Is Guanosine-5'-triphosphate Involved in Calcium-Activation of Contractile Proteins in Vascular Smooth Muscle?†

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ABSTRACT—Isometric tension was measured to investigate the effects of guanosine-5'-triphosphate (GTP) on the run-down of myofilament Ca2+ sensitivity in isolated rat mesenteric arteries permeabilized with β-escin. The Ca2+ sensitivity assessed by the EC50 value for the Ca2+ (0.1–100 μM)-tension relationship progressively runs down in the control strips, while it was well-preserved for 5-successive Ca2+ applications in the presence of GTP (50 μM); no significant difference was found in the Ca2+ sensitivity observed with the 1st Ca2+ application between the control and GTP-treated strips. Guanosine-5'-(2-thio)diphosphate (GDPpS, 100 μM) significantly decreased the Ca2+ sensitivity with the 1st Ca2+ application and eliminated the run-down of Ca2+ sensitivity. GTP (3–150 μM), applied to the strips submaximally precontracted with Ca2+, had a little effect on the Ca2+ contractions in the early stage of experiments, but dramatically enhanced the Ca2+ contractions in their later stage; its latter effect was mimicked by guanosine-5'-(3-thio) triphosphate (GTPiS) and reversed by GDPpS (100 μM). The results suggest: 1) loss of endogenous GTP following permeabilization is involved in the run-down of Ca2+ sensitivity; and 2) activation of G-proteins is involved in Ca2+-activation of contractile proteins.

Keywords: Guanosine-5'-triphosphate (GTP), GTP-binding protein (G-protein), Myofilament Ca2+ sensitivity, Membrane-permeabilization, Vascular smooth muscle

It is believed that changes in intracellular free Ca2+ concentration ([Ca2+]i) are the principal mechanisms that regulate the contractile state of smooth muscle (1–3). An increase in [Ca2+]i, in response to stimuli leads to formation of the Ca2+-calmodulin complex, which in turn activates myosin light-chain kinase (MLCK) (3). The activated MLCK subsequently phosphorylates the 20-kD regulatory light chain of myosin (MLC20), allowing the myosin ATPase to be activated by actin and the muscle to contract as a result of MgATP-dependent cyclic interactions of myosin with actin (3, 4). On the other hand, a decrease in [Ca2+]i, inactivates MLCK and permits dephosphorylation of MLC20 by myosin light-chain phosphatase (MLCP), thereby inactivating the actomyosin ATPase and causing relaxation (3). However, many investigators have documented dissociation between force levels, [Ca2+]i, and MLC20 phosphorylation levels during maintenance of contraction, proposing the existence of a second regulatory mechanism for smooth muscle contraction (1, 5–8).

The force/[Ca2+]i ratio during contractile responses to receptor agonists has been shown to be much higher than that during contractile responses to high K+ in intact smooth muscle (9–12). In support of this, recent studies have unequivocally demonstrated increases in Ca2+ sensitivity caused by agonists in smooth muscle permeabilized by either staphylococcus aureus α-toxin or β-escin (13–17). These agonist-induced increases in Ca2+ sensitivity were inhibited by guanosine-5'- (2-O-thio) diphosphate (GDPpS), a non-hydrolyzable analogue of guanosine-5'-diphosphate (GDP) that competitively inhibits the binding of guanine nucleotides to G-proteins (18), and mimicked by guanosine-5'-triphosphate (GTP).
or guanosine-5’-(3-O-thio) triphosphate (GTP\textsubscript{1S}), a non-hydrolyzable GTP analogue known as a potent and stable activator of G-proteins (19), suggesting involvement of activation of G-proteins (14, 16, 17, 20, 21).

In preliminary experiments with \(\beta\)-escin-permeabilized smooth muscle designed to confirm the recently-reported GTP-induced increases in Ca\textsuperscript{2+} sensitivity (14, 21), differential effects of GTP on the Ca\textsuperscript{2+} sensitivity were found between the early and later time points after the \(\beta\)-escin-permeabilization, i.e., a very small, if any, effect of GTP on Ca\textsuperscript{2+} sensitivity immediately after the permeabilization, but a significant large enhancing effect of GTP on Ca\textsuperscript{2+} sensitivity at the later time points similar to recent observations (14, 21). In addition, it was noticed that the Ca\textsuperscript{2+} sensitivity is well-preserved in the presence of GTP, but runs down progressively in the absence of GTP. These results implying loss of endogenous GTP with the progress of membrane permeabilization prompted us to investigate the role of GTP in the Ca\textsuperscript{2+}-activation of contractile proteins or the rundown of Ca\textsuperscript{2+} sensitivity after permeabilization.

