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Novel marine-derived halogen-containing gramine analogues induce vasorelaxation in isolated rat aorta

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Abstract

We examined the effects of 2,5,6-tribromo-1-methylgramine (TBG), isolated from bryozoan, and its derivative, 5,6-dibromo-1,2-dimethylgramine (DBG), on the contraction of rat aorta. TBG and DBG decreased the high-K^+-induced increase in muscle contraction and cytosolic Ca^{2+} level ($[Ca^{2+}]_i$), respectively. The inhibitory effects of TBG and DBG on high-K^+-induced contraction were antagonized by increasing the external Ca^{2+} concentration or by 1,4-dihydro2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid (Bay k8644). The high-K^+-induced increase of Mn^{2+} influx was completely blocked by 10 μM TBG or 10 μM DBG. In the Ca^{2+}-free solution, 30 μM TBG or 30 μM DBG inhibited the phenylephrine-induced transient increase in $[Ca^{2+}]_i$ and muscle tension, while scarcely affecting caffeine-induced transient changes. TBG and DBG significantly increased the cyclic AMP content at 30 μM, but not at 10 μM. These results suggest that TBG and DBG inhibit the smooth muscle contraction by inhibiting Ca^{2+} entry, and at higher concentrations, the increase in intracellular cyclic AMP content also contributes to their inhibitory effect.

Keywords: Gramine analogue; Marine natural product; Ca^{2+} channel blocker; Aorta, rat; cAMP

1. Introduction

It is well known that many marine invertebrates, such as sponges and corals, remain remarkably free from settlement by fouling organisms. It has been suggested that they contain biologically active compounds that prevent other marine organisms from settling and attaching to their bodies. While seeking an inhibitor of barnacle settlement, we isolated 2,5,6-tribromo-1-methylgramine (TBG; Fig. 1A) from a bryozoan *Zoobotryon pellucidum* (Kon-ya et al., 1994). The structure of TBG is unique in that it possesses three bromides at C-2, C-5 and C-6 of gramine, the toxic ingredient found in several plants (Goelz et al., 1980).

Since it was shown that biogenic amines, such as histamine, dopamine and serotonin, play an important role in the settlement of the barnacle cyprid larvae (Kawahara et al., 1997), the antifouling effect of TBG is interpreted as inhibiting signal transduction for regulating barnacle settlement. On the other hand, in mammals, biogenic amines elicit physiological responses as neurotransmitters or neuromodulators in the central nervous system, and as regulators of smooth muscle tension. Therefore, it was expected that TBG could also show some useful effects in mammals.

Numerous marine natural products have been found useful as tools for physiological and biological studies because of their actions on specific sites of functional proteins (Ohizumi, 1997). Recently, several natural products have been isolated in our laboratory, such as goniodomin A, which modulates actomyosin ATPase activity (Furukawa et al., 1993) and xestoquinone, which releases Ca^{2+} from skeletal muscle sarcoplasmic reticulum (Ito et al., 1999), suggesting that marine products are attractive resources in the search for physiologically active agents.

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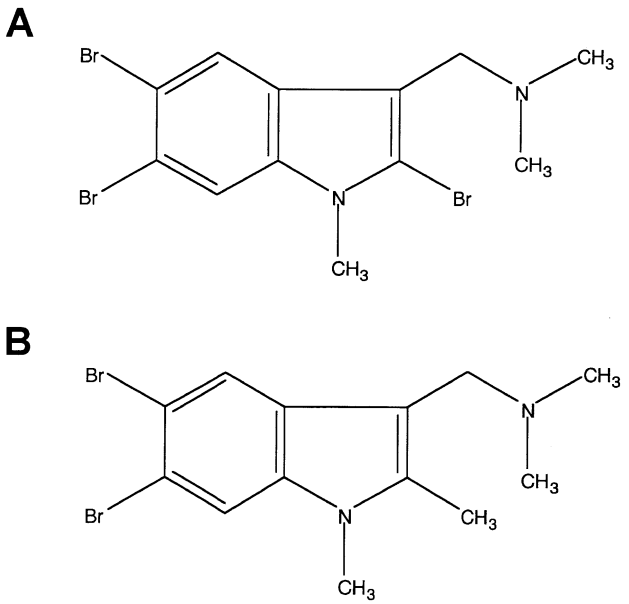

Fig. 1. Chemical structures of TBG (A) and DBG (B).

This is the first pharmacological study of the effects of a halogen-containing gramine analogue, TBG, and its derivative, 5,6-dibromo-1,2-dimethylgramine (DBG), on signal transduction in mammals, using rat aortic smooth muscle tissue.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (9–10 weeks old) were used for this study. The thoracic aorta was dissected and connective tissues were carefully removed. The aorta was cut into helical strips, 2–3 mm wide and 10–15 mm long. The endothelium was removed from the tissues by gently rubbing the endothelial surface with a cotton swab.

