Adenosine Triphosphate Induces a Low [Ca\textsuperscript{2+}], Sensitivity of Phosphorylation and an Unusual Form of Receptor Desensitization in Smooth Muscle* 

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The contractile sensitivity of smooth muscle to changes in myoplasmic [Ca\textsuperscript{2+}] is dependent on the form of stimulation. Both myosin phosphorylation and force are less sensitive to increases in [Ca\textsuperscript{2+}], derived from Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels than to increases in [Ca\textsuperscript{2+}] induced by agents which release internal Ca\textsuperscript{2+} stores. 

Both myosin phosphorylation and force were rapidly reduced. Stimulation of a P2x receptor-operated cation channel in smooth muscle, ATP, via activation of a P2x purinergic receptor, induced large, transient increases in [Ca\textsuperscript{2+}]; yet only small transient increases in [Ca\textsuperscript{2+}], cyt, yet still transient increases in [Ca\textsuperscript{2+}], phosphorylation, and force; BW A1433U, a P1 (adenosine) receptor antagonist, enhanced ATP-induced contractions; and 3) ATP, but not a-β-methylene ATP increased bath [adenosine]. The [Ca\textsuperscript{2+}], sensitivity of phosphorylation during P2x receptor activation was similar to that observed with KCl-depolarization-induced opening of L channels, supporting the hypothesis that transplasmalemmal Ca\textsuperscript{2+} influx produces less phosphorylation and force than mobilization of intracellular Ca\textsuperscript{2+} stores. Cumulative additions of higher α-β-methylene ATP concentrations induced repeated transient contractions, indicative of an unusual form of receptor desensitization which could be explained if the affinity of the P2x receptor for ATP, but not the receptor number were rapidly reduced.

1) Ca\textsuperscript{2+} was added as the Mg\textsuperscript{2+} salt to prevent ATP chelation of [Mg\textsuperscript{2+}], caused by hydrolysis of ATP into adenosine since 1) α-β-methylene ATP (a poorly hydrolyzable analog of ATP) produced larger, yet still transient increases in [Ca\textsuperscript{2+}], phosphorylation, and force; 2) BW A1433U, a P1 (adenosine) receptor antagonist, enhanced ATP-induced contractions; and 3) ATP, but not α-β-methylene ATP increased bath [adenosine]. The [Ca\textsuperscript{2+}], sensitivity of phosphorylation during P2x receptor activation was similar to that observed with KCl-depolarization-induced opening of L channels, supporting the hypothesis that transplasmalemmal Ca\textsuperscript{2+} influx produces less phosphorylation and force than mobilization of intracellular Ca\textsuperscript{2+} stores. Cumulative additions of higher α-β-methylene ATP concentrations induced repeated transient contractions, indicative of an unusual form of receptor desensitization which could be explained if the affinity of the P2x receptor for ATP, but not the receptor number were rapidly reduced. 

MATERIALS AND METHODS

Swine common carotid arteries were obtained from a slaughterhouse and transported at 2 °C in physiological salt solution (PSS). Dissection of medial strips and mounting were performed as illustrated by Driska (17). The intimal surface was mechanically rubbed to remove the endothelium. PSS contained (mm): NaCl, 140; KCl, 5; MOPS, 2; CaCl\textsubscript{2}, 1.6; MgCl\textsubscript{2}, 1.2; Na\textsubscript{2}HPO\textsubscript{4}, 1.2; glucose, 5.6 (pH adjusted to 7.4 at 37 °C). The Ca\textsuperscript{2+}-free solution was PSS with 1 mM EGTA and no added CaCl\textsubscript{2}. Agonist stimulation was performed by injecting an appropriate volume of 10 mM stock histamine into the tissue bath. Histamine stock solutions were prepared daily. ATP and α-β-methylene ATP were added as the Mg\textsuperscript{2+} salt to prevent ATP chelation of [Mg\textsuperscript{2+}] (18–20). Aequorin (batch 2, obtained from Dr. John Blinks, Mayo Medical School, Rochester, MN) was loaded intracellularly by isosmotic hyperpermiabilization (1). Simultaneous light and force measurements were made in a light-tight enclosure. Force was measured and muscle length controlled with a Cambridge Technology 300H Servo in the isometric mode. A Metabyte DASH 16 AD board in an AT&T personal computer was used to store force measurements. Stress was calculated as force per cross-sectional area, which was estimated from 2) Histamine or phenylephrine (1, 6). Regardless of the activating agent when released with norepinephrine, acetylcholine, or other substances from nerve terminals (15, 16). The goal of this study was to evaluate the mechanism of ATP-induced smooth muscle contraction in swine carotid smooth muscle.

1) The abbreviations used are: PSS, physiological salt solution; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, [ethylenbis(oxyethylenenitrilo)tetraacetic acid, BW A1433U, 1,3-dipropyl-8-(p-acrylate)-phenylxanthine].

