Agonist-Induced Vascular Tone

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The cellular mechanisms underlying the agonist-induced sustained contraction of the vascular smooth muscle are reviewed in the light of the use of Ca\(^{2+}\) and the change of Ca\(^{2+}\) sensitivity of the contractile apparatus. It is generally accepted that the main trigger for contraction of vascular smooth muscle is the elevation of intracellular Ca\(^{2+}\) concentration. However, the measurement of intracellular Ca\(^{2+}\) concentration during the sustained phase of agonist-induced contraction is reported to be lower than that of high K\(^+\) stimulation or the value obtained by the experiments with chemically skinned smooth muscle preparations. These observations indicate that a second regulatory system may exist. One possible mechanism is the effectiveness of Ca\(^{2+}\) use. Agonist-induced Ca\(^{2+}\) influx may be more effective in raising the intracellular Ca\(^{2+}\) in the bulk of the cytoplasm than is Ca\(^{2+}\) entry induced by depolarization by the inhibition of a putative sarcoplasmic reticulum buffer barrier. Another possibility is the change of Ca\(^{2+}\) sensitivity of the contractile apparatus. Although the survey of the recent literature concerning the phorbol ester-induced vasoconstriction tends to support a role for protein kinase C in the change of Ca\(^{2+}\) sensitivity of the contractile proteins, it fails to establish a clear link between receptors, protein kinase C, and myofilaments. By using new methods for permeabilizing smooth muscle fibers, which retain the function of receptors and signal transduction systems, we now provide direct evidence that the activation of G protein by norepinephrine or guanosine 5\'-0-(3-triphosphate) (GTP-\(\gamma\)-S), nonhydrolyzable GTP analogue, enhances myofilament sensitivity to Ca\(^{2+}\). (Hypertension 1989;13:835-844)

Elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) is the main trigger for contraction of smooth muscle. The [Ca\(^{2+}\)\(_i\)], is regulated by the fluxes of Ca\(^{2+}\) between three anatomic compartments, the extracellular space (ECS), cytoplasm, and sarcoplasmic reticulum (SR). The following mechanisms mediate these Ca\(^{2+}\) flows: Ca\(^{2+}\) enters the cells passively through 1) a sizable Ca\(^{2+}\) leak that may be the simple consequence of disruption of the bimolecular lipid leaflet by a wide variety of functional membrane proteins, 2) voltage-gated channels (VGC) of which two types, "L" and "T", have been identified, and 3) receptor-operated channels (ROC), one of which has been identified electrophysiologically. The reverse flow of Ca\(^{2+}\) from the cytoplasm to the ECS is driven by adenosine triphosphate (ATP) hydrolysis through a 130 kDa calmodulin and cyclic guanosine monophosphate (cGMP)–stimulated Ca\(^{2+}\),Mg\(^{2+}\)-adenosine triphosphatase (ATPase) and to a variable extended by the inward Na\(^+\) gradient through the Na\(^+\),Ca\(^{2+}\) antiporter. The situation in the SR is analogous to the plasma membrane in that three passive pathways exist for Ca\(^{2+}\) release into the cytoplasm: 1) a passive leak, 2) inositol 1,4,5-trisphosphate (IP\(_3\))-activated channels, and 3) Ca\(^{2+}\)-activated channels. Ca\(^{2+}\) entry into the SR is energetically uphill and affected by a 100 kDa Ca\(^{2+}\),Mg\(^{2+}\)-ATPase, which is stimulated by cyclic adenosine monophosphate (cAMP) and cGMP.

Of these 10 transport mechanisms, only the L type VGC are directly modulated therapeutically for the control of hypertension. The ROC and Ca\(^{2+}\) pumps are affected indirectly through receptor agonists and antagonists. Several reviews have been written recently concerning the above transport mechanisms. For this reason the present study will address the specific question: how do agonists affect extracellular Ca\(^{2+}\)-dependent vascular tone?