2.2. Solutions

The modified Krebs–Ringer-bicarbonate solution contained (in mM), NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25.2, HEPES 20, KH_2PO_4 1.2, and glucose 5.8. A high K^+ solution was made by replacing 60 mM NaCl in the modified Krebs–Ringer-bicarbonate solution by equimolar KCl. The Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1 mM ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). These solutions were saturated with a mixture of 95% O_2 and 5% CO_2 at 37 °C (pH 7.4).

In some experiments, $NaHCO_3$ was omitted from all these solutions in order to prevent the precipitation of $MnCO_3$. These HEPES-buffered solutions were saturated with 100% O_2 at 37 °C (pH 7.4).

2.3. Force measurement

Muscle tension was recorded isometrically with a force-displacement transducer connected to a polygraph (Nihon Koden, Tokyo, Japan). A passive tension of 1 g was initially applied and tissues were allowed to equilibrate in a 5-ml bath for 60 min. After equilibration, the strips were precontracted with high K^+ until the response reached a steady level.

2.4. Cytosolic Ca^{2+} levels

For measuring cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) simultaneously with muscle contraction, we used a fluorescent Ca^{2+} indicator, fura-2 (Grynkiewicz et al., 1985). Muscle strips were loaded with 10 μM acetoxymethyl ester of fura-2 for 4 h at room temperature and then placed

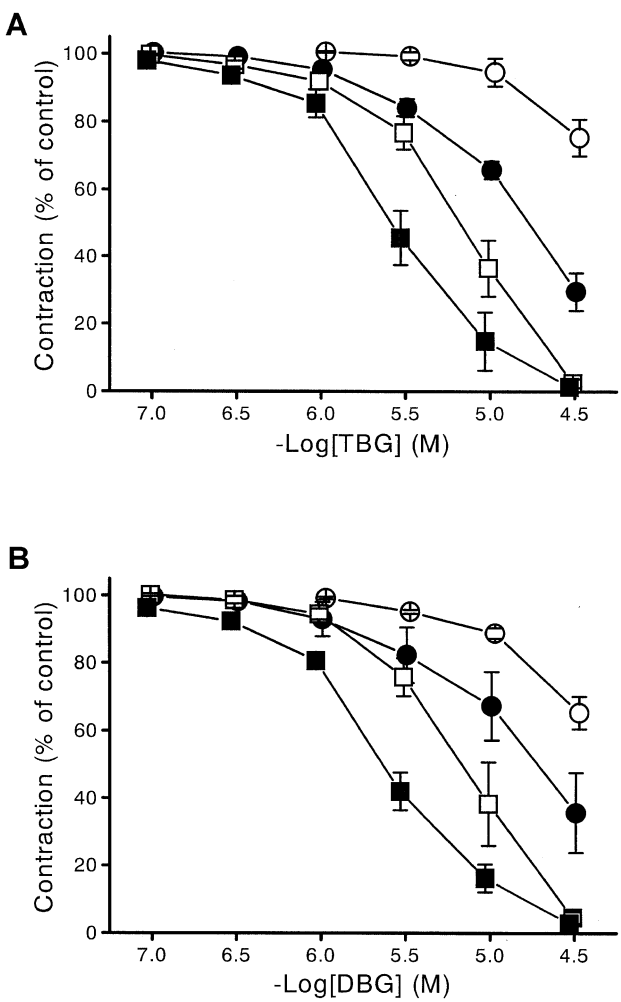

Fig. 2. Concentration–inhibition curves for the effects of TBG and DBG on the contractions induced by 64.8 mM K^+ (■), 10 μM phenylephrine (□), 10 μM prostaglandin $F_{2\alpha}$ (●) or 0.1 μM PDBu (○). During the sustained contraction, TBG and DBG were cumulatively added when the decrease reached a plateau; 100% represents the level of sustained contraction before the addition of TBG and DBG. Each value is the means ± S.E.M. of four to six experiments.

in a tissue bath at 37 °C. The muscle strips were alternately illuminated (48 Hz) with 340 and 380 nm light, and 500 nm emission was detected with a fluorometer (CAF-100, JASCO, Tokyo, Japan). The 500-nm fluorescence induced by the 340-nm excitation (F340) and that induced by the 380-nm excitation (F380) were measured, and the ratio of these two fluorescences (R340/380) was calculated. The absolute amount of $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} in the smooth muscle cytoplasm may be different from that obtained in vitro (Konishi et al., 1988). Instead, R340/380 was used as a relative indicator of $[Ca^{2+}]_i$ (Ozaki et al., 1987), taking the ratio R340/380 in the resting muscle as 0% and that in high-K^+-stimulated muscle as 100%. As Mitsui et al. (1993) reported previously, fura-2 leaks out from the rat aorta during the experiment. Therefore, we always monitored F340 and F380, and finished each experiment before the mirror image response of F340 and F380 signals was lost.