MATERIALS AND METHODS

Tension measurement experiments

After receiving institutional approval, adult male Sprague-Dawley rats were preoxygenated with 100% \(O_2\) for 2–3 min and anesthetized with halothane (halothane /\(O_2\)). The mesenteric arteries were then exposed, rapidly excised and immediately placed in a dissecting chamber filled with 4-(2-hydroxyethyl) piperazine-l-ethanesulfonic acid (HEPES)-buffered physiological salt solution (PSS). Endothelium-denuded transverse strips (150–200 \(\mu\)m in width, 250–400 \(\mu\)m in length) were prepared from the second or third order branch of the artery (150–200 \(\mu\)m in diameter) with the method described elsewhere (22, 23). The strip was then horizontally mounted in a small chamber (0.9 ml) for tension measurement, and the resting tension was adjusted to obtain a maximal contractile response to high K\textsuperscript{+} as previously described (23, 24). The solution was changed by perfusing it rapidly from one end while aspirating it simultaneously from the other end. Functional removal of the endothelium was confirmed by lack of acetylcholine (10 \(\mu\)M)-induced endothelium-dependent relaxation in each strip. Membrane permeabilization was achieved by incubating the strips with \(\beta\)-escin (50 \(\mu\)M, 22–25 min [see the Results section]) at 22°C in relaxing solution after measuring steady contractions induced by high K\textsuperscript{+}. Ionomycin (0.3 \(\mu\)M) was present throughout the experiments to eliminate the influence of intracellular Ca\textsuperscript{2+} stores as used previously (17, 25).

Solutions

The ionic concentrations of the HEPES-buffered PSS were as follows: 138 mM NaCl, 5.0 mM KCl, 1.2 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 10 mM HEPES and 10 mM glucose. The pH was adjusted with NaOH to 7.35 at 22°C. The high K\textsuperscript{+} solutions were prepared by replacing NaCl with KCl isosmotically.

The composition of relaxing or activating solutions used in the membrane-permeabilized muscle experiments were determined by solving multiequilibrium equations using a hydrogen ion activity coefficient of 0.75 and association constants for the various ions as detailed elsewhere (25). The composition of the relaxing solution was 80 mM potassium methansulfonate (KMS), 20 mM piperazine-1,4-\textit{bis}-(2-ethanesulfonic acid) (PIPES), 7 mM Mg(MS)\(_2\), 5 mM ATP, 10 mM creatinine phosphate (CP) and 4 mM ethyleneglycol-\textit{bis}-(\(\beta\)-aminoethylether) N,N,N,N-tetraacetic acid (EGTA). The 4 mM EGTA-containing activating solution was prepared by adding a specific amount of Ca(MS)\(_2\) to obtain the desired concentration of free Ca\textsuperscript{2+} ions based on the previously reported calculations (25). The pH was adjusted with KOH to 7.00 at 22°C, and the ionic strength was kept constant at 0.2 M by adjusting the concentration of KMS.

Drugs

ATP, CP, EGTA, \(\beta\)-escin, ionomycin, GTP, GDP\textsubscript{\(\beta\)S} and GTP\textsubscript{1S} were obtained from Sigma Chemical Co., St. Louis, MO, USA. The PIPES-K\(_2\) and methanesulfonic acid were obtained from Fluka Chemie AG, Buchs, Switzerland. All other reagents were of the highest grade commercially available.

Calculation and statistical analyses

Data were expressed as the mean±S.E.M. The \(n\) denotes the number of strips (=the number of animals). The concentration-response data for the Ca\textsuperscript{2+}-tension relationship were fitted according to a four parameter logistic model as described by De Lean et al. (26), and the EC\textsubscript{50} (the concentration that produced 50% of the maximal response) values were derived from the least squares fit using the above model. Statistical analyses were performed by one- or two-factor ANOVA with repeated measures and Student’s paired or unpaired \(t\)-test, when appropriate. A \(P\) level of <0.05 was considered significant.

RESULTS

Ca\textsuperscript{2+}-tension relationship in \(\beta\)-escin-permeabilized muscle

Recent studies have shown that \(\beta\)-escin, a saponin analogue, at concentrations of 20–50 \(\mu\)M for 20–30 min

Drugs

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provided satisfactory permeabilization of the smooth
muscle cell membrane (15, 17, 21, 22, 25, 27). Figure 1
shows the Ca\textsuperscript{2+}-tension relationship obtained after ex-
posure to 50 pM \(\beta\)-escin for 22-24 min at 22°C in relax-
ning solution. To obtain this Ca\textsuperscript{2+}-tension relationship,
various concentrations (0.1-100 pM) of Ca\textsuperscript{2+} were cu-
mulatively applied 3 min after washout of \(\beta\)-escin. The
obtained EC\textsubscript{50} value was 1.88 ± 0.09 pM (n = 5), and the
amplitude of Ca\textsuperscript{2+}-activated contractions was normalized
to the 143 mM K\textsuperscript{+}-induced phasic contraction before permeabilization. Each value is a mean ± S.E.M., n = 5.

