeci. They proposed that this inhibition could account for the suppression of smooth muscle contraction. However, no direct evidence of whether BDM specifically suppresses Ca^{2+} influx has yet been obtained. In the present study, to obtain such direct evidence, cytosolic Ca^{2+} transients were simultaneously recorded with force in fura-2-loaded intact preparations, and the effects of BDM on the contractile elements were also examined in skinned preparations obtained with α-toxin treatment. The results indicated that (a) BDM actually suppresses voltage-dependent cytosolic Ca^{2+} transients, as proposed by Lang and Paul [13], and (b) BDM also suppresses the force-enhancing effect of phenylephrine on the contractile elements. Preliminary reports have been communicated [21, 22].

Materials and methods

Preparations of muscle strips. The dissection and experiments were performed at room temperature (20–25°C). Guinea-pigs (Hartley, female, 400–600 g) were sacrificed by cervical bleeding under deep anaesthesia with ethyl ether. The portal vein was dissected and immersed in normal external solution [NES; in mM: 150 NaCl, 4 KCl, 2 $Ca(Ms)_2$, 1 $Mg(Ms)_2$, 5.6 glucose, and 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) adjusted with TRIS/H_2O to pH 7.4 at 20°C, where (Ms) stands for the methanesulphonate group]. After the perivenous tissues and endothelium had been cleaned, small strips (0.1–0.2 mm wide and 1.5–2 mm long) were separated.

Intact preparations. To load fura-2, muscle strips were treated for 2–3 h with 5 μM fura-2AM (the acetoxymethyl ester of fura-2: Dojin, Kumamoto, Japan) dissolved in NES with 0.5% dimethylsulphoxide and 0.02% cremophor EL. After the strip had been washed with plain NES for 1 h, it was tied at both ends with silk monofilaments to stainless-steel hooks in an experimental chamber (stretched to 1.2–1.5 times the slack length). One of the hooks was attached to a force transducer (BG-10, Kulite Semiconductor, N. J., USA). The strip was alternately (50 Hz) exposed to 340-nm and 380-nm UV light to excite fura-2; the fluorescence at 510 nm

was detected by a fluorometer (CAM-220, Jasco, Tokyo, Japan). At the end of the experiment, each preparation was treated with Triton X-100 (0.5% w/w) to quench the fura-2 photosignal, and the autofluorescence of the preparation was measured [24]. It was noted that 20 mM BDM did not influence the autofluorescence. The ratio (R) of the fluorescence at 340 nm to that at 380 nm was adopted as an indicator of intracellular Ca^{2+} concentration [6]. To evaluate R and the force of each preparation, $(R-R_0)$ and the force were normalized to values at the peak of K^+ contracture, where R_0 denotes the R values at rest [19].

High-K^+ solution was prepared by replacing NaCl in NES with K(Ms) so as to keep $[K^+]\times[Cl^-]$ at 616 mM. To prepare Ca^{2+}-free high-K^+ solution, $Ca(Ms)_2$ of the high-K^+ solution was replaced by [ethylenebis(oxonitrilo)]tetraacetic acid (EGTA). Phenylephrine and BDM were purchased from Sigma (Missouri, USA) and from Nacarai Tesque (Kyoto, Japan) respectively.

Skinned preparations. To prepare a receptor-coupled skinned preparation, α-toxin was used [11, 16]. The toxin was purified from culture media of *Staphylococcus aureus* according to Sakurada et al. [18], and its activity was determined by haemolysis [4]. The protocol of skinning was the same as that described by Kitazawa et al. [11]. In brief, the strips were skinned with 400−1200 units/ml α-toxin for 60−90 min. After the skinning, the strips were treated with 20 μM A23187 (a Ca^{2+} ionophore; Sigma) to destroy intracellular Ca^{2+} storage sites [12].

Solutions for skinned preparations were according to Horiuti [9]. All the solutions contained in common (mM) 1.5 Mg^{2+}, 3.5 MgATP, 10 creatine phosphate and 20 1,4-piperazinediethanesulphonic acid (PIPES). The concentration of EGTA was 2 mM in standard relaxing solution (G2), 10 mM in Ca^{2+} washing solution (G10), 0.1 mM in pre-activating solution (G0.1), and 10 mM in activating solution, which contained various concentrations of $Ca(Ms)_2$ (pCa = 6.5−4.4; pCa denotes the negative logarithm of the estimated Ca^{2+} concentration). The apparent dissociation constant of CaEGTA was assumed to be $10^{-6.4}$ M. K(Ms) was added to keep the ionic strength at 0.20 M, and KOH to adjust the pH to 7.0 at 20°C. The bubble plate system [9] was used to change the incubating solution quickly.