2.5. Mn^{2+} influx

Fura-2 loaded strips were held horizontally in a tissue bath filled with normal HEPES-buffered solution to prevent the precipitation of $MnCO_3$. The muscle strips were illuminated with 360 nm light and the intensity of the fluorescence at 500 nm (F360) was measured. The F360 intensity measured before the administration of 20 μM $MnCl_2$ and 10 min after the administration of 3 mM $MnCl_2$ were taken as 100% and 0%, respectively.

2.6. Cyclic AMP content

The tissue cyclic AMP content was measured with a radioimmunoassay. A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 1 mM), was added 5 min before the treatment with forskolin (1 μM), TBG and DBG (10 and 30 μM). The strips were treated with these compounds for 5 min, and at the end of treatment, the muscle strips were frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation, trichloroacetic acid in the supernatant was removed by washing with water-saturated ether, and the succinylated cyclic AMP was assayed in a competitive radioimmunoassay (Yamasa Shoyu, Tokyo, Japan).

2.7. Chemicals

2,5,6-tribromo-1-methylgramine and 5,6-dibromo-1,2-dimethylgramine were supplied by the Marine Biotechnology Institute (Shimizu, Japan). Fura-2 acetoxymethyl ester was purchased from Dojindo (Japan). All other chemicals were purchased from Wako (Osaka, Japan).

2.8. Statistics

Results of the experiments are expressed as means ± S.E.M. Significance was tested with Student's *t*-test, or Dunnett's multiple comparison test when comparisons involved more than two groups. *P* values smaller than 0.05 were considered significant.

3. Results

In rat aorta, high K^+ (64.8 mM) induced a sustained contraction. Cumulative addition of TBG and DBG (0.1–30 μM) inhibited the contraction in a concentration-dependent manner (Fig. 2). The concentrations needed to induce

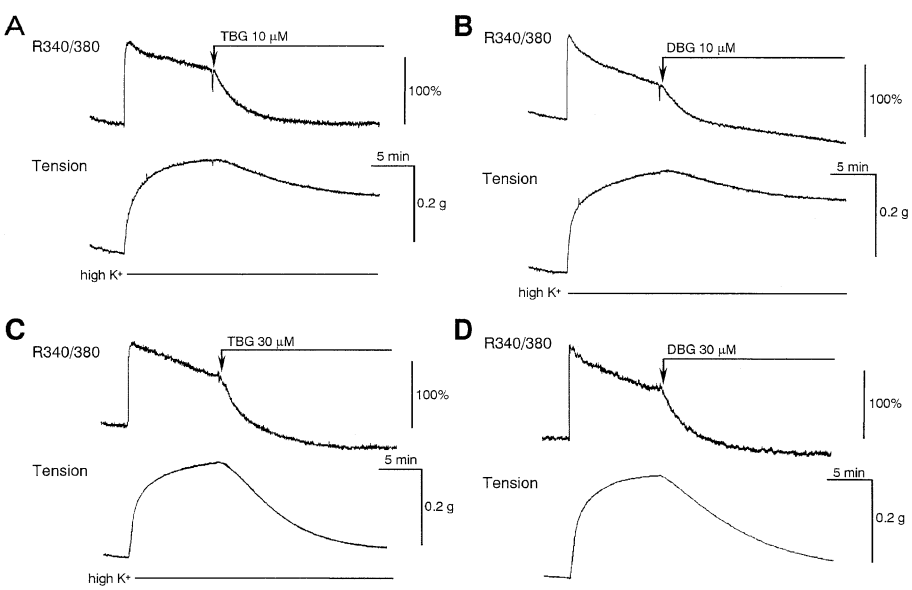

Fig. 3. Inhibitory effects of 10 μM (A, B) or 30 μM (C, D) TBG (A, C) and DBG (B, D) on $[Ca^{2+}]_i$ (shown as R340/380; upper trace) and muscle tension (lower trace) in rat aorta stimulated by high K^+. Traces from typical experimental results.

50% inhibition (IC_{50}) were 2.3 ± 0.6 μM for TBG ($n = 4$) and 2.7 ± 0.5 μM for DBG ($n = 4$). After several washes, contraction never recovered (data not shown). This suggests that the inhibitory effects of TBG and DBG are irreversible.

TBG and DBG also inhibited the sustained contraction induced by phenylephrine (10 μM) in rat aorta (Fig. 2). The IC_{50} values of TBG and DBG were 6.5 ± 0.7 and 6.8 ± 2.5 μM, respectively ($n = 6$). The maximal concentration (30 μM) of TBG and DBG completely reduced the high-K^+- and phenylephrine-induced contraction.