**Effects of GTP and GDP\textsubscript{S} on the rundown of Ca\textsuperscript{2+}-
tension relationship**

Figure 2A shows the experimental protocols to system-
atically investigate the role of GTP in the rundown of the Ca\textsuperscript{2+} sensitivity. The effects of GTP on the changes in
Ca\textsuperscript{2+} sensitivity following permeabilization (50 μM \(\beta\)-
escin for 25 min) were examined by measuring the Ca\textsuperscript{2+}
(0.1–100 μM)-tension relationship in the presence and absence of GTP or GDP\textsubscript{S} (Fig. 2). The concentration of
GTP, 50 μM, was chosen from its ability to maximally
increase the Ca\textsuperscript{2+} sensitivity, and the concentration of
GDP\textsubscript{S}, 100 μM, was determined from its ability to com-
pletely reverse the maximal effect of GTP (50 μM) on the
Ca\textsuperscript{2+} sensitivity as shown below. The Ca\textsuperscript{2+} sensitivity in
each Ca\textsuperscript{2+} application was assessed after the Ca\textsuperscript{2+}-acti-
vated maximal contraction in each Ca\textsuperscript{2+} application was
normalized to 100%.

As shown in the Fig. 2B, the Ca\textsuperscript{2+} sensitivity progressively ran down in the absence of GTP for the first 4 suc-
cessive Ca\textsuperscript{2+} applications; however, GTP, applied in the
5th Ca\textsuperscript{2+} application, restored the Ca\textsuperscript{2+} sensitivity to the
control Ca\textsuperscript{2+} sensitivity observed in the 1st Ca\textsuperscript{2+} application. The EC\textsubscript{50} value for the Ca\textsuperscript{2+}-tension relationship in
the 1st Ca\textsuperscript{2+} application without GTP and in the 5th
Fig. 2. Effects of GTP and GDPβS on the rundown of Ca²⁺ sensitivity. A: Protocols to examine the effects of GTP or GDPβS on the rundown of the Ca²⁺-tension relation. In protocol #1 (n=6), GTP was applied to the muscle only at the 5th Ca²⁺ application. In protocol #2 (n=6), GTP was present throughout the experiments after measuring the control 30 μM Ca²⁺-induced contraction. In protocol #3 (n=5), GDPβS was present from the 1st to 4th Ca²⁺ applications, and GTP was applied after washout of GDPβS at the 5th Ca²⁺ application. B: Significant decreases in the Ca²⁺ sensitivity were observed at the 2nd-4th Ca²⁺ applications. GTP, applied on the 5th Ca²⁺ application, restored the Ca²⁺ sensitivity. *P<0.05 vs 2nd, 3rd and 4th Ca²⁺ applications; tP<0.05 vs 4th Ca²⁺ application. C: Preservation of the Ca²⁺ sensitivity in the presence of GTP. D: Impairment of the Ca²⁺ sensitivity and lack of the rundown of Ca²⁺ sensitivity in the presence of GDPβS (100 μM). Again, GTP, applied after washout of GDPβS, restored the Ca²⁺ sensitivity. *P<0.05 vs 2nd, 3rd and 4th Ca²⁺ applications. E: Impairment of the Ca²⁺ sensitivity and lack of the rundown of Ca²⁺ sensitivity in the presence of GDPβS (100 μM). Again, GTP, applied after washout of GDPβS, restored the Ca²⁺ sensitivity. *P<0.05 vs 2nd, 3rd and 4th Ca²⁺ applications. E: Rundown of the Ca²⁺-activated maximal contraction, which was not significantly affected by the presence of either GTP or GDPβS. *P<0.05 vs control 30 μM Ca²⁺-induced contraction (see panel A). *P<0.05 vs the previous application within each group. Each value is a mean±S.E.M., n=5-6.
Ca\textsuperscript{2+} application with GTP were 3.61 and 3.08 \(\mu\)M, respectively, and no significant difference was observed in the Ca\textsuperscript{2+}-tension relationship between the 1st and 5th Ca\textsuperscript{2+} applications. Moreover, the Ca\textsuperscript{2+} sensitivity was almost perfectly preserved in the presence of GTP (50 \(\mu\)M) for the 5 successive Ca\textsuperscript{2+} applications, and the obtained EC\textsubscript{50} values were constant (\(\approx 3 \mu\)M) (Fig. 2C); no significant differences were observed in the Ca\textsuperscript{2+}-tension relationship among the 5 Ca\textsuperscript{2+} applications. The Ca\textsuperscript{2+} sensitivity observed in the 1st Ca\textsuperscript{2+} application after exposure to GDP\textsubscript{S} was already impaired with the EC\textsubscript{50} value of 5.57 \(\mu\)M, and the progressive rundown of Ca\textsuperscript{2+} sensitivity was not observed for the 4 successive Ca\textsuperscript{2+} applications, with EC\textsubscript{50} values being constant (\(\approx 6 \mu\)M) (Fig. 2D); no significant differences were observed in the Ca\textsuperscript{2+-tension relationship among the 4 Ca\textsuperscript{2+} applications. However, application of GTP after washout of GDP\textsubscript{S} in the 5th Ca\textsuperscript{2+} application restored the Ca\textsuperscript{2+} sensitivity to the Ca\textsuperscript{2+} sensitivity observed in the 1st and 5th Ca\textsuperscript{2+} applications in the control group or that observed in the

