Facilitation of the Purinergic Contractile Response of the Guinea Pig Vas Deferens by Sodium Orthovanadate

Latchezar D. Todorov, Svetlana T. Mihaylova-Todorova, Sophie M. Choe, and David P. Westfall

Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada

Received June 18, 2004; accepted October 18, 2004

ABSTRACT

Experiments were carried out to examine the effects of protein tyrosine kinase (PTK) and protein tyrosine phosphatase inhibitors on the purinergic contraction of the guinea pig vas deferens. Sodium orthovanadate (SOV) produced a robust increase of the amplitude of contractions evoked by both neurogenic electrical field stimulation and exogenous ATP. This effect of SOV was concentration- and time-dependent, as well as, reversible and reproducible. Genistein, a PTK inhibitor, but not its inactive structural analog daidzein, inhibited the SOV-induced facilitation of the purinergic contraction. Another PTK inhibitor, 2,5-dihydroxycinnamic acid methyl ester, which is structurally unrelated to genistein, also inhibited the facilitation effects of SOV. Although an application of as low as 3 μM of these inhibitors significantly decreased the effect of SOV, other PTK inhibitors, namely, butein, levandustin C, and thyrphostin 23, were less effective even at concentrations of 100 μM. Western blot experiments showed that the facilitation of the purinergic contraction by SOV is associated with a prominent increase in the level of tyrosine phosphorylation of proteins with estimated molecular sizes of 180 and 123 kDa, which was reversed in the presence of genistein. Evidence is also presented that argue against the possibility that inhibition of the Na+ /K+ -ATPase or ATPases, responsible for the clearance of ATP is involved in the SOV-induced facilitation of the purinergic contraction. Together, these results suggest that the responsiveness of the smooth muscle of the vas deferens to the actions of ATP is modulated via a previously unidentified mechanism, which may involve protein tyrosine phosphorylation.

Electrical stimulation of the sympathetic nerves innervating the guinea pig vas deferens causes the release of two cotransmitters, ATP and norepinephrine (NE) (Sneddon and Westfall, 1984). These cotransmitters seem to be stored separately and released via independent mechanisms (Todorov et al., 1996, 1999). The ATP-induced smooth muscle contractile response is mediated via P2X1 receptors, which are ligand-gated cationic channels (Benham and Tsien, 1987; Bean, 1992). When activated, the P2X1 receptors produce excitatory junction potentials that summate allowing the opening of L-type voltage-dependent calcium channels (L-VDCC). The resulting influx of extracellular Ca^{2+} triggers action potentials and a twitch-like contraction. NE acts via α1-adrenergic receptors to stimulate formation of inositol triphosphate, leading to a release of Ca^{2+} from intracellular stores, which contributes to the tonic phase of the contractile response.

It has been clearly shown that variations of the free intracellular calcium concentration regulates the contraction of the smooth muscle via a calcium-calmodulin stimulation of myosin light chain kinase and subsequent phosphorylation of myosin regulatory light chain (Kamm and Stull, 1989; Somlyo and Somlyo, 1994). There is a consensus that the signaling pathways involved in the process of smooth muscle contraction are driven by phosphorylation of proteins on serine and/or threonine. However, an accumulating body of evidence suggests that in addition to cell proliferation, differentiation, and migration (Ullrich and Schlessinger, 1990; Schieffer et al., 1997), signal transduction cascades that involve protein tyrosine phosphorylation may also regulate a number of processes leading to smooth muscle contraction (Hollenberg, 1994; Hughes and Wijetunge, 1998). For example, growth factors such as epidermal growth factor and platelet-derived growth factor, known activators of protein tyrosine kinases have been found to produce smooth muscle contraction (Burk et al., 1985, 1986). Moreover, tyrosine kinase inhibitors attenuate the contractile responses of vascular preparations to several G protein-coupled agonists, such as angiotensin-II, NE, and...
Materials and Methods

Tissue Preparation. Male guinea pigs (350–450 g) were sacrificed by decapitation. Vasa deferentia were dissected out of the body and placed in Krebs’ solution of the following composition: 150 mM NaCl, 4.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, and 5.6 mM glucose, which was constantly bubbled with 95% O₂ and 5% CO₂. The tissue preparations were cleaned of connective tissues, and the prostatic portion of the vas deferens was set up for isotonic registration of contractile activity. The smooth muscle contractile responses to neurally released ATP were studied under superfusion experimental conditions, whereas the effects of exogenously applied ATP were studied in incubation.

Superfusion. Tissue preparations were placed in a horizontally oriented organ chamber equipped with two platinum ring electrodes for producing electrical field stimulation (EFS). One end of the tissue was fixed in place, and the other was attached by silk surgical suture to a Grass force displacement transducer (FTO3). The tissues were superfused (2 ml/min) with oxygenated Krebs’ solution (37°C). After 20 min of superfusion, an initial load of 0.5 g was applied to each tissue. The preparations were allowed to equilibrate for another 40 min before recording of stimulated contractile responses. The average of three control neurogenic responses evoked by a single electrical impulse with duration of 0.1 ms and supramaximal voltage were calculated and compared with that of neurogenic responses after pretreatment with drugs. In some experiments, the tissue preparations were subjected to a 20-s-long train of electrical impulses delivered at a frequency of 8 Hz.