Results are presented as means ± standard errors of the mean (SEM). Statistical hypotheses on the differences between means were tested with Student's t-test for paired samples. The null hypotheses was rejected when P was less than 0.05.

Results

Effect of BDM on the fluorescence of fura-2

Borzak et al. [3] reported that high concentrations (40 mM) of BDM suppressed the fluorescence of fura-2. Therefore, the effect of BDM on the fluorescence of 1 μM fura-2 was examined. Although the fluorescence was appreciably suppressed by BDM (10 mM BDM suppressed about 20% of the fluorescence both at 340 nm and 380 nm emission; data not shown), the extent of the suppression was almost independent of Ca^{2+} concentration. Therefore, the ratio R at any concentration of Ca^{2+} was relatively insensitive to BDM (in G10 and pCa 4.4, R values at 10 mM BDM were 97% and 91% respectively of those in the absence of BDM). Thus, R mainly reflects cytosolic Ca^{2+} concentration: the larger the R value, the higher the Ca^{2+} concentration. Detailed quantitative estimation of the Ca^{2+} concentration is impossible at present because of the lack of reliable calibration. Instead, any change in R with contraction was expressed relative to the value at the peak of K^+ contracture as described in Materials and methods.

Experiments in intact preparations

K^+ contracture. Transfer of an intact preparation into high-K^+ solution (154 mM) induced K^+ contracture (Fig. 1 A). In the presence of BDM, changes in R and force during K^+ contracture were suppressed in a dose-dependent manner (Fig. 1 B). The suppression was significant at concentrations of 5 mM BDM and above ($P < 0.05$) and was completely reversible (data not shown). Note that the extent of suppression of changes in R and force were almost parallel with each other.

Phenylephrine-induced contractions. In the absence of BDM, phenylephrine (0.1 mM) induced a transient force development forming a spike on the trace (initial spike contraction; Fig. 2 A). Following this, the force slowly increased to a plateau level that was about 50% larger than the peak force of K^+ contracture (after contraction).

Regarding R, phenylephrine induced a spontaneous oscillation of R without an accompanying force oscillation, which was observed in one-third of the preparations (Fig. 2 A). In these cases, three series of analyses were performed to analyse R levels during phenylephrine-induced contractions: by adopting the maximum, middle and minimum points of the oscillation a baseline for R levels was drawn. In Fig. 2 B, C, the results obtained with the middle-point analyses are shown. However, the subsequent analyses were found to be valid if maximum or minimal points were adopted instead.

At the peak of the initial spike contraction, R reached 85%−90% of the value at the peak of K^+ contracture. At the plateau phase of the after contraction, R was not significantly different from that at the peak of the initial spike contraction, while the force level was 100%−110% larger at the plateau than at the spike peak ($P < 0.05$). These findings are consistent with those of Himpens and Somlyo [8] obtained in guinea-pig small intestine.

BDM suppressed changes in R and force at the initial spike contraction was well as during the after-contraction in a dose-dependent manner (Fig. 2 B, C). The suppression of changes in R and force was found significant at 10 mM BDM at the spike peak, and at or above 5 mM during the after-contraction ($P < 0.05$). It seemed that 5 mM BDM selectively suppressed the after-contraction without appreciably affecting the initial spike contraction. BDM also suppressed the spontaneous oscillation of R as shown in Fig. 2 A.

Voltage-independent Ca^{2+} release. During the phenylephrine-induced contractions (Fig. 2 A), some of the cytosolic Ca^{2+} may be released in a voltage-independent manner from the sites of intracellular Ca^{2+} stores, since Himpens and Somlyo [8] reported voltage-independent Ca^{2+} release from the intracellular Ca^{2+} stores by chol-