Prostaglandin $F_{2\alpha}$ (10 μM) induced a sustained contraction. TBG and DBG inhibited the contraction in a concentration-dependent manner, but differently from high K^+ and phenylephrine, TBG and DBG were less effective on the prostaglandin $F_{2\alpha}$-induced contraction (Fig. 2).

The phorbol 12,13-dibutyrate (PDBu, 0.1 μM)-induced sustained contraction was scarcely affected by TBG and DBG (Fig. 2).

In the fura-2-loaded rat aorta, high K^+ induced a sustained increase in both muscle tension and $[Ca^{2+}]_i$. TBG and DBG (10 μM) inhibited the high-K^+-induced increase in $[Ca^{2+}]_i$ to near the resting level (Fig. 3A and B). Addition of 30 μM TBG and DBG during the high-K^+-induced sustained contraction decreased both muscle tension and $[Ca^{2+}]_i$ to their respective resting levels (Fig. 3C and D).

As shown in Fig. 4, an increase in external Ca^{2+} concentration from the control level of 1.2 to 5 mM increased the high-K^+-induced contraction ($123.9 \pm 1.4\%$). This increase in external Ca^{2+} antagonized the inhibitory effects of TBG and DBG on the high-K^+-induced sustained contraction (IC_{50}: TBG, 10.2 ± 5.3 μM; DBG, 7.4 ± 1.3 μM; $n = 6$; $P < 0.05$).

Fig. 4 also shows the effect of a Ca^{2+} channel activator, 1,4-dihydro2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]pyridine-3-carboxylic acid (Bay k8644; 0.1 μM), on the inhibitory effects of TBG and DBG on high-K^+-induced contraction. Addition of Bay k8644 during high-K^+-induced contraction caused an increase in muscle tension ($133.0 \pm 4.8\%$). Cumulative addition of TBG and DBG (0.1–30 μM) inhibited the contraction in a concentration-dependent manner, but Bay k8644 antagonized the inhibitory effects of TBG (IC_{50}, 8.7 ± 2.6 μM; $n = 5$; $P < 0.05$) and DBG (IC_{50}, 9.3 ± 3.6 μM; $n = 5$; $P < 0.01$).

Fig. 5 shows the effects of 10 μM TBG and 10 μM DBG on the rate of Mn^{2+} influx measured by the quenching of fura-2 fluorescence. High K^+ increased the Mn^{2+} influx immediately after its administration (Fig. 5A). TBG and DBG (10 μM), applied 15 min before the addition of Mn^{2+} completely inhibited the high-K^+-induced increase of Mn^{2+} influx (Fig. 5B and C).

The half time of F360-decrease was calculated from the F360 intensity curve under all conditions (Fig. 5D). The half time was shortened by the addition of high K^+ (control, 23.7 ± 5.6 min; high K^+, 16.0 ± 1.3 min). In the presence of TBG or DBG , however, high K^+ did not lengthen the half time (TBG, 24.2 ± 2.8 min; DBG, 23.6 ± 2.0 min).