5407
measured length, weight, and a density of 1.050 g/cm³. Aequorin light signals were collected digitally, counted by the Metrabyte DASH 16 AD board, and were presented in the form log L/Lmax, where L is the photon count (in counts/s) and Lmax is an estimate of the peak light intensity that would be recorded if all of the aequorin in the tissue were instantaneously exposed to 5 mM CaCl₂ (18). Aequorin light emission was calibrated in series of Ca²⁺/EGTA buffers with 0.5 mM Mg²⁺ at 37 °C (1).

Phosphorylation was estimated in a second set of tissues that underwent identical protocols except the tissues were not loaded with aequorin. Aequorin loading has been previously shown not to change the time course of phosphorylation or stress development in the swine carotid (9, 19). Tissues were frozen by immersion in a dry ice/acetone slurry (20 g/20 ml) at −78 °C. Phosphorylation of the smooth muscle-specific 20-kDa myosin light chain was determined by the method of Driuka et al. (17) with precautions noted by Aksoy et al. (20). Phosphorylation is reported as mol P/mol total smooth muscle specific light chain. Force was measured isometrically with Grass FT.03 (Quincy, MA) force transducers in the tissues that were assayed for phosphorylation.

Back [adenosine] was measured in a second set of tissues that underwent identical protocols except that these tissues were not loaded with aequorin. The contractile response of these tissues to histamine, ATP, and α,β-methylene ATP was similar to that observed in the aequorin-loaded tissues. Twenty-five ml of fresh PSS was added at the beginning of each 10 min experimental period. After 8 min of exposure to the tissue, an aliquot of the bath solution was removed and assayed for [adenosine] with a highly sensitive radioimmunoassay procedure. Antibodies were prepared to N⁶-carboxybenzyladenosine coupled to methylated bovine serum albumin (21). N⁶-substituted adenosine analogs were found to bind to the antisera with 50 times higher affinity than adenosine. Hence, N⁶-$\text{F}$-aminobenzyladenosine was synthesized as a radioligand (22). Samples of medium (0.5 ml) were mixed with 0.1 ml each of 0.3 M zinc sulfate and 0.3 M barium hydroxide to coprecipitate adenosine nucleotides which weakly cross-reacted with the antisera. Following centrifugation, 0.4 ml of the supernatant was evaporated to dryness overnight at 50 °C. Adenosine in the sample was derivatized to C⁶-benzyladenosine by incubation for 3 h at room temperature with 0.2 ml benzylobromide/dimethylacetamide (16). The nucleoside product was separated from unreacted benzylobromide upon the addition of 0.4 ml each of 1,1,2-trichloro-1,2,2-trifluoromethane and 50 mM ammonium hydroxide in water. Following centrifugation, the aqueous (top) phase was warmed to 80 °C for 2 h to promote the alkali-catalyzed conversion of C⁶-benzyladenosine to N⁶-benzyladenosine, and evaporated to dryness under vacuum. Following resuspension of the pellet residue in phosphate-buffered saline, samples were clarified by centrifugation and the N⁶-benzyladenosine quantitated by automated radioimmunoassay (23). The assay could detect derivatized adenosine in the range of 2–250 nM, and endogenous [adenosine] in PSS containing 1 mM ATP which was not incubated with smooth muscle tissues was 12 nM. This very small increase was either caused by contaminating adenosine, interference by residual ATP with the radioimmunoassay, or nonenzymatic breakdown of ATP to adenosine. Thus, the ATP added to tissue baths without tissues was not significantly hydrolyzed to adenosine.

Changes in log L/Lmax and active stress were compared between groups using Student's unpaired t test and significance was defined as p < 0.05.

RESULTS

Stimulation with 1 mM ATP induced a large [Ca²⁺], transient that was associated with only a small increase in phosphorylation and stress (Fig. 1, left panel). [Ca²⁺], phosphorylation, and stress levels quickly returned to resting values. In the absence of extracellular CaCl₂, 1 mM ATP induced only a small [Ca²⁺], transient and no detectable contraction, suggesting that ATP induced [Ca²⁺], transients are primarily caused by [Ca²⁺], influx and not release of the intracellular Ca²⁺ stores (Fig. 1, center panel).