The main difference between depolarization and agonist-induced activation appears to be related to the activation of membrane bound enzymes, which are stimulated by the latter but not the former. The consequences of this appear to be twofold: 1) Agonist-induced influx is more effective in raising the [Ca\(^{2+}\)] in the bulk of the cytoplasm than is Ca\(^{2+}\) entry induced by depolarization per se and 2) agonists enhance the sensitivity of the myofilaments to Ca\(^{2+}\), whereas this probably does not occur if the smooth muscle cells are activated solely by depolarization.
The hypothesis of this study is that, to varying degrees, both these mechanisms contribute to more tension development per unit Ca\(^{2+}\) influx during tonic agonist-induced contraction than during high K\(^{+}\)-induced depolarization.

**Superficial Buffer Barrier**

Besides increasing Ca\(^{2+}\) entry through ROC and VGC, agonists may increase the effectiveness of Ca\(^{2+}\) influx to induce smooth muscle contraction by inhibition of the putative SR Ca\(^{2+}\) buffer barrier.\(^{11}\) According to the buffer barrier hypothesis, the superficial SR will take up Ca\(^{2+}\) as it enters the smooth muscle cell and then release it preferentially toward the plasmalemma to be extruded via the plasmalemmal Ca\(^{2+}\) pump. This hypothetical model predicts that 1) the proportion of Ca\(^{2+}\) entering across the surface membrane and taken up by the superficial SR is crucial in determining the threshold value of Ca\(^{2+}\) influx that activates tension, 2) the state of the SR with respect to its permeability to Ca\(^{2+}\) and its rate of Ca\(^{2+}\) ATPase activity partly determines the steady-state [Ca\(^{2+}\)]\(_i\), and 3) a variable outwardly directed Ca\(^{2+}\) gradient exists in a narrow region (in the order of tenths of nanometers) just underneath the plasmalemma.

In the last decade a growing body of evidence has been accumulating to support this model. Earlier studies were based on measurements of tension and \(^{45}\)Ca influx. One of these studies has shown that when a certain amount of Ca\(^{2+}\) is introduced rapidly into the smooth muscle cell in a short period of time, it produces a faster and larger contraction than when the same net quantity of Ca\(^{2+}\) enters more slowly over a long period of time.\(^{12}\) It has also been found that as much as 200 \(\mu\)mol Ca\(^{2+}\) may be transferred from the extracellular space to the SR within minutes without causing contraction.\(^{13}\) Thus, the magnitude of contraction depends on the rate of net cellular Ca\(^{2+}\) gain rather than on the magnitude of this gain.\(^{12}\) These observations were reinforced by the finding that depletion of the SR Ca\(^{2+}\) by exposing aortic rings bathed in Ca\(^{2+}\)-free solution to maximal concentrations of norepinephrine (NE) could diminish the ability of a subsequent high K\(^{+}\)-stimulated Ca\(^{2+}\) influx to activate tension. It was found that high K\(^{+}\) depolarization could then induce tension only if the NE-releasable Ca\(^{2+}\) store or the SR was refilled to near its physiological resting level. In other words, tension development derived from activation of VGC is dependent on the state of loading of the SR.\(^{14}\)

In another study, a correlation was made between \(^{45}\)Ca influx and tension measured in rabbit aorta under various conditions to estimate the threshold Ca\(^{2+}\) influx that initiates smooth muscle contraction.\(^{15}\) It was concluded that under resting conditions Ca\(^{2+}\) entry through the leak (approximately 14 \(\mu\)mol/kg aorta) would be buffered by the SR and plasmalemma Ca\(^{2+}\), Mg\(^{2+}\)-ATPase, and therefore no smooth muscle contraction would be observed. On smooth muscle depolarization, the opening of VGC enhances Ca\(^{2+}\) entry to an extent that exceeds the Ca\(^{2+}\) influx threshold and activates the myofilaments. Conversely, stimulation of the SR Ca\(^{2+}\) pump by cAMP enhances the effectiveness of the buffer barrier to accumulate a greater portion of Ca\(^{2+}\) entry and shifts the Ca\(^{2+}\) influx threshold for tension development to higher values.\(^{15}\) On the other hand, a reduction in Ca\(^{2+}\) influx threshold is demonstrated when the SR is prevented from accumulating Ca\(^{2+}\) by the continuous presence of the agonist. However, these latter data could also be explained by changes in the myofilaments' sensitivity to Ca\(^{2+}\) (e.g., its decrease by cAMP and its increase by agonists as described below).