Fig. 3. Effects of GTP and GDP\textsubscript{S} on pCa-tension relation at each Ca\textsuperscript{2+} application. A: The Ca\textsuperscript{2+} sensitivity was already impaired in the presence of GDP\textsubscript{S} at the 1st Ca\textsuperscript{2+} application. No significant differences were observed in the Ca\textsuperscript{2+} sensitivity between the 1st and 5th Ca\textsuperscript{2+} applications. The Ca\textsuperscript{2+} sensitivity observed in the 1st Ca\textsuperscript{2+} application after exposure to GDP\textsubscript{S} was already impaired with the EC\textsubscript{50} value of 5.57 \(\mu\)M, and the progressive rundown of Ca\textsuperscript{2+} sensitivity was not observed for the 4 successive Ca\textsuperscript{2+} applications, with EC\textsubscript{50} values being constant (\(\approx 6 \mu\)M) (Fig. 2D); no significant differences were observed in the Ca\textsuperscript{2+}-tension relationship among the 4 Ca\textsuperscript{2+} applications. However, application of GTP after washout of GDP\textsubscript{S} in the 5th Ca\textsuperscript{2+} application restored the Ca\textsuperscript{2+} sensitivity to the Ca\textsuperscript{2+} sensitivity observed in the 1st and 5th Ca\textsuperscript{2+} applications in the control group or that observed in the
GTP-treated group. These results indicate that GTP is effective in preventing the rundown of Ca$^{2+}$ sensitivity. However, GTP was not effective in preventing the rundown of the "maximal" Ca$^{2+}$-activated contraction; as shown in Fig. 2E, neither GTP nor GDP$\gamma$S significantly affected the rundown of the Ca$^{2+}$-activated maximal contraction.

Figure 3 compares the Ca$^{2+}$ sensitivity at each Ca$^{2+}$ application among the control, GTP-treated and GDP$\gamma$S-treated groups, in which protocols #1, #2 and #3 (Fig. 2A) were used, respectively. No significant difference was observed in the Ca$^{2+}$ sensitivity at the 1st Ca$^{2+}$ application between the control (GTP-untreated) and GTP-treated groups; however, the Ca$^{2+}$ sensitivity at the...
1st Ca²⁺ application in the GDP;βS-treated group was significantly decreased as compared to both the control and GTP-treated groups (by 2-factor ANOVA with repeated measures) (Fig. 3A). In the 2nd, 3rd and 4th Ca²⁺ applications, no significant differences were observed in the Ca²⁺ sensitivity between the control and GDP;βS-treated groups, while the Ca²⁺ sensitivity in both the control and GDP;βS-treated groups were significantly decreased as compared to that in the GTP-treated group (Fig. 3: B-D). At the 5th Ca²⁺ application, in which GTP was present in all three groups, no significant difference was observed in the Ca²⁺ sensitivity among the three groups (Fig. 3E).

Differential effects of GTP on Ca²⁺ sensitivity between early and late stages of experiments and similarity in the effects between GTP and GTP;βS

Figure 4 demonstrates differential effects of GTP on the Ca²⁺ sensitivity between early and later time points after washout of β-escin (50 µM, 25 min). GTP was applied to the strips after 3 µM (≈EC₅₀) Ca²⁺-induced contraction had reached a steady state (10 min after Ca²⁺ application) in either the early (3 min after washout of β-escin) or later (10 min after washout of the 1st 3 µM Ca²⁺) stage of the experiments. As shown in Fig. 4 (B and C), GTP (3–150 µM), applied in the early stage, had little apparent effect on the maintenance of 3 µM Ca²⁺-induced contraction; however, comparison of the data with the time control data (Fig. 4A) revealed slight (at most ≈15%), but significant increases in Ca²⁺ contraction by GTP in the early stage (Fig. 5). In contrast, GTP (3–150 µM), applied at later time points, distinctly increased the Ca²⁺ contraction (Fig. 4C), and comparison of the data with the time control data revealed significant increases (≈75%) in Ca²⁺ contraction in the late stage (Fig. 5).

To examine if GTP;βS affects the Ca²⁺ sensitivity like GTP, GTP;βS was applied to the strips in the later time points. GTP;βS concentration (3–150 µM)-dependently increased the Ca²⁺ contraction (Fig. 4D); the maximum increase in Ca²⁺ contraction obtained with GTP;βS was significantly larger than that by GTP (Fig. 5). When GTP (10–150 µM) was applied after the Ca²⁺ contraction had been increased by 3 µM GTP;βS (i.e., to the same level as the Ca²⁺ contraction was maximally increased by GTP in prior experiments), GTP failed to further increase the Ca²⁺ sensitivity in the presence of 3 µM GTP;βS (Figs. 4E and 5). This suggests that the mechanism behind the observed GTP;βS-induced increases in Ca²⁺ contraction is identical to that behind the GTP-induced increases in Ca²⁺ contraction.