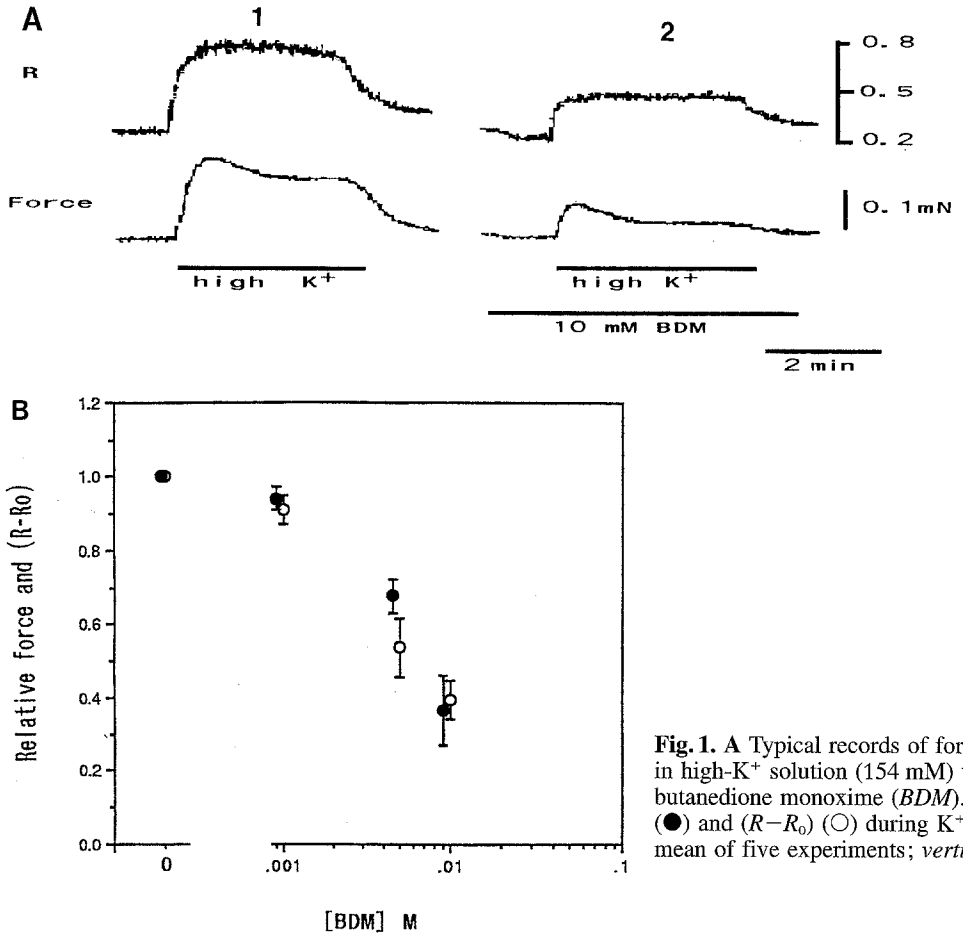

Fig. 1. A Typical records of force (*lower traces*) and *R* (*upper traces*) in high-K^+ solution (154 mM) without (*1*) and with (*2*) 10 mM 2,3-butanedione monoxime (*BDM*). **B** Effects of BDM on the peak force (●) and $(R-R_0)$ (○) during K^+ contracture. Each point represents the mean of five experiments; *vertical bar*, SEM

inergic agents in guinea-pig small intestine. To examine the effect of BDM on this mechanism, the following experiments were performed.

After evoking K^+ contracture in high-K^+ (154 mM) solution (for 3 min), an intact preparation was transferred to Ca^{2+}-free high-K^+ solution. Two minutes after the transfer, *R* and the force completely returned to their original resting levels. Then 0.1 mM phenylephrine was introduced, which induced a transient increase of *R* and force, both of which reached about the level induced by phenylephrine in NES. This increase in *R* would have been due mainly to Ca^{2+} released in a voltage-independent manner from intracellular Ca^{2+} store sites since the specimen was kept depolarized after the K^+ contracture and was in the Ca^{2+}-free solution. This protocol depletes extracellular Ca^{2+} while maintaining Ca^{2+} in the intracellular stores [2]. The changes in both *R* and force were not affected by 10 mM BDM ($P > 0.05$, data not shown). Therefore, BDM would have had little effect on the voltage-independent Ca^{2+} release from the intracellular Ca^{2+} stores.