Recently, electrophysiological studies have provided evidence to support the existence of a Ca\(^{2+}\) gradient underneath the plasmalemma. It has been found that Ca\(^{2+}\)-sensitive K\(^{+}\) channels of smooth muscle plasmalemma may show spontaneous activation while no contractile activity is stimulated.\(^{16}\) A subplasmalemmal Ca\(^{2+}\) gradient attributed to SR Ca\(^{2+}\) uptake has also been suggested by Rembold and Murphy.\(^{17}\) They have observed that Ca\(^{2+}\) depletion of the SR by repetitive agonist applications in Ca\(^{2+}\)-free solution causes temporal dissociation of Ca\(^{2+}\)-induced aequorin luminescence from myosin light chain phosphorylation and smooth muscle tension when Ca\(^{2+}\) influx is stimulated by readdition of Ca\(^{2+}\).

Inasmuch as agonist activation involves many Ca\(^{2+}\) regulatory mechanisms, a more direct way to establish that the SR can function to extrude Ca\(^{2+}\) from the cytoplasm under steady-state conditions would be to test an agent that selectively modulates the capacity of the SR to accumulate Ca\(^{2+}\). Although caffeine is potent in releasing the SR Ca\(^{2+}\), it also inhibits cAMP phosphodiesterase\(^{18}\) and Ca\(^{2+}\) influx.\(^{19}\) Ryanodine, on the other hand, releases Ca\(^{2+}\) efficiently from the SR but does not affect Ca\(^{2+}\) influx\(^{20}\) nor Ca\(^{2+}\) extrusion by the plasmalemmal Ca\(^{2+}\), Mg\(^{2+}\)-ATPase.\(^{21}\) Although ryanodine induces Ca\(^{2+}\) release from the smooth muscle SR, it does not cause contraction by itself but enhances the tonic depolarization-induced tension.\(^{20}\) This effect was explained as ryanodine interference with the SR Ca\(^{2+}\)-buffering process, thus allowing a greater proportion of the depolarization-stimulated Ca\(^{2+}\) influx to activate the myofilaments. In a recent study the ryanodine-impaired SR function, as measured by the inhibition in caffeine-induced tension, was correlated directly with [Ca\(^{2+}\)]\(_i\) as measured by the fluorescent Ca\(^{2+}\) indicator fura-2 (R.A. Khalil and C. van Breemen, unpublished observations). In the presence of external Ca\(^{2+}\), ryanodine (30 \(\mu\)M) caused a maintained increase in [Ca\(^{2+}\)]\(_i\) of fura-2–loaded rabbit inferior vena cava (Figure 1, upper trace). Parallel tension measurements showed that ryanodine caused a time-dependent inhibition of caffeine-induced contraction. Ryanodine inhibited both the peak tension and the area under the tension curve,
FIGURE 1. Effect of ryanodine on intracellular calcium concentration ([Ca^{2+}]_{i}) and sarcoplasmic reticulum (SR) Ca^{2+} stores of rabbit inferior vena cava. Upper panel, ryanodine (30 µM) was applied to fura-2-loaded tissue and changes in fluorescence were monitored for 30 minutes in physiological saline solution (PSS). After subtraction of autofluorescence, changes in 340/380 ratio were converted into corresponding levels of [Ca^{2+}]_{i}. Lower panel, rings of rabbit inferior vena cava were equilibrated for 1 hour in PSS. After a 25 mM caffeine control contraction, tissue was allowed to recover and SR Ca^{2+} stores were allowed to replenish for 1 hour. Tissue was preincubated with ryanodine (30 µM) for specified periods of time and then challenged with a mixture of 25 mM caffeine and 30 µM ryanodine. Remaining caffeine contractile response was represented as percent of caffeine control contraction.