Consistent with the above results showing the lack of effect of GTP on the Ca²⁺-activated maximal contraction

![Fig. 5. Effects of GTP, GTP;βS or GTP;βS + GTP on the 3 µM (submaximal) Ca²⁺-induced contractions at either the early or late stage of the experiments. The original traces were shown in the Fig. 4. In the control #1 group, neither GTP nor GTP;βS was applied in either stage (see Fig. 4A). In all other groups, GTP (3–150 µM) was cumulatively applied in the early stage; as shown in the Fig. 4, B–E, 3, 10, 50 and 150 µM GTP were applied 10, 13, 16, 19 min after application of the 3 µM Ca²⁺, respectively. In the control #2 group, neither GTP nor GTP;βS was applied in the late stage, while GTP (3–150 µM), GTP;βS (3–100 µM) and GTP;βS (3 µM) + GTP (10–150 µM) were applied in the late stage in the groups #1, #2 and #3, respectively (see Fig. 4: B–E). Each value is a mean ± S.E.M., n=4. left panel: Effects of GTP on the submaximal Ca²⁺ contraction at the early stage. GTP only slightly, although significantly, enhanced the contraction. No significant differences were observed in the effects of GTP among all the groups in which GTP was applied. *P<0.05 vs the GTP-treated groups (control #2 group and groups #1–3). right panel: Effects of GTP or GTP;βS on the Ca²⁺ contraction at the late stage. Please note the much more distinct and larger enhancing effects of GTP on the Ca²⁺ contraction at the late stage as compared to those at the early stage. *P<0.05 vs Groups #1–3, *P<0.05 vs Groups #2 and #3, *P<0.05 vs Group #2.}
(Fig. 2E), GTP (up to 150 μM) did not have a significant effect on the 30 μM Ca2+-induced maximal contraction in both early and late stages of the experiments (Fig. 6).

Finally, the GTP (50 μM)-induced maximal increase in Ca2+ sensitivity at the later time points were completely reversed by GDPΔS (100 μM) (n=5) (Fig. 7A), while the GTPγS (3 μM)-induced increase in Ca2+ sensitivity in the later time points were not significantly affected even with 1 mM GDPΔS (n=3) (Fig. 7B).

**Effects of receptor stimulation on Ca2+ sensitivity in the presence of GTP**

Phenylephrine (PE, 10–100 μM), applied after Ca2+ (3 μM)-induced contraction reached steady state, failed to increase Ca2+ sensitivity in the presence of 50 μM GTP in either the early or late stage of experiments (n=33). In addition, PE did not evoke any contraction in the presence of GTP (50 μM) in Ca2+-free (0.05 mM EGTA-buffered, pCa 7.17) solution after the intracellular stores were loaded with Ca2+ at pCa 6.5 for 5–8 min in the ionomycin-untreated strips (n=17), in which both caffeine and inositol 1,4,5-trisphosphate (IP3) constantly evokes contraction in Ca2+-free solution as we previously demonstrated (28). These results suggest that receptor-coupling was not retained in the β-escin-permeabilized strips used in this study. Perhaps, the concentration of β-escin used in this study (50 μM) might have been too high to retain receptor coupling.

**Fig. 6.** Effects of GTP on Ca2+-induced maximal contraction in either the early or late stage of the experiments. A: Control 30 μM Ca2+-induced contraction. 1= Control injection of 30 μM Ca2+ solution without GTP. B: Effects of GTP on the 30 μM Ca2+-induced contraction in both early and late stages of experiments. C: No significant differences were observed in tension development between the control and GTP groups. Each value is a mean±S.E.M., n=4.
Fig. 7. Effects of GDP\(\beta\)S on either GTP (A) or GTP\(\gamma\)S (B)-induced increases in Ca\(^{2+}\) sensitivity in the later time points. The \(\beta\)-escin-permeabilized strips were submaximally precontracted with 3 \(\mu\)M Ca\(^{2+}\) before application of GTP or GTP\(\gamma\)S. The GTP-induced increase in Ca\(^{2+}\) sensitivity was completely reversed by 100 \(\mu\)M GDP\(\beta\)S (A), while the increase in Ca\(^{2+}\) sensitivity caused by GTP\(\gamma\)S (3 \(\mu\)M) was not affected even by 1 mM GDP\(\beta\)S (B). Identical results were obtained in several other strips (n = 3–5).