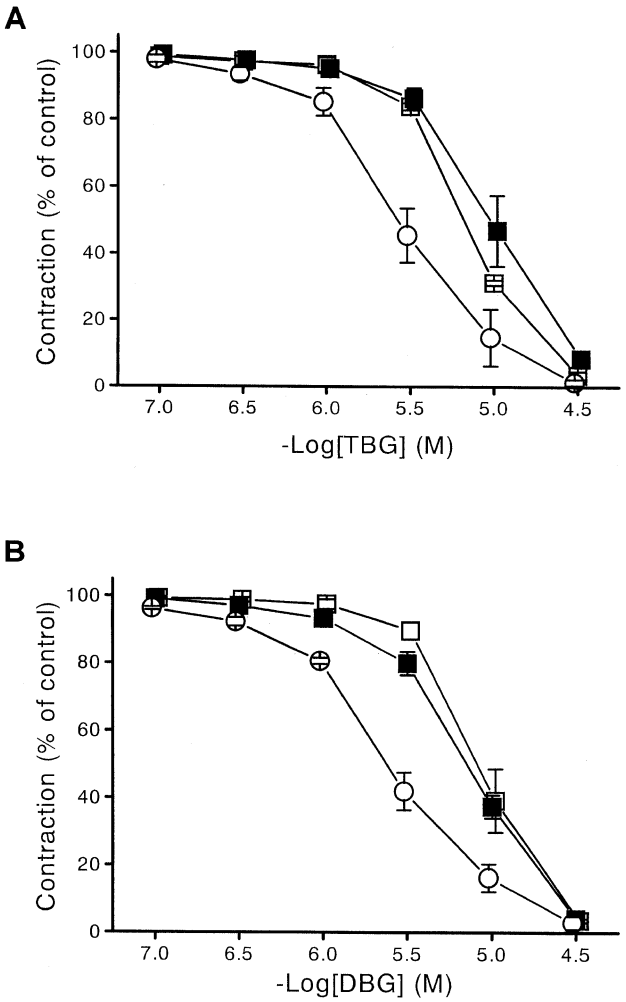

Fig. 4. Effects of external Ca^{2+} concentration and Bay k8644 on the concentration–inhibition curves for TBG (A) and DBG (B) on the contractions induced by high K^+. The concentration of Ca^{2+} (5 mM, ■) was changed 2 min before the addition of high K^+. Bay k8644 (0.1 μM, □) was added when the high-K^+-induced contraction was sustained. Control (○) is the same as in Fig. 2. Each value is the means ± S.E.M. of four experiments.

Phenylephrine also induced a sustained increase in $[Ca^{2+}]_i$ accompanied by a sustained contraction. TBG and DBG (10 μM) reduced the phenylephrine-induced increase in $[Ca^{2+}]_i$ to the resting level (Fig. 6A and B). However, muscle tension was only partially reduced. The remaining portion(s) of these contractions were further inhibited by higher concentrations of TBG and DBG (30 μM) without an additional decrease in $[Ca^{2+}]_i$ (Fig. 6C and D). TBG and DBG (10 and 30 μM) also inhibited the prostaglandin $F_{2\alpha}$-induced $[Ca^{2+}]_i$ increase to the resting level, although some contractions still remained (data not shown).

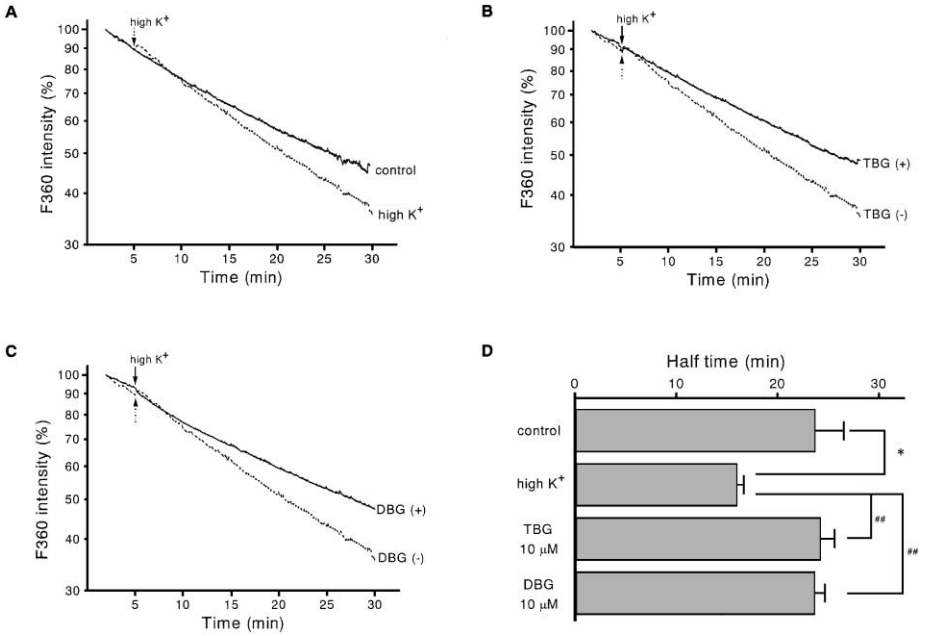

Fig. 5. Effects of TBG and DBG on Mn^{2+}-induced quenching of fura-2 fluorescence. (A–C) Fluorescence decreases are compared with those in normal solution and in high K^+ (A) or compared with effects in the absence or presence of TBG (B) or DBG (C) in high K^+. The 500-nm fluorescence, excited at 360 nm (F360) before starting the experiment, and F360, obtained 10 min after administration of 3 mM Mn^{2+}, were taken as 100% and 0%, respectively. Each trace shows the mean of four experiments. (D) Half time of the F360 decrease. Slopes of change in F360 were calculated by linear regression of the data obtained 5 min after the addition of $MnCl_2$. $^{*}P < 0.05$, Student's *t*-test; $^{\#\#}P < 0.01$, Dunnett's multiple comparison test.

In the Ca^{2+}-free solution, phenylephrine induced a transient increase in $[Ca^{2+}]_i$, followed by a decrease to the resting level (Fig. 7A). TBG and DBG (10 μM) did not inhibit the phenylephrine-induced transient contraction or the transient increase in $[Ca^{2+}]_i$ (Fig. 7B and C). However, 30 μM TBG and 30 μM DBG reduced the transient contraction, and completely blocked the increase of $[Ca^{2+}]_i$ (Fig. 7D and E).