strongly suggesting that it decreased the SR Ca^{2+} content to a new steady-state level of 50% in 20 minutes (Figure 1, lower panel). The ryanodine-induced increase in [Ca^{2+}], was not due to stimulation of Ca^{2+} influx through VGC or ROC, because it was not inhibited by a high concentration of the Ca^{2+} channel blocker diltiazem. The steady-state increase in [Ca^{2+}], by ryanodine was dependent on Ca^{2+} influx. It was abolished when Ca^{2+} influx was blocked by La^{3+} and was enhanced when Ca^{2+} influx was stimulated by 20 mM K^{+}. These results indicate that the ryanodine-induced reduction in the ability of the SR to accumulate Ca^{2+} partially impairs the capability of the smooth muscle cell to extrude Ca^{2+} from the cytoplasm.

Since agonists also enhance SR permeability, one element of their ability to maintain vascular tone may be ascribed to inhibition of the SR to function as a Ca^{2+} sink under steady-state conditions. Another element of agonist-induced tone is due to their effect on myofilament Ca^{2+} sensitivity as described below.

The presently accepted theory for Ca^{2+} activation of smooth muscle myofilament holds that actomyosin cross-bridge cycling is controlled by the degree of myosin light chain phosphorylation.\textsuperscript{22} This 20 kDa light chain is phosphorylated by the calcium-calmodulin-sensitive myosin light chain kinase. According to Rembolt and Murphy,\textsuperscript{23} two contractile states exist that have different dependencies on myosin light chain phosphorylation: 1) a state characterized by rapid cross-bridge cycling rates and shortening velocity regulated myosin light chain ratios of 0.3:1 (mol P/mol) and 2) a steady state or latch state characterized by equal active stress but low rates of cross-bridge cycling and unloaded shortening velocity, regulated myosin light chain ratios between 0.08 and 0.3 (mol P/mol). However, in the light of recent evidence that agonists are able to change the Ca^{2+} sensitivity of the contractile apparatus, our present understanding of Ca^{2+} activation may still be incomplete.

Measurement of [Ca^{2+}]

A few years ago Morgan and Morgan\textsuperscript{24,25} and De Feo and Morgan\textsuperscript{26} introduced a procedure for simultaneous recording of [Ca^{2+}], and tension development in vascular smooth muscle strips from ferret portal vein and aorta by use of the photoprotein aequorin. They found that α-adrenergic activation resulted in a peak of light emission during the period of force development, which fell to levels close to the basal values during force maintenance. In both ferret aorta and portal vein, Ca^{2+}-force curves were obtained by plotting the steady-state level of tone induced by different degrees of K^{+} depolarization or in the presence of various concentrations of phenylephrine at different concentrations of extracellular Ca^{2+}. The curve for phenylephrine was shifted to the left compared with the curve for K^{+}-induced depolarization, which was used as the control. In addition, these curves were much steeper, ranging from 200 to 400 nM Ca^{2+}, than has been reported for chemically skinned smooth muscle preparations. From these results, they concluded that phenylephrine increases the sensitivity of the contractile apparatus to Ca^{2+} and suggested that protein kinase C might be the cellular mediator for this sensitivity change. Other recently developed fluorescent indicators have been used to measure [Ca^{2+}], in cultured vascular smooth muscle cells\textsuperscript{27-30} or freshly isolated single vascular smooth muscle cells.\textsuperscript{31} Although there were some differences in the size of the transient increases of [Ca^{2+}], induced by agonists and the steady-state levels of the [Ca^{2+}], due to both agonists and high K^{+}, the results in general confirmed the original observations by Morgan and Morgan\textsuperscript{24,25} and De Feo and Morgan.\textsuperscript{26}
Ca$^{2+}$ transient was observed in the presence or following observations. When the relation between absence of 10 nM TPA (parallel measurements), acetylcholine-induced contraction amplitude and the acetate (TPA), a potent phorbol ester, enhances the contractility of vascular smooth muscle from porcine carotid arteries was associated with low values of myosin light chain phosphorylation. Itoh et al$^{45}$ also concluded that phorbol ester activation of protein kinase C contracts vascular smooth muscle by a mechanism that is not directly related to the regulation of transmembrane Ca$^{2+}$ flux, since phorbol ester exhibited the same Ca$^{2+}$-phosphorylation relation as agonists.