Purinergic receptors have been subdivided into several types: P1 receptors which are responsive to adenosine > ATP and linked to adenylyl cyclase, P2X receptors which are responsive to α,β-methylene ATP > ATP > adenosine and are linked to ion channels, and P2Y receptors which are responsive to ATP > α,β-methylene ATP > adenosine and stimulate inositol 1,4,5-trisphosphate production (13, 14). It is known that ATPases are present in the extracellular matrix of smooth muscle (24, 25). These ATPases can convert some of the ATP to adenosine which is known to produce Ca²⁺, dependent relaxation of smooth muscle. α,β-methylene ATP (0.1 mM, a poorly hydrolyzable analog of ATP) produced a larger [Ca²⁺], transient than was observed with 1 mM ATP (Fig. 1, right panel). This [Ca²⁺], transient was associated with a larger increase in phosphorylation and stress than was observed with 1 mM ATP, suggesting that 1) the ATP receptor is of the P2x class, and/or 2) production of adenosine by hydrolysis of ATP may be attenuating the ATP contraction. With sustained α,β-methylene ATP stimulation, [Ca²⁺], phosphorylation, and stress all returned to resting values. Subsequent stimulation with 100 μM histamine induced a large [Ca²⁺], phosphorylation, and phosphorylation transient and rapid development of maximal stress, suggesting that α,β-methylene ATP induced homologous receptor desensitization.

The [Ca²⁺] and stress transients from Fig. 1 are shown with greater time resolution in Fig. 2. For the first 30 s after stimulation, the histamine-induced [Ca²⁺], transient was less than the induced by 1 mM ATP or 0.5 mM α,β-methylene ATP, however, the increase in stress in the first 30 s was greater with histamine. These data confirm that the [Ca²⁺], sensitivity of stress production was lower with ATP or α,β-methylene ATP stimulation. ATP and α,β-methylene ATP also induced a low [Ca²⁺], sensitivity of phosphorylation, similar to that induced by depolarization (Fig. 3). In contrast, histamine induced a higher [Ca²⁺] sensitivity of phosphoryl-
Bath adenosine was slightly, but not significantly increased by 1 µM ATP. Ten µM ATP induced a small [Ca^{2+}]; transient that was followed by a decrease in [Ca^{2+}]; and stress to near resting values. The 100 µM and 1 mM ATP doses induced large [Ca^{2+}]; transients with only small contractions similar to that observed in Fig. 1. Bath [adenosine] was significantly increased by the 10 µM, 100 µM, and 1 mM ATP doses. Subsequent stimulation with 100 µM histamine overcame the relaxation induced by the lower doses of ATP and increased both [Ca^{2+}]; and stress.

α-β-Methylene ATP (a poorly hydrolyzable analog of ATP) induced larger contractile effects and smaller relaxant effects than were observed with ATP (Fig. 5). No substantial decrease in [Ca^{2+}]; or relaxation was noted until the 100 µM dose of α-β-methylene ATP. Bath [adenosine] was not increased by any of the tested α-β-methylene ATP doses. α-β-Methylene ATP induced more prolonged [Ca^{2+}]; transients and accentuated both the peak and duration of the contraction. These data suggest that adenosine, produced by hydrolysis of ATP, may be responsible for the relaxation at lower ATP concentrations and the inhibition of the contractile effects of ATP at higher concentrations.

A similar protocol was performed in the presence of 10 µM BW A1433U (1,3-dipropyl-8-(p-acryloyl)phenylxanthine), a specific acidic P1 (adenosine) receptor antagonist which does not activate phosphodiesterase in intact tissues (26). The ATP-dependent relaxation was attenuated by the adenosine antagonist, and stress did not approach resting values until the 100 µM ATP dose (Fig. 6). The peak of the ATP-induced [Ca^{2+}]; transients were not affected by BW A1433U. However, the [Ca^{2+}]; transients had a longer duration, and the peak and duration of the ATP induced contraction were potentiated by BW A1433U. Since the response to ATP in the presence of adenosine receptor blockade (BW A1433U) was less than the response to α-β-methylene ATP, we can identify the ATP receptor present in the swine carotid as the P2x subtype.
The ability of a contractile agent to release Ca\(^{2+}\) from intracellular stores appears to correlate with the [Ca\(^{2+}\)], sensitivity of phosphorylation, however, Ca\(^{2+}\) influx is indispensable for sustained smooth muscle contractions regardless of the stimulus (2, 33, 34). In the absence of extracellular Ca\(^{2+}\), histamine or phenylephrine-induced contractions quickly relax as the intracellular Ca\(^{2+}\) pool is depleted (35). Sustained agonist induced contractions are associated with increased Ca\(^{2+}\) influx (as measured with 45Ca\(^{2+}\) (36, 37)) and with sustained elevation of myoplasmic [Ca\(^{2+}\)] (1, 5, 38-40), although this is not reported by all investigators (30, 31, 41). To account for the sustained phase of contraction, the existence of histamine and phenylephrine activated receptor-operated Ca\(^{2+}\) permeable channels have long been hypothesized (42), however, such channels have yet to be identified. Norpinephrine has been shown to increase L channel current without changing the channel’s voltage sensitivity (43). However, this cannot explain norepinephrine induced Ca\(^{2+}\) influx in the absence of a change in membrane potential (i.e. pharmacomechanical coupling (44)).