**DISCUSSION**

Both the minimal effect of GTP on the Ca\(^{2+}\)-sensitivity and the significantly inhibited Ca\(^{2+}\) sensitivity in the GDP\(\beta\)S-treated muscle observed in the earlier time points after washout of \(\beta\)-escin suggest that GTP is involved in contractile response to a rise in [Ca\(^{2+}\)], through activation of G-proteins under the \(\beta\)-escin-permeabilized condition. The rundown of Ca\(^{2+}\) sensitivity in the absence of GTP, the well-preserved Ca\(^{2+}\) sensitivity in the presence of GTP, the lack of the rundown of Ca\(^{2+}\) sensitivity with impaired EC\(_{50}\) values in the presence of GDP\(\beta\)S, and the distinct enhancing effects of GTP on Ca\(^{2+}\) sensitivity in the later time points (i.e., larger than those in the earlier time points) all suggest that endogenous GTP is being lost possibly by leaking out or being consumed following or in the progress of membrane-permeabilization. The enhancing effect of GTP on Ca\(^{2+}\) sensitivity was reversed by GDP\(\beta\)S, a hydrolysis-resistant guanine nucleotide known to competitively inhibit G-protein activation by binding to the \(\alpha\)-subunit of G-proteins (18), and the effect was mimicked by GTP\(\gamma\)S, a nonhydrolyzable guanine nucleotide, an irreversible activator of all types of G-proteins. Furthermore, no additional effect with GTP was observed after the Ca\(^{2+}\) sensitivity had already been increased by GTP\(\gamma\)S to the same level as that of the Ca\(^{2+}\) sensitivity maximally increaseable by GTP. These results also suggest that the observed effect of GTP on Ca\(^{2+}\) sensitivity is due to activation of G-proteins, and these results rule out the possibility that energy derived from hydrolysis of GTP, a high energy phosphate, is required for the effect of GTP on the Ca\(^{2+}\)-activated contractions.

It is believed that receptor activation by the Ca\(^{2+}\) sensitizing agonists activates heterotrimeric (\(\alpha\beta\gamma\)) G-protein by catalyzing the exchange of GDP bound to the \(\alpha\)-subunit of the G-protein for GTP (19), activating phospholipase C and thereby promoting production of IP\(_{3}\) and diacylglycerol from hydrolysis of phosphatidylinositol 4,5-bisphosphate. Previous studies in membrane-permeabilized muscle have shown that the agonist-induced Ca\(^{2+}\) sensitization requires the presence of GTP, is mimicked by GTP\(\gamma\)S, and is inhibited by GDP\(\beta\)S, indicating that the agonist-induced Ca\(^{2+}\) sensitization is mediated through activation of G-proteins, presumably the heterotrimeric G-protein (14–17, 20). In addition to the heterotrimeric G-protein, a large number of proteins that bind and hydrolyze GTP such as Rho- or Ras proteins have recently been discovered (29), and some recent studies have proposed that activation of these small G-proteins increases the Ca\(^{2+}\) sensitivity of contractile proteins and/or MLC phosphorylation in smooth muscle and is possibly involved in the agonist-induced Ca\(^{2+}\) sensitization (21, 30–32). Our results suggest that GTP is involved in Ca\(^{2+}\)-activation of contractile proteins through activation of some G-proteins independent of agonist-induced Ca\(^{2+}\) sensitization. Since receptor stimulation failed to further increase the Ca\(^{2+}\) sensitivity in the presence of GTP, there was no evidence that receptor-coupling was retained, and thus the G-proteins specifically involved in the receptor-mediated responses may not be activated as a result of the receptor stimulation in our \(\beta\)-escin-permeabilized muscle. Therefore, our data do not conflict with the widely accepted idea that activation of G-proteins is involved in the agonist-induced Ca\(^{2+}\) sensitization. Another potential explanation for the observed effects of GTP would be that certain G-proteins, irrelevant to the
Ca\textsuperscript{2+}-activation of contractile proteins under physiological conditions, had been constitutively activated as a result of the unphysiological intervention, i.e., membrane-permeabilization with \( \beta \)-escin. Further investigations would be needed to identify the G-proteins involved in activation of contractile proteins in response to a rise in \([\text{Ca}\textsuperscript{2+}])

Membrane-permeabilization of smooth muscle has been considered a valuable tool to investigate the mechanisms of smooth muscle contraction and/or relaxation by directly controlling cytoplasmic solute composition and by utilizing cell membrane-impermeable pharmacological agents. However, one limitation of this method, as has been recognized among investigators (3, 17, 33, 34), is the loss of important diffusible endogeneous cofactors or regulators of contractile proteins, which could result in the impaired Ca\textsuperscript{2+} sensitivity or the early rundown of Ca\textsuperscript{2+} sensitivity. A recent study comparing Ca\textsuperscript{2+} sensitivity obtained by simultaneous measurement of \([\text{Ca}\textsuperscript{2+}])\textsuperscript{2+} and force in fura-2-loaded intact cardiac muscle with that obtained in Triton X-permeabilized muscle has implied that membrane-permeabilization may impair myofilament Ca\textsuperscript{2+} sensitivity, although this proposal was based on the assumed validity regarding the employed in vivo calibration of fura-2 and the calculation of free Ca\textsuperscript{2+} ionic concentrations contained in solutions used in the membrane-permeabilized muscle experiments (34). The present study has demonstrated that GTP is quite effective in preventing the rundown of myofilament Ca\textsuperscript{2+} sensitivity following permeabilization, suggesting that GTP (MW \( \approx 512 \)) is an important endogenous factor essential for activation of contractile proteins by Ca\textsuperscript{2+}. The solutions to obtain Ca\textsuperscript{2+}-activated contraction which have long been used in membrane-permeabilized muscle experiments normally contain Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, K\textsuperscript{+}, EGTA, ATP, CrP and a certain buffer such as PIPES or HEPES, but not GTP. We would propose to consider adding GTP to the solutions to obtain Ca\textsuperscript{2+}-activated contraction to prevent the rundown of Ca\textsuperscript{2+} sensitivity. Previously reported mean concentrations of total GTP in intracellular water of smooth muscle cells have been 120 \( \mu \)M in bladder (35), 180 \( \mu \)M in the uterus (35) and 270–290 \( \mu \)M in the aorta (36), although the fraction of total cellular GTP that is bound is currently unknown (36). It is also known that most G-proteins exhibit some basal turnover that is independent of receptor stimulation (19).