Although the above survey of the recent literature tends to support a role for protein kinase C in modulating agonist-induced vasoconstriction, it fails to establish a clear link between receptors, protein kinase C, and myofilaments. A definitive resolution of this problem requires a skinned smooth muscle preparation that responds to receptor activation. This has now become possible as described below.

### Effects of Phorbol Esters

Tumor-promoting phorbol esters have been shown to dramatically increase the affinity of protein kinase C for Ca$^{2+}$ from the 10$^{-6}$ to the 10$^{-7}$ level.$^{36}$ These esters are found to induce contractions of different arterial smooth muscles,$^{37-40}$ through the activation of protein kinase C. In addition, they increase the amplitude of contractions induced by high K$^+$. A role of protein kinase C in the sustained agonist-induced contraction of smooth muscle has been supported by the finding that the combined use of Ca$^{2+}$ ionophores and phorbol esters can produce a response comparable with that produced by carbachol in tracheal smooth muscle.$^{42}$

To elucidate the mechanism of phorbol ester-induced contractions it is again critical to correlate Ca$^{2+}$ concentration, force development, and the myosin light chain phosphorylation. Jiang and Morgan$^{43}$ reported that the resting [Ca$^{2+}$], is both sufficient and required to support phorbol ester-induced contractions in vascular smooth muscles from ferret and rat aorta. Chatterjee and Tejadai$^{44}$ reported that high stress in the skinned muscles from porcine carotid artery was associated with low values of myosin light chain phosphorylation. They concluded that phorbol ester activation of protein kinase C contracts vascular smooth muscle by a mechanism that is not directly related to myosin light chain phosphorylation. Itok et al$^{45}$ also concluded that 12-0-tetradecanoylphorbol-13-acetate (TPA), a potent phorbol ester, enhances the Ca$^{2+}$ sensitivity of the contractile proteins from the following observations. When the relation between acetylcholine-induced contraction amplitude and the Ca$^{2+}$ transient was observed in the presence or absence of 10 nM TPA (parallel measurements), TPA greatly reduced the Ca$^{2+}$ transient but did not modify the amplitude of contraction. In saponin-treated skinned muscle strips, TPA with phosphatidylserine increased the amplitude of contraction evoked by various concentrations of Ca$^{2+}$. TPA with phosphatidylserine also increased the amplitude of contraction evoked by 10 μM IP$_3$ in chemically skinned muscle strips. On the other hand, Rembold and Murphy$^{46}$ reported that low doses of phorbol dibutyrate induced a modest contraction, associated with small, yet significant elevations in [Ca$^{2+}$], and myosin phosphorylation. They concluded that protein kinase C may be involved in the force relations. 47-49 Unfortunately these preparations no longer respond to agonists$^{46,49}$ due to the loss of important proteins for stimulus contraction coupling. For example, it has been reported that saponin treatment causes a significant loss of calmodulin from the fibers.$^{40}$

A number of new methods for permeabilization have been tried in other cell types, by means of streptolysin O,$^{51,52}$ Sendai virus,$^{3}$ electrical permeabilization,$^{54,55}$ pseudomonas aeruginosa cytotoxin,$^{56}$ or α-toxin.$^{57-59}$ We employed α-toxin to permeabilize the arterial smooth muscle fibers, since Hohman$^{50}$ reported that rat basophilic leukemia cells can be permeabilized to small molecules by α-toxin while maintaining the ability to degranulate in response to aggregation of immunoglobulin (Ig)E receptors.

Initially, Cassidy et al$^{62}$ used α-toxin to permeabilize rabbit ileum strips, but they did not use agonists to activate the muscle. α-Toxin is a cytolytic exoprotein produced by staphylococcus aureus. This toxin binds to the cell surface and forms hexamers with other toxin molecules, which insert into the plasma membrane to form pores of 2-3 nm diameter.$^{58}$ This limited pore size allows equilbrium of the cytoplasm with inorganic ions and small molecules but prevents the passage of proteins, including α-toxin itself, into or out of the cells.