Experiments with skinned preparations

Direct effects on the contractile elements. The direct effects of BDM on the contractile elements were examined in skinned preparations. When the skinned preparation was transferred from G0.1 to the activating solution (pCa 6.5−4.4), the force rapidly developed to a peak level and then gradually declined to a steady level (Fig. 3 A). Both the peak and the steady force were not significantly affected by BDM up to 20 mM (Fig. 3 B, C; $P > 0.05$); therefore, it is considered that BDM has no direct effect on the contractile elements.

Indirect effects on the contractile elements. It has been reported that α_1-adrenergic agents modulate the force responses of the contractile elements to Ca^{2+} in skinned vascular smooth muscle prepared with α-toxin [11, 16]. In the present skinned preparations, 0.1 mM phenylephrine enhanced the peak and steady force by about 40% at pCa 6.5−4.4 (Fig. 4 A, B). Although the enhancement of the peak force was not significantly affected by BDM up to 10 mM ($P > 0.05$), that of the steady force was significantly suppressed in the presence of 10 mM BDM ($P < 0.05$).

Discussion

BDM has been known to suppress the contraction of various smooth muscles. The main interest in the present

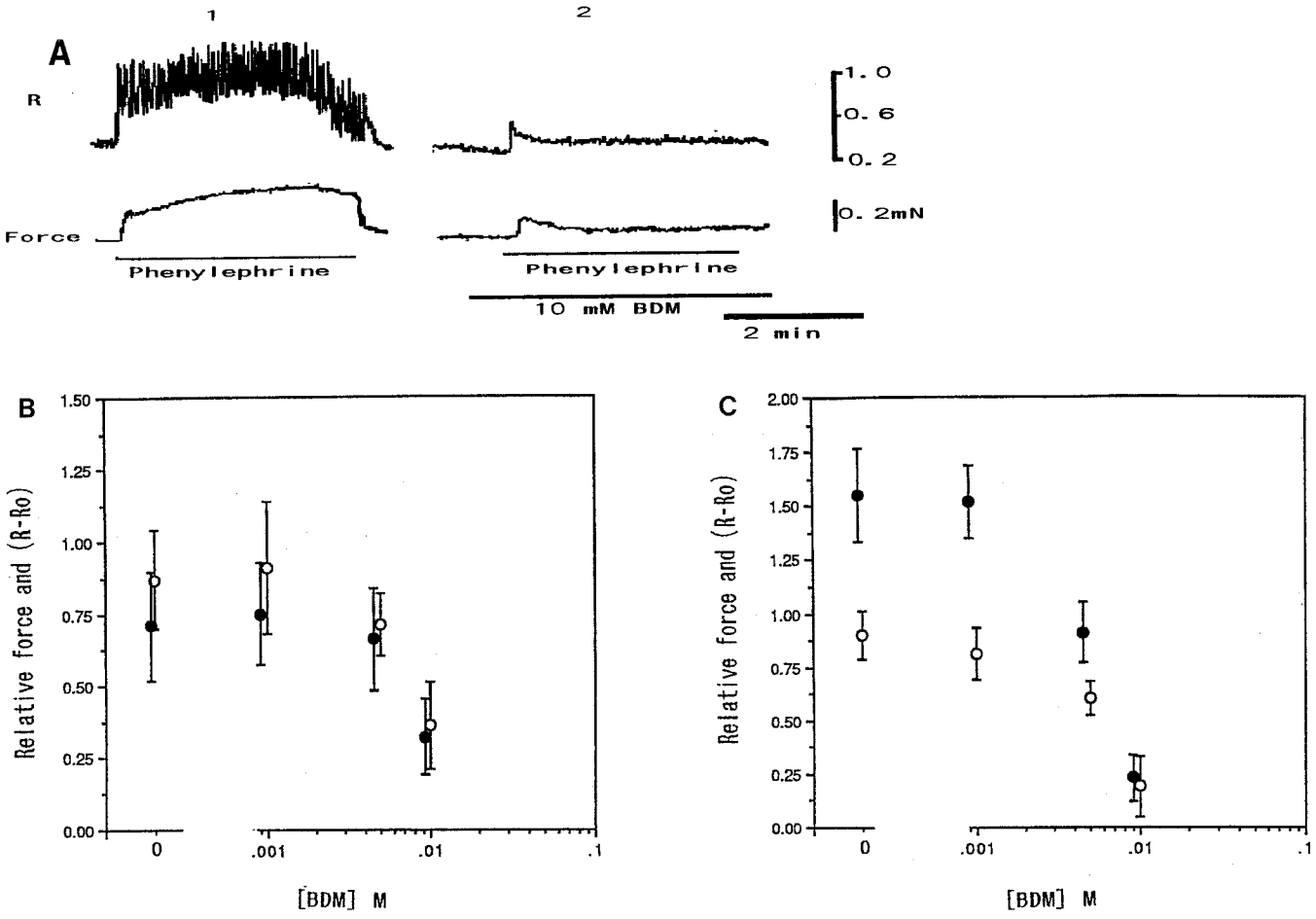

Fig. 2. A Typical records of force (*lower trace*) and *R* (*upper trace*) in 0.1 mM phenylephrine without (*1*) and with (*2*) 10 mM BDM. In this preparation, *R* spontaneously oscillated in the absence of BDM. **B** BDM effects on the initial peak force (●) and accompanying ($R-R_0$) (○) in 0.1 mM phenylephrine. **C** BDM effects on the steady force (●) and ($R-R_0$) (○) in 0.1 mM phenylephrine. The force and *R* are normalized to the values during K^+ contracture. Each point represents the mean of five experiments; *vertical bar*, SEM