The mechanism for the variable [Ca\(^{2+}\)], sensitivity of phosphorylation is not yet clear. In vitro, the activity of myosin kinase is a function of both [Ca\(^{2+}\)], and [calmodulin] (45). Thus, myosin kinase activity and therefore phosphorylation levels should be proportional to myoplasmic [Ca\(^{2+}\)]. Myosin kinase is phosphorylated at several sites in intact tissues (46). It appears that only phosphorylation of myosin kinase at one site (termed “peptide A”) is associated with a decreased [Ca\(^{2+}\)], sensitivity for myosin kinase activity, phosphorylation at other sites has no effect (47). Neither agonist stimulation nor depolarization alter cAMP levels in the swine carotid (48), suggesting that protein kinase A-dependent phosphorylation of the peptide A site is not responsible for the variable [Ca\(^{2+}\)], sensitivity of phosphorylation. However, preliminary data from another laboratory (47) suggests that the amount of peptide A phosphorylation can change during agonist stimulation of the canine trachealis. Potentially, other kinases may phosphorylate myosin kinase at the peptide A site. An alternative explanation is that myosin phosphatase is regulated in smooth muscle (49); however, there is no convincing evidence for the existence of a regulated myosin phosphatase.

At least some of the effect of exogenous ATP in the swine
carotid was caused by hydrolysis of ATP, formation of adenosine, and activation of P1 (adenosine) receptors (50). Low doses of ATP increased bath [adenosine] and induced relaxation of submaximally stimulated tissues (Fig. 4). ATP-induced relaxation was attenuated by P1 receptor blockade (Fig. 6) or the use of α-β-methylene ATP (a poorly hydrolyzable analog of ATP which did not increase bath [adenosine]; Fig. 5). The [Ca\(^{2+}\)]\(_{i}\) sensitivity of phosphorylation was similar despite the use of ATP or α-β-methylene ATP (Fig. 3), suggesting that the effect of adenosine was predominantly to decrease myoplasmic [Ca\(^{2+}\)]\(_{i}\), and not an alteration in the [Ca\(^{2+}\)] sensitivity of phosphorylation. The [Ca\(^{2+}\)]\(_{i}\) transient was larger and more prolonged with α-β-methylene ATP than with ATP supporting the contention that formation of adenosine from ATP can attenuate the ATP response. Since adenosine is rapidly formed and metabolized by smooth muscle, bath [adenosine] does not necessarily equal the [adenosine] present near the P1 receptor. The measured bath [adenosine] should be considered more as an index of adenosine production.

When the effects of adenosine were blocked, both ATP and α-β-methylene ATP induced an unusual form of receptor desensitization. Low doses of ATP and α-β-methylene ATP induced transient elevations in [Ca\(^{2+}\)]\(_{i}\), and force with rapid desensitization (Figs. 5 and 6). Higher doses of ATP and α-β-methylene ATP induced similar [Ca\(^{2+}\)]\(_{i}\) transients, with force transients with rapid desensitization. This pattern of desensitization could be explained if the receptor affinity is rapidly decreased, without a substantial change in receptor number. However, this hypothesis requires further evaluation.

ATP has been hypothesized to function as a cotransmitter when released with norepinephrine or other substances from nerve terminals (16). The results of this study indicate that ATP induced contractions in the swine carotid are limited by the low [Ca\(^{2+}\)]\(_{i}\), sensitivity of phosphorylation, the production of adenosine, and rapid homologous desensitization. ATP, per se, did not interfere with high dose histamine contractions, however, the adenosine produced from ATP hydrolysis relaxed low dose histamine contractions (Fig. 4). Norepinephrine or phenylephrine were not used in these studies because the contractions elicited by these agents in the swine carotid are quite variable. However, histamine and phenylephrine both produce tonic contractions with a high [Ca\(^{2+}\)]\(_{i}\), sensitivity of phosphorylation in the swine carotid (1). It is reasonable to hypothesize that nerve terminals can release two types of contractile agents: 1) tonic contractile agonists, such as norepinephrine, and 2) phasic contractile agonists, such as ATP. These two different types of contractile agents could allow more versatility in the contractile response of the smooth muscle to neural stimuli.

In conclusion, ATP appears to contract swine carotid media by activation of P2x receptors resulting in opening of plasma membrane cation channels. The resulting Ca\(^{2+}\) mobilization occurs predominantly from the extracellular space and is associated with a low [Ca\(^{2+}\)]\(_{i}\), sensitivity of phosphorylation. Two mechanisms prevent sustained contraction: 1) hydrolysis of ATP into adenosine which activates P1 receptors and 2) a unusual form of receptor desensitization in which the receptor affinity for the agonist appears to be reduced.

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REFERENCES