Figure 2 shows α-toxin-mediated permeabilization of the rabbit mesenteric artery smooth muscle to Ca$^{2+}$ and ATP.$^{60}$ After α-toxin treatment, the fibers developed tension as a function of Ca$^{2+}$.
concentration. The fibers did not contract without added ATP, which indicates loss of endogenous ATP from the permeabilized cells. In addition, the permeabilized artery responded repeatedly to caffeine if the SR was previously loaded, which indicates that the function of SR was preserved while the plasmalemma was rendered freely permeable to Ca^{2+} and ATP.

Norepinephrine-Induced Increase of Myofilament Sensitivity to Ca^{2+}

As shown in Figure 3, application of NE to permeabilized smooth muscle bathed in 2 mM EGTA 0 M Ca^{2+} solution induced a transient contraction if the SR had been previously loaded with Ca^{2+}. When NE was added to a 5 \times 10^{-7} M Ca^{2+} solution, the response was biphasic with maintained elevated tension after a transient peak. A small maintained contraction was obtained even at 10^{-7} M Ca^{2+} solution after depletion of the SR by caffeine (Figure 6). These responses were not related to an artificial remnant of intact cells since the latter do not respond to such low [Ca^{2+}] and, without replenishment of mM Ca^{2+}, do not refill their SR. We routinely discharged the SR of the permeabilized cells with caffeine before subjecting them to submicromolar [Ca^{2+}], NE, and various guanosine nucleotides. In addition the NE-induced contractions could be abolished by guanosine 5'-0-(2-thiodiphosphate) (GDP-\beta-S), a compound that does not enter intact cells. We could thus conclude that the permeabilization of rabbit mesenteric artery by \alpha-toxin does not comprise the \alpha-adrenergic receptor nor its signal transduction system.

The sustained NE-induced contraction of the permeabilized mesenteric artery, clamped at [Ca^{2+}] of 10^{-7} M, therefore establishes that \alpha-adrenergic receptor mediates enhancement of myofilament Ca^{2+} sensitivity. The quantitative Ca^{2+}-force relations are shown in Figure 4. NE plus 100 \mu M guanosine triphosphate (GTP) causes a profound increase in myofilament sensitivity to Ca^{2+}, decreasing the EC_{50} from 890 nM to 280 nM. In addition, 100 \mu M GTP alone has small effect on the Ca^{2+}-force curve.

GTP-\gamma-S–Induced Increase of Myofilament Sensitivity to Ca^{2+}

Many hormones stimulate phospholipase C-catalyzed phosphatidylinositol 4,5-bisphosphate hydro-
lysis, which yields two intracellular messengers, IP₃ and diacylglycerol. IP₃ releases Ca²⁺ from intracellular store sites and diacylglycerol activates protein kinase C. Recent reports have shown that guanine nucleotide binding proteins (G protein) are involved in phospholipase C activation.

To obtain further information regarding the transduction of the agonist-receptor interaction to the change in myofilament Ca²⁺ sensitivity, we used guanosine 5’-O-(3-thiotriphosphate) (GTP-γ-S) as the irreversible activator of G protein. As shown in Figure 5, 10 μM GTP-γ-S induced large contractions, the pattern of which depended on the experimental conditions. The contraction was phasic followed by maintained tension when GTP-γ-S was applied to a 1.8×10⁻⁷ M Ca²⁺ solution after the SR was loaded with Ca²⁺. The contraction was transient when GTP-γ-S was added to a solution containing 2 mM EGTA, 0 M Ca²⁺ after the SR was loaded. It was monophasic tonic when GTP-γ-S was added to 1.8×10⁻⁷ M Ca²⁺ solution after the SR Ca²⁺ was depleted. The latter contraction could be reversibly inhibited by 20 μM H-7, a compound that is reported to inhibit protein kinase C. This concentration of H-7 caused only a small relaxation if the muscle had not been stimulated by NE or GTP-γ-S. Furthermore, the effect of GTP-γ-S was preserved after washing out of GTP-γ-S, probably by guest on July 10, 2017 http://hyper.ahajournals.org/ Downloaded from
due to the inability of guanosine triphosphatase (GTPase) to hydrolyze GTP-γ-S. Figure 4 shows the quantitative Ca²⁺-force relation in the presence of 10 μM GTP-γ-S. Direct activation of G protein by GTP-γ-S caused an even greater increase in Ca²⁺ sensitivity, shifting the EC₅₀ from 890 nM to 160 nM. Thus, GTP-γ-S mimics the effect of NE in releasing the Ca²⁺ from the SR and in causing an increase of myofilament sensitivity to Ca²⁺, indicating that G protein is involved in α-adrenergic receptor-mediated activation of phospholipase C in vascular smooth muscle.