study is whether BDM affects excitation/contraction coupling and/or contractile elements of guinea-pig portal vein, because BDM might affect both of these in smooth muscles as in striated muscles.

Before discussing the effects of BDM on the cytosolic Ca^{2+} transients, the effects of the agent on fura-2 signals must be noted. BDM slightly decreased the *R* value of artificial solutions. However, this side-effect of BDM has little influence on the present analyses, since the effects of BDM on *R* in intact preparations (Figs. 1 B, 2 B, 2 C) were much greater than this side-effect. Thus, it seems likely that *R* mainly reflects cytosolic Ca^{2+} concentration.

In the fura-2-loaded intact preparations BDM never suppressed force development without suppressing *R*. BDM showed no appreciable effect on the Ca^{2+}-activated force of skinned preparations at less than 20 mM. These facts support the idea of Lang and Paul [13] that BDM suppresses smooth-muscle contraction by the inhibition of voltage-dependent Ca^{2+} channel currents. However, these facts do no necessarily indicate that BDM suppresses contraction entirely, or even mainly, by its action on the Ca^{2+} transient. Let us discuss the suppressive mechanism in more detail.

Suppression of K^+ contracture

In K^+ contracture, BDM was found to suppress the changes in *R* and force almost in parallel with each other. Such parallelism between the changes in *R* and force was also observed when K^+ contracture was induced by various concentrations of K^+ (unpublished). If some effect of BDM on the contractile elements modifies K^+ contracture, the relationship between the changes in *R* and force under the effect of BDM should differ from that obtained with various K^+ concentrations without BDM. Therefore, it can be assumed that BDM suppresses K^+ contracture mainly by the suppression of the cytosolic Ca^{2+} transient.