Caffeine (20 mM) also induced a transient increase in $[Ca^{2+}]_i$ and muscle tension. These changes were scarcely

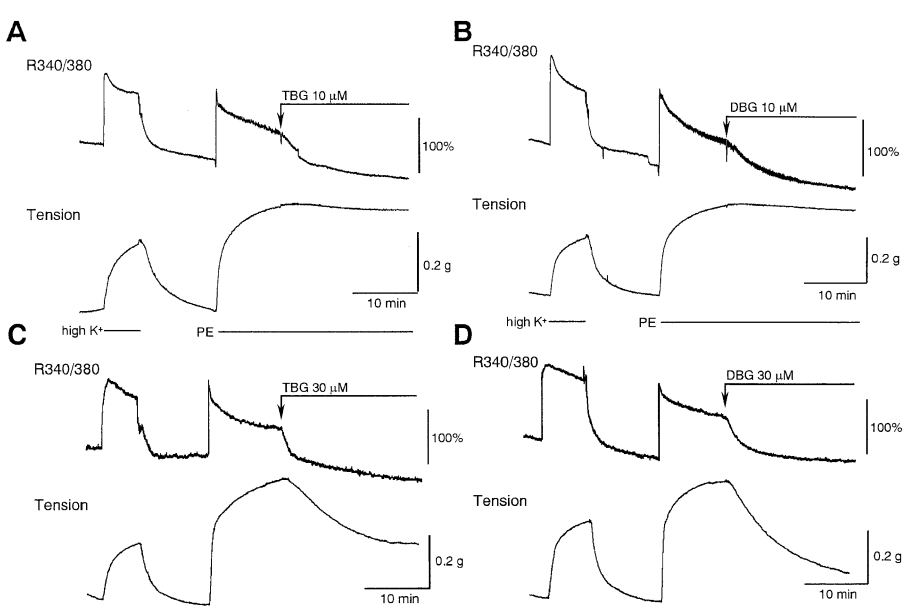

Fig. 6. Inhibitory effects of 10 μM (A, B) or 30 μM (C, D) TBG (A, C) and DBG (B, D) on $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace) in rat aorta stimulated with phenylephrine (10 μM). Traces from typical experimental results.