The present study has demonstrated that the time stage of membrane-permeabilized muscle experiments can significantly affect the effect of agents (e.g., GTP), suggesting the importance of careful consideration of time points when the experiments are carried out following the permeabilization before interpreting the data. The early stage of the present experiments when GTP failed to exert a distinct effect on the Ca\textsuperscript{2+} sensitivity was probably a non-steady state in which the membrane-permeabilization was still ongoing and not yet completed.

Although GTP was effective in preventing the rundown of Ca\textsuperscript{2+} sensitivity assessed after the Ca\textsuperscript{2+}-induced maximal contraction at each Ca\textsuperscript{2+} application was normalized to 100\%, it was not effective in preventing the rundown of the Ca\textsuperscript{2+}-activated maximal contraction. The loss of other important components or regulators of contractile proteins, desensitization to Ca\textsuperscript{2+} or deterioration of muscle due to activation of proteases might possibly be involved in this rundown. It was previously reported that contractions of \( \beta \)-escin-permeabilized muscle were affected by the presence of calmodulin (17-kD), implying that calmodulin could leak out of \( \beta \)-escin-permeabilized muscle and that its loss might contribute to the rundown of the Ca\textsuperscript{2+} sensitivity (15). In support of this, a recent study proposed that permeabilization of smooth muscle with \( \beta \)-escin allows the transmembrane passage of 150-kD proteins (37). In addition, some of previous studies have used leupeptin, a protease inhibitor, to prevent the early deterioration of muscle (15, 21). Further investigations would be necessary to clarify the mechanisms of the rundown of the Ca\textsuperscript{2+}-activated "maximal" contraction utilizing calmodulin or leupeptin. The lack of effect of GTP on the Ca\textsuperscript{2+}-activated "maximal" contraction observed in this study appears to be consistent with previous reports showing the lack of effect of GTP\textsuperscript{3S} on the Ca\textsuperscript{2+}-activated maximal contraction (21, 30).

In conclusion, this study has demonstrated that GTP is effective in preventing the rundown of myofilament Ca\textsuperscript{2+} sensitivity in \( \beta \)-escin-membrane-permeabilized vascular smooth muscle. In addition, GTP has only a minimal effect on contractile response to Ca\textsuperscript{2+} in the early time points after permeabilization, but it has a dramatic increasing effect on contractile response to Ca\textsuperscript{2+} at the later time points. The effect of GTP observed in the later time points was mimicked by GTP\textsuperscript{3S}, and reversed by GDP\textsuperscript{3S}. These results suggest that loss of endogenous GTP following membrane-permeabilization is involved in the rundown of the Ca\textsuperscript{2+} sensitivity, and that activation of G-proteins is involved in Ca\textsuperscript{2+}-activation of contractile proteins under the \( \beta \)-escin-membrane-permeabilized condition.

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REFERENCES

1 Kamm KE and Stull JT: The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Annu Rev Pharmacol Toxicol 25, 593–620 (1985)

2 Itoh T, Ikebe M, Kargacin GJ, Hartshorne DJ, Kemp BE and Fay FS: Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. Nature 338, 164–167 (1989)

3 Somlyo AP and Somlyo AV: Signal transduction and regulation in smooth muscle. Nature 372, 231–236 (1994)

4 Kamm K and Stull J: Regulation of smooth muscle contractile elements by second messengers. Annu Rev Physiol 59, 299–313 (1997)

5 Dillon PF, Aksoy MO, Driska SP and Murohy RA: Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science 211, 495–497 (1981)

6 Morgan JP and Morgan KG: Vascular smooth muscle: the first recorded Ca2+ transient. Pflugers Arch 395, 75–77 (1982)

7 Hai C-M and Murphy RA: Ca2+, crossbridge, phosphorylation, and contraction (review). Annu Rev Physiol 51, 285–298 (1989)

8 Morgan KG, Khalil RA, Suematsu E and Katsuyama H: Calcium-dependent and calcium-independent pathways of signal transduction in smooth muscle. Jpn J Pharmacol 58, Suppl II, 47–53 (1992)

9 Bradley AB and Morgan KG: Alteration in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. J Physiol (Lond) 385, 437–448 (1987)

10 Rembold CM and Murphy RA: Myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. Circ Res 63, 593–603 (1988)

11 Sato K, Ozaki H and Karaki H: Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent indicator fura-2. J Pharmacol Exp Ther 246, 294–300 (1988)