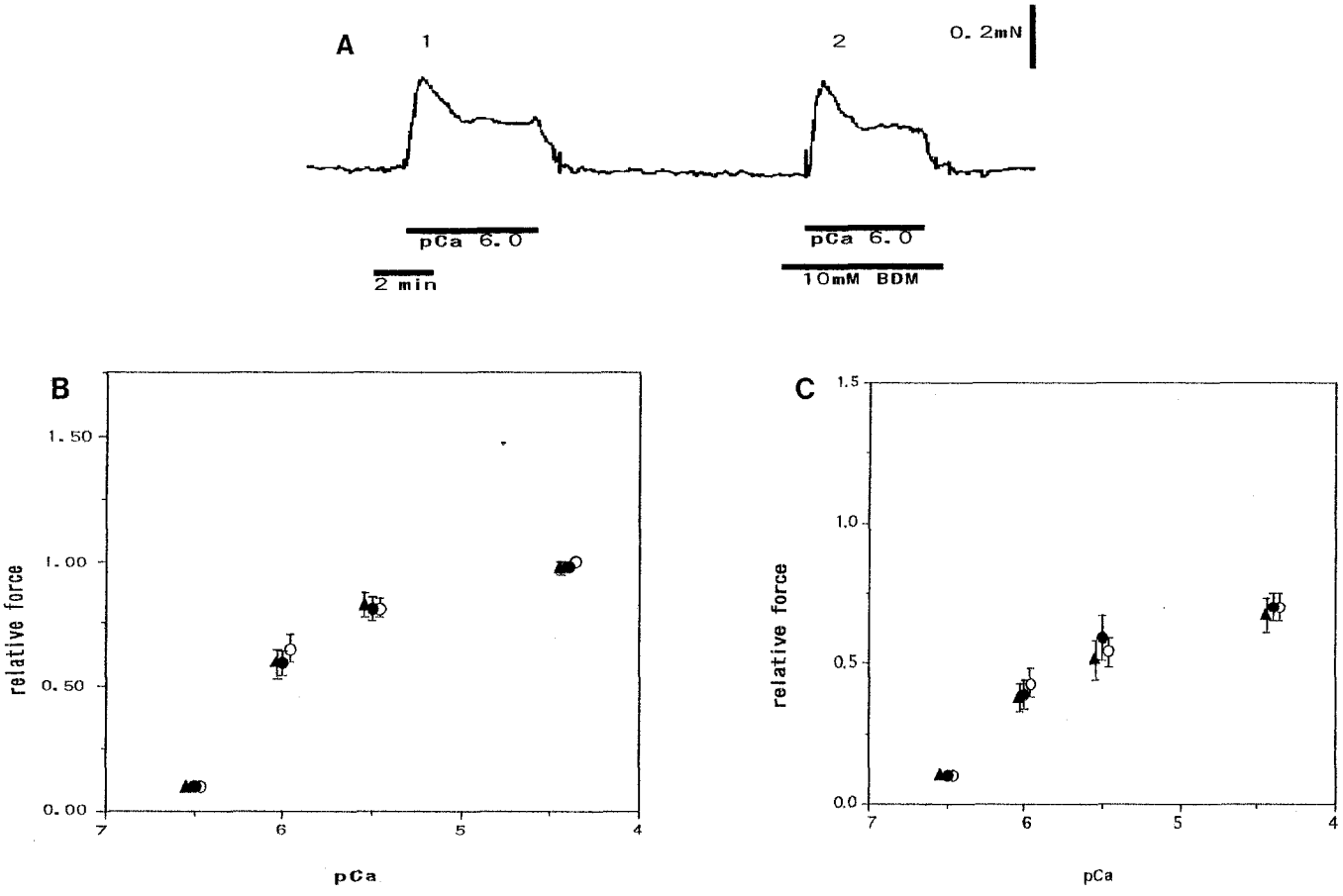

Fig. 3. A A typical force record of a skinned preparation obtained with α-toxin treatment, without (*1*) and with (2) 10 mM BDM. **B, C** BDM effect on the relationship between pCa and the force in skinned preparations. The force was normalized to the peak force at pCa 4.4 in the absence of BDM. **B** Peak force and **C** steady force in 0 mM (○), 10 mM (●) and 20 mM BDM (▲). Each point represents the mean of five experiments; *vertical bar*, SEM

Suppression of phenylephrine-induced contraction

BDM suppressed both Ca^{2+} transient and force development of phenylephrine-induced contractions. But the mechanisms of the inhibition must be much more complicated than that of K^+ contracture.

The suppression of the initial spike contraction would appear to be mainly due to the BDM effect on the cytosolic Ca^{2+} transient, because *R* and force were observed to change in parallel (see above, Fig. 2 B).

BDM suppressed the initial spike contraction less than K^+ contracture (Figs. 1 B, 2 B). That is, BDM affected the Ca^{2+} transient in K^+ contracture more than that in the initial spike contraction induced by phenylephrine. This seems to suggest that the main site of BDM action in excitation/contraction coupling resides in the voltage-dependent Ca^{2+} influx through Ca^{2+} channels, as Lang and Paul [13] discussed. The reasons are as follows. (a) K^+ contracture must be triggered by voltage-dependent Ca^{2+} influx. Therefore, the Ca^{2+} channel, which Lang and Paul [13] found susceptible to BDM, plays a critical role in K^+ contracture. (b) Phenylephrine-induced contraction, on the other hand, should not critically depend on the Ca^{2+} channel, since phenylephrine-induced contraction in Ca^{2+}-free depolarizing solution was comparable to that in NES. From the simultaneous measurement of force and intracellular Ca^{2+} concentration in fura-2-loaded guinea-pig small intestine, Himpens and Somlyo [8] also concluded that the initial spike contraction primarily resulted from the Ca^{2+} release from the intracellular Ca^{2+} storage sites. In addition, the voltage-independent Ca^{2+} release from the intracellular Ca^{2+} sites induced by phenylephrine was shown to be unsusceptible to BDM in the present study.