The Ca²⁺-sensitizing effects of NE and GTP-γ-S were observed as increases in tonic tension even when the concentration of the Ca²⁺-EGTA buffer was raised to 10 mM, ruling out a possible contribution of the SR in this aspect of receptor or G protein activation. Figure 4 also shows that the extent of change of sensitivity may be graded rather than “all or none” in character. In other words, the extent of leftward shift of the Ca²⁺-force curves may be variable. The potency order of the G protein activation illustrated in Figure 4 (i.e., 10 μM GTP-γ-S, 100 μM GTP plus NE, or 100 μM GTP alone) could be explained by nonselective activation of G proteins associated with a number of receptor types (e.g., angiotensin or vasopressin), by GTP-γ-S, selective activation by NE and GTP, or only partial activation by GTP by itself. Accordingly, the extent of G protein activation may determine the extent of the leftward shift of the Ca²⁺-force curve. However, it is unlikely that G protein activation directly sensitizes the contractile proteins, since the α-toxin-permeabilized fiber sensitized by GTP-γ-S loses the high sensitivity state to Ca²⁺ after the subsequent treatment by saponin (J. Nishimura and C. van Breemen, unpublished data). Thus, a factor that is permeable after the treatment with saponin but not after α-toxin may be playing an important role in the change of sensitivity of the contractile apparatus. Protein kinase C or the substrates of protein kinase C are probable candidates. The inhibitory effect by H-7 on GTP-γ-S-induced contraction suggests a role for protein kinase C in the agonist-induced myofilament sensitization.

**Coupling of α-Adrenergic Receptors to G Proteins**

As mentioned above, the effect of GTP-γ-S may be nonselective, and we don’t know at present how many sets of G proteins are present in vascular smooth muscle cells. For example, Kobayashi et al. reported that GTP-γ-S induces Ca²⁺ release from the SR of rabbit main pulmonary artery permeabilized by saponin. For this reason, the next experiment was designed to show that G protein is coupled to cell surface α-adrenergic receptors.
Figure 6 shows the effect of GTP and GDP-β-S on NE-induced contractions of α-toxin-permeabilized rabbit mesenteric artery (J. Nishimura and C. van Breemen, unpublished data). A sustained contraction was caused by 10⁻⁴ M NE in 10⁻⁷ M Ca²⁺ solution buffered by 2 mM EGTA after the SR was depleted by 25 mM caffeine (Figure 6A). However, the size of NE-induced contraction in the absence of GTP was small and variable. Addition of 100 μM GTP consistently increased this NE-induced contraction whereas 100 μM GDP-β-S inhibited it (Figure 6B and 6C).

The variation of the NE-induced contraction without GTP is likely to be due to the variability of the endogenous GTP. Thus, the inhibitory effect induced by GDP-β-S on NE-induced contraction and the preferential requirement of GTP for NE-induced contraction indicated that G protein couples α-adrenergic receptors to contraction.

In conclusion, it is now established that receptor activation by at least one agonist enhances myofilament sensitivity to calcium ions. It remains to be determined whether the mechanism underlying this sensitization maintains an invariant relation between myosin light chain phosphorylation and tension or involves the phosphorylation of cytoskeletal proteins and caldesmon as proposed by Rasmussen et al.

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