12 Himpens B, Kitazawa T and Somlyo AP: Agonist-dependent modulation of Ca2+ sensitivity in rabbit pulmonary artery smooth muscle. Pflugers Arch 417, 21–28 (1990)

13 Nishimura J, Kolber M and van Breemen C: Norepinephrine and GTPγS increase myofilament Ca2+ sensitivity in α-toxin-permeabilized arterial smooth muscle. Biochem Biophys Res Commun 157, 677–683 (1988)

14 Kitazawa T, Kobayashi S, Horiiuchi K, Somlyo AV and Somlyo AP: Receptor-coupled, permeabilized smooth muscle. J Biol Chem 264, 5339–5342 (1989)

15 Kobayashi S, Kitazawa T, Somlyo AV and Somlyo AP: Cytosolic heparin inhibits muscarinic and alpha-adrenergic Ca2+ release in smooth muscle. J Biol Chem 264, 17997–18004 (1989)

16 Nishimura J, Moreland S, Ahn H, Kawase T, Moreland R and van Breemen C: Endothelin increases myofilament Ca2+ sensitivity in α-toxin-permeabilized rabbit mesenteric artery. Circ Res 71, 951–959 (1992)

17 Yoshida M, Suzuki A and Itoh T: Mechanism of vasoconstriction induced by endothelin-1 in smooth muscle of rabbit mesenteric artery. J Physiol (Lond) 477, 253–265 (1994)

18 Eckstein F, Cassel D, Levkoviz H, Lowe M and Selinger Z: Guanosine 5′-O-(2-thiodiphosphate). J Biol Chem 254, 9829–9834 (1979)

19 Andrade R: Infusion of guanine nucleotides through recording electrodes for studies on G-proteins of ion currents and channels. In Heterotrimeric G-protein Effectors, Edited by Iyengar R, pp 348–356, Academic Press, Inc, San Diego (1994)

20 Nishimura J, Khalil RA and van Breemen C: Agonist-induced vascular tone. Hypertension 13, 835–844 (1989)

21 Satoh S, Rensland H and Pfizer G: Ras proteins increase Ca2+-responsiveness of smooth muscle contraction. FEBS Lett 324, 211–215 (1993)

22 Akata T, Yoshitake J, Nakashima M and Itoh T: Effects of promazine on vascular smooth muscle of rabbit mesenteric artery. Anesthesiology 75, 833–846 (1991)

23 Akata T, Kodama K and Takahashi S: Effects of heparin on the vasodilator action of promazine in the rabbit mesenteric artery. Br J Pharmacol 109, 1247–1253 (1993)

24 Akata T, Nakashima M, Kodama K, Boyle III WA and Takahashi S: Effects of volatile anesthetics on ACh-induced relaxation in the rabbit mesenteric resistance artery. Anesthesiology 82, 188–204 (1995)

25 Akata T and Boyle III WA: Volatile anesthetic actions on contractile proteins in membrane-permeabilized small mesenteric arteries. Anesthesiology 82, 700–712 (1995)

26 De Lean AP, Munson PJ and Rodbard D: Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235, E97–E102 (1978)

27 Ito S, Kajikuri J, Itoh T and Kuriyama H: Effects of lemakalim on changes in Ca2+ concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. Br J Pharmacol 104, 227–233 (1991)

28 Akata T and Boyle III WA: Dual actions of halothane on intracellular calcium stores of vascular smooth muscle. Anesthesiology 84, 580–595 (1996)

29 Bourne HR, Sanders DA and McCormick F: The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125–132 (1990)

30 Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibichi K, Matsura Y, Seki H, Saika K and Takai Y: Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem 267, 8719–8722 (1992)

31 Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Fung J, Nakano T, Okawa K, Iwamatsu A and Kaibuchi K: Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245–248 (1996)

32 Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsura Y and Kaibuchi K: Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271, 20246–20249 (1996)

33 Itoh T, Suzuoka A and Watanabe Y: Effect of a peptide inhibitor of protein kinase C on G-protein-mediated increase in myofilament Ca2+-sensitivity in rabbit arterial wall muscle. Br J Pharmacol 111, 311–317 (1994)

34 Gao W, Backx P, Azan-Backx M and Marban E: Myofilament...
Ca$^{2+}$ sensitivity in intact versus skinned rat ventricular muscle. 
Circ Res 74, 408–415 (1994)

35 Kushmerick MJ, Dillon PF, Meyer RA, Brown TR, Krisanda JM and Sweeney HL: $^{31}$P NMR spectroscopy, chemical analysis, and free Mg$^{2+}$ of rabbit bladder and uterine smooth muscle. J Biol Chem 261, 14420–14429 (1986)

36 Cobrun RF, Azim S, Fillers WS and Baron CB: Smooth muscle guanine nucleotides and receptor-effector coupling following inhibition of oxidative energy production. Am J Physiol 264 (Lung Cell Mol Physiol 8), L1–L6 (1993)

37 Izuka K, Ikebe M, Somlyo AV and Somlyo AP: Introduction of high molecular weight (IgG) proteins into receptor coupled, permeabilized smooth muscle. Cell Calcium 16, 431–445 (1994)