The suppression of the after-contraction, on the other hand, appears to be partly due to the indirect effect of BDM on the contractile elements, even though the main cause of the suppression of the contraction is the inhibition of the cytosolic Ca^{2+} transient (Fig. 2 C). This is because BDM markedly suppressed the force-enhancing effect of phenylephrine at the steady state of Ca^{2+}-activated contraction in the skinned preparations (Fig. 4). Note that intracellular Ca^{2+} stores of the present skinned preparations were destroyed with A23187. Investigation

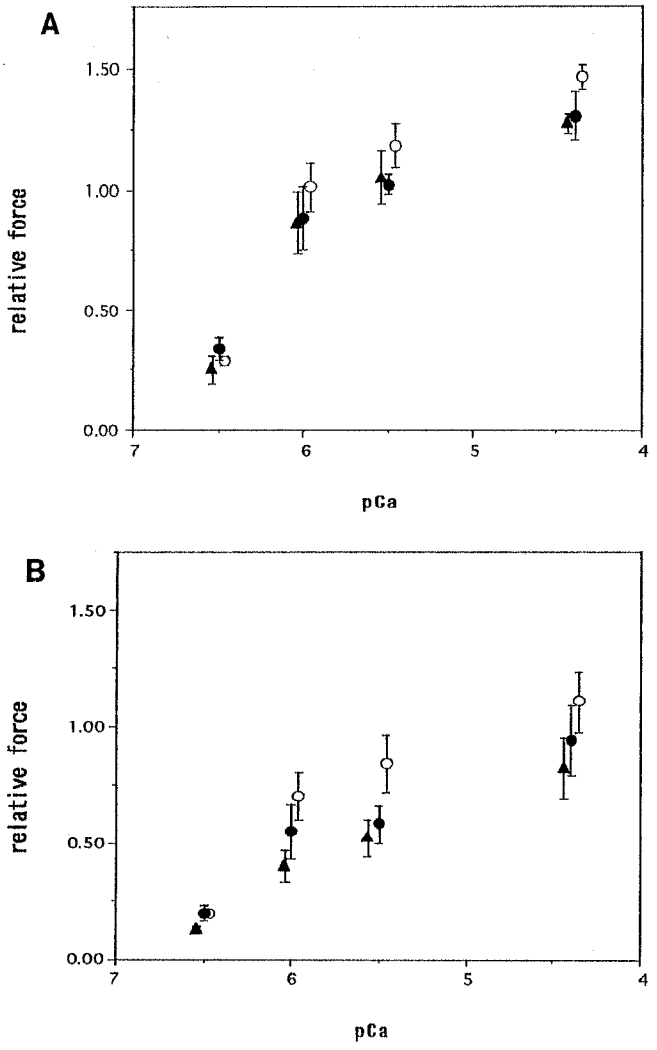

Fig. 4A, B. The relationship between force and pCa in skinned preparations under 0.1 mM phenylephrine. The force was normalized to the peak force at pCa 4.4 in the absence of phenylephrine and BDM. **A** Peak force and **B** steady force in 0 mM (○), 10 mM (●) and 20 mM BDM (▲). Each point represents the mean of five experiments; *vertical bar*, SEM

of the mechanism of this indirect effect of BDM on the contractile elements is now in progress.

In conclusion, BDM might suppress contraction of guinea-pig portal vein by the inhibition of (a) the voltage-dependent cytosolic Ca^{2+} transient, and (b) the force-enhancing effect of phenylephrine on the contractile elements.

Acknowledgements. I would like to thank Dr. J. Sakurada, Professor S. Masuda, Dr. M. Konishi and Professor S. Kurihara for their help with the experiments, Dr. K. Horiuti and Dr. S. Takemori for their valuable discussions and comments, Miss K. Yoshida for her help with manuscript preparation and Mr. W. Elliott for his help with manuscript editing. Also I am grateful to Professor Y. Umazume for his constant encouragement. This study was partly supported by a grant-in-aid for scientific research from Ministry of Education, Science and Culture, Japan.