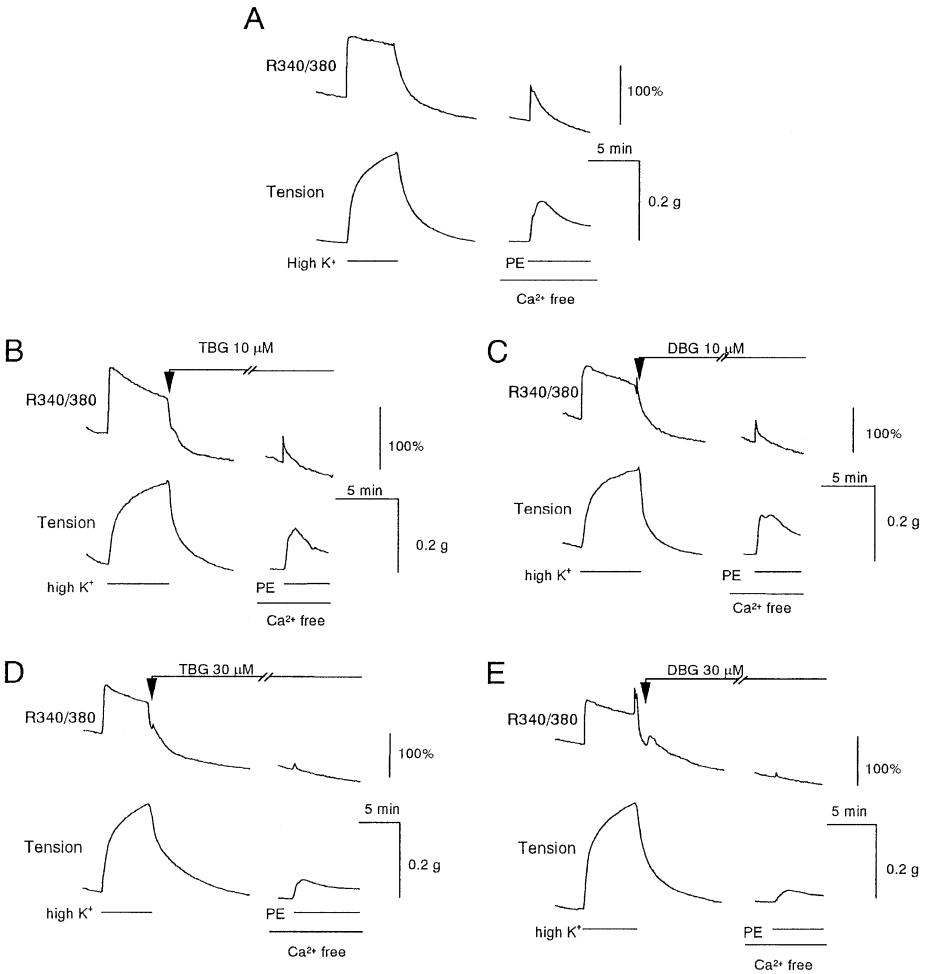

Fig. 7. Effects of TBG and DBG on the transient increase in $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace) induced by phenylephrine (10 μM) in rat aorta in Ca^{2+}-free solution. TBG (10 μM, B; 30 μM, D) and DBG (10 μM, C; 30 μM, E) were added 15 min before the addition of phenylephrine. Traces from typical experimental results.

inhibited by a higher concentration of TBG and DBG (30 μM) (data not shown).

In the presence of 1 μM *N*-(2-[*p*-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide (H-89), a protein kinase A inhibitor, 10 μM phenylephrine-induced contraction was decreased to 63.5 ± 6.3% of the high-K^+-induced contraction, while 10 μM phenylephrine induced 107.8 ± 3.3% of the high-K^+-induced contraction without inhibitor. TBG and DBG inhibited this contraction in a concentration-dependent manner, and the inhibitory effects were not affected by H-89 (IC_{50}: TBG, 3.9 ± 0.5 μM; DBG, 4.7 ± 0.9 μM). One micromolar of 3-[*N*-(dimethylamino)propyl-3-indoyl]-4-[3-indoyl]maleimide (GF109203X), a protein kinase C inhibitor, showed no inhibitory effect on the 10 μM phenylephrine-induced contraction (90.4 ± 5.7% of high K^+). TBG and DBG inhibited this contraction in a concentration-dependent manner, and the inhibitory effects of TBG and DBG were not affected by GF109203X (IC_{50}: TBG, 5.1 ± 0.7 μM; DBG, 7.4 ± 1.1 μM).

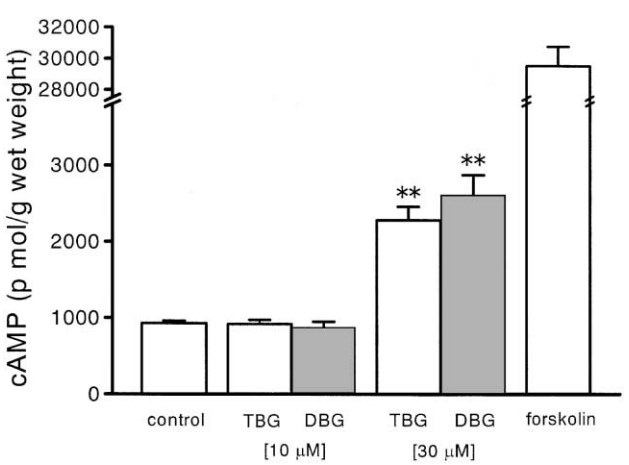

Fig. 8. Effects of TBG and DBG on cyclic AMP content in rat aorta. TBG and DBG were added in the presence of IBMX (1 mM) for 5 min. One micromolar forskolin was used as positive control. Each column represents the means ± S.E.M of four experiments. $^{**}P < 0.01$; Dunnett's multiple comparison test.

The effect of TBG or DBG on cyclic AMP content was measured in the rat aorta. The results indicated that neither 10 μM TBG nor 10 μM DBG increased the cyclic AMP content, whereas 30 μM TBG and 30 μM DBG significantly increased the cyclic AMP content (Fig. 8).

4. Discussion

Membrane depolarization increases Ca^{2+} influx by opening the L-type Ca^{2+} channel to elevate $[Ca^{2+}]_i$ and finally induces contraction (Karaki et al., 1997). TBG and DBG inhibited the high-K^+-stimulated muscle contraction and $[Ca^{2+}]_i$ in isolated rat aorta (Fig. 2). The inhibitory effect of TBG and DBG on high-K^+-induced contraction was antagonized by the increase in external Ca^{2+} concentration and addition of Bay k8644 (Fig. 4), a Ca^{2+}-channel activator (Schramm et al., 1983). These results are similar to those obtained with verapamil and other Ca^{2+} channel blockers (Hof and Vuorela, 1983; Hagiwara et al., 1993).

The measurement of the rate of fura-2 quenching by Mn^{2+} gives a direct estimate of Mn^{2+} influx through voltage-dependent Ca^{2+} channels (Chen and van Breemen, 1993). The high-K^+-induced acceleration of Mn^{2+} influx was completely inhibited by TBG and DBG, suggesting that TBG and DBG may inhibit Ca^{2+} entry by blocking voltage-dependent Ca^{2+} channels.