References

1. Bergey JL, Reiser J, Wiggins JR, Freeman AR (1981) Oximes: 'enzymatic' slow channel antagonists in canine cardiac purkinje fibers? Eur J Pharmacol 71:307–319
2. Bond M, Kitazawa T, Somlyo AP, Somlyo AV (1984) Release and recycling of calcium by the sarcoplasmic reticulum in guinea-pig portal vein smooth muscle. J Physiol (Lond) 355:677–695
3. Borzak S, Kelly RA, Krämer BK, Matoba Y, Marsh JD, Reers M (1990) In situ calibration of fura-2 and BCECF fluorescence in adult rat ventricular myocytes. Am J Physiol 259:H973–H981
4. Dalen AB (1973) Production of staphylococcal α-toxin in a defined medium and identification of a stimulating factor from yeast extract. J Gen Microbiol 74:53–60
5. Fryer MW, Neering IR, Stephenson DG (1988) Effects of 2,3-butanedione monoxime on the contractile activation properties of fast- and slow-twitch rat muscle fibres. J Physiol (Lond) 407:53–75
6. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
7. Higuchi H, Takemori S (1989) Butanedione monoxime suppresses contraction and ATPase activity of rabbit skeletal muscle. J Biochem (Tokyo) 105:638–643
8. Himpens B, Somlyo AP (1988) Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. J Physiol (Lond) 395:507–530
9. Horiuti K (1988) Mechanism of contracture on cooling of caffeine-treated frog skeletal muscle fibres. J Physiol (Lond)398:131–148
10. Horiuti K, Higuchi H, Umazume Y, Konishi M, Okazaki O, Kurihara S (1988) Mechanism of action of 2,3-butanedione 2-monoxime on contraction of frog skeletal muscle fibres. J Muscle Res Cell Motil 9:156–164
11. Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV, Somlyo AP (1989) Receptor-coupled, permeabilized smooth muscle. J Biol Chem 264:5339–5342
12. Kitazawa T, Somlyo AP (1990) Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca^{2+} in smooth muscle. Biochem Biophys Res Commun 172:1291–1297
13. Lang RJ, Paul RJ (1991) Effects of 2,3-butanedione monoxime on whole-cell Ca^{2+} channel currents in single cells of the guinea-pig taenia caeci. J Physiol (Lond) 433:1–24
14. Li T, Sperelakis N, Teneick RE, Solaro RJ (1985) Effects of diacetyl monoxime on cardiac excitation-contraction coupling. J Pharmacol Exp Ther 232:688–695
15. Mulieri LA, Alpert NR (1984) Differential effects of 2,3-butanedione monoxime (BDM) on activation and contraction. Biophys J 45:47a
16. Nishimura J, Kolber M, Breemen C van (1988) Norepinephrine and GTP-γ-S increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. Biochem Biophys Res Commun 157:677–683
17. Packer CS, Kagan ML, Kagan JF, Robertson SA, Stephens NL (1988) The effect of 2,3-butanedione monoxime (BDM) on smooth muscle mechanical properties. Pflügers Arch 412:659–664
18. Sakurada J, Seki K, Ogasawara M, Murai M, Arai Y, Edamura K, Masuda S (1990) Adhesion of protein A-bearing *Staphylococcus aureus* organisms to staphylococcal alpha-toxin-treated Ehrlich tumor cells mediated by anti-alpha-toxin-antibodies. Jikeikai Med J 37:15–20

19. Sato K, Ozaki H, Karaki H (1988) Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. J Pharmacol Exp Ther 246:294–300
20. Warren TB, Butler TM, Siegman MJ, Mooers SU (1985) Effects of 2,3-butanedione monoxime (BDM) on force production and myosin light chain phosphorylation (MYLCP) in mammalian smooth and skeletal muscle. Biophys J 47:295a
21. Watanabe M (1991) Mechanism of the inhibitory effects of 2,3-butanedione monoxime (BDM) on force development in phasic smooth muscle. Jpn J Physiol 41:S299
22. Watanabe M (1992) The effects of 2,3-butanedione monoxime on smooth muscle contraction of guinea pig portal vein. Jpn J Pharmacol 58 [Suppl II]:311P
23. West JM, Stephenson DG (1989) Contractile activation and the effects of 2,3-butanedione monoxime (BDM) in skinned cardiac preparations from normal and dystrophic mice (129/ReJ). Pflügers Arch 413:546–552
24. Yanagisawa T, Kawada M, Taira N (1989) Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2. Br J Pharmacol 98:469–482