The 10 μM TBG and 10 μM DBG reduced the phenylephrine- and prostaglandin $F_{2\alpha}$-induced increase in $[Ca^{2+}]_i$ to the resting level, however, muscle tension was only partially reduced. Smooth muscle contraction is not only regulated by $[Ca^{2+}]_i$, since membrane receptor stimulation increases the isometric force developed at a constant submaximal $[Ca^{2+}]_i$, i.e., increases Ca^{2+} sensitivity (Himpens et al., 1988; Horowitz et al., 1996). Compared to receptor agonist- and phorbol ester-induced ones, high-K^+-induced contraction is considered to be due to a relatively simple mechanism, an increase in $[Ca^{2+}]_i$, without changes in other signal transduction systems, including phosphatidylinositol turnover and Ca^{2+} sensitization (Karaki et al., 1997). Verapamil showed only a small inhibitory effect on norepinephrine-(Sato et al., 1988), prostaglandin $F_{2\alpha}$-(Hori et al., 1992) or phorbol ester-induced contraction (Sato et al., 1988). Contractions induced by agonists are less sensitive to Ca^{2+} channel blockers than the high-K^+-induced contraction, possibly because these blockers do not inhibit agonist-induced Ca^{2+} sensitization (Karaki et al., 1997). In fact, as we have observed in the case of 10 μM phenylephrine- or 10 μM prostagrandin $F_{2\alpha}$-induced contraction, receptor agonist-induced contractions still remained in the presence of 10 μM TBG or 10 μM DBG, although the $[Ca^{2+}]_i$ was greatly decreased.

However, a higher concentration (30 μM) of TBG and DBG inhibited portions of the phenylephrine and prostaglandin $F_{2\alpha}$-induced contraction without further decreasing $[Ca^{2+}]_i$. These results suggest that higher concentrations of TBG and DBG may have other mechanisms for inhibiting smooth muscle contraction.

Some intracellular enzymes modify the Ca^{2+} sensitivity of contractile elements and regulate the smooth muscle contractile mechanisms. Protein kinase C activation elicits Ca^{2+} sensitization (Jiang et al., 1994; Satoh et al., 1994), whereas protein kinase A and protein kinase G activation causes desensitization of contractile elements to Ca^{2+} (Karaki et al., 1997). The inhibitory effects of TBG and DBG on the smooth muscle contraction induced by phenylephrine were not significantly different in the presence or absence of H-89, a protein kinase A inhibitor, or of absence or presence of GF109203X, a protein kinase C inhibitor.

In rat aorta, stimulation of the α_1-adrenoceptors by phenylephrine induced a transient contraction in Ca^{2+}-free solution, corresponding to a transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from intracellular Ca^{2+} pools by inositol 1,4,5-trisphosphate (IP_3) (Hisayama et al., 1990). The phenylephrine-induced transient increases in $[Ca^{2+}]_i$ and muscle tension were not affected by 10 μM TBG and DBG, but a higher concentration of TBG and DBG significantly inhibited the transient changes. In contrast with this, the caffeine-induced transient increase in $[Ca^{2+}]_i$ and muscle tension which may have been due to activation of the Ca^{2+}-induced Ca^{2+} release mechanisms (Karaki and Weiss, 1988) were scarcely inhibited by a higher concentration of TBG and DBG. These results suggest that TBG and DBG may affect the signal transduction of the IP_3-induced Ca^{2+} release mechanism, but may have little or no effect on the Ca^{2+}-induced Ca^{2+} release pathway.

Accumulation of cyclic AMP in smooth muscle leads to inhibition of contraction by decreasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contractile elements of smooth muscle (De Feo and Morgan, 1989; Tajimi et al., 1991; Chen and Rembold, 1992; Ito et al., 1999). TBG, 10 μM, and DBG, 10 μM, did not change the intracellular cyclic AMP concentration, whereas TBG (30 μM) and DBG (30 μM) slightly increased the cyclic AMP content, indicating that any further inhibition of smooth muscle contraction induced by TBG and DBG at a high concentration is partially due to the cyclic AMP-dependent signaling pathway. In addition, the inhibitory effects of 30 μM TBG and 30 μM DBG on phenylephrine-induced Ca^{2+} release can be explained by an increase in intracellular cyclic AMP content (Abdel-Latif, 1991; Ahn et al., 1992).

In conclusion, we have now demonstrated that TBG and DBG inhibit smooth muscle contraction by inhibiting Ca^{2+} entry, and only at higher concentrations does an increase of intracellular cyclic AMP content also contribute to the inhibitory effects of these compounds.

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