Incubation. Vas deferens tissue preparations were fixed at one end to a holder and suspended vertically in an organ chamber filled with 5 ml of Krebs’ solution (37°C). The other end of each tissue was attached to a force displacement transducer (Grass FTO3) by silk surgical suture. After 20 min of incubation, an initial load of 0.5 g was applied to each tissue by the means of a micromanipulator, and the tissues were equilibrated for another 40 min. The Krebs’ solution was replaced at 15-min intervals. Three control contractile responses to ATP (100 μM) or 3 μM α,β-methylene ATP (α,β mATP) were recorded at the beginning of each experiment. The average amplitude of these responses was compared with that of ATP and α,β mATP-induced contractile responses after treatment with drugs.

Recording and Processing of Data from Contraction Experiments. The signals from the force displacement transducers were recorded, displayed, and analyzed using a Power Lab system consisting of Power Lab Bridge Amplifier, Power Lab 8SP high-speed recorder, and a Pentium IV PC equipped with Power Lab Chart 4.2 software (ADInstruments Pty Ltd., Castle Hill, Australia). The areas under the waveforms were calculated and entered in spreadsheets of the Prism 3 (GraphPad Software Inc., San Diego, CA) software for statistical evaluation, pharmacological analysis, and graphic presentation. The results are presented as the mean ± S.E.M. One-way analysis of variance was applied to compare variables, and P values of less than 0.05 were considered to represent statistically significant differences.

Breakdown of Extracellular ATP by the Guinea Pig Vas Deferens. Guinea pig vas deferens tissue preparations were set up for recording contractile responses under incubation conditions as described above. After an equilibration period of 40 min, one of the vasa deferentia dissected from each animal was treated with phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO) diluted 1:100, whereas the other served as a control. The phosphatase inhibitor cocktail 2 contains sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole. The final concentration of sodium orthovanadate (SOV) in these experiments was 1 mM. After an incubation period of 15 min, the tissue chambers were drained and refilled with buffer containing the fluorescent analog of ATP, 1N⁶-etheno-ATP (e-ATP) or buffer containing e-ATP and phosphatase inhibitor II, respectively. Ten minutes after the addition of the substrate, aliquots of 50 μl were taken, acidified with 100 μl of citric phosphate buffer (pH 4), and stored on ice. The tissue preparations were washed three times and left to equilibrate for another 20 min before repeating the same procedures with higher concentration of e-ATP (0.1–1000 μM). An HPLC-based assay was used to quantify the adenine nucleotides present in each of the samples.

HPLC-Based Assay for Nucleotidase Activity. The fluorescent 1N⁶-etheno analog of ATP was used as substrate in these experiments designed to study the breakdown of ATP by the guinea pig vas deferens. The rationale of this assay is based on the fact that a sequential dephosphorylation of e-ATP results in formation of 1N⁶-etheno ADP followed by formation of 1N⁶-etheno AMP, and finally formation of 1N⁶-etheno adenosine. The substrate and the resulting metabolites were simultaneously quantified by HPLC with fluorescence detection.

HPLC Technique. The e-purines were separated on a gradient HPLC system equipped with a Waters Resolve radial pack cartridge (8NV Ph 4, 4 μm; 8 × 10 mm). The amount of each compound was quantified by means of a Waters 474 fluorescent detector at an excitation wavelength of 230 nm and an emission wavelength of 420 nm. Buffer solutions consisted of 0.1 M phosphate (KH₂PO₄), pH 6.0 (buffer A) and 75% 0.1 M phosphate and 25% methanol (buffer B). The nucleotides and adenosine were separated using a gradient in which the concentration of buffer B was increased from 0 to 100% in 8 min according to Waters gradient profile 7. Identification of individual peaks was by comparison with the retention times of known e-purines standards, and the concentration was determined by peak area per picomole compared with standards. Standards were run with each set of samples. The HPLC equipment was controlled by, and data collected by, a Pentium II-based desktop computer equipped with an LAC/E card and Millennium 2010 Chromatography Manager software from Waters (Milford, MA). Results were normalized for volume and tissue weight and the data calculated as picomoles per milligram.

Protein Separation and Western Blots Experiments. Guinea pig vasa deferentia were set up for experiments under incubation conditions, and the contractile responses of each of the tissues to a bolus injection of ATP (100 μM) were recorded as described above. The tissue preparations were divided in three groups: 1) preparations treated only with ATP (control), 2) preparations treated for 15 min with SOV (300 μM) before the application of ATP, and 3) preparations treated for 15 min with genistein (100 μM) before the application of SOV and ATP. Immediately after recording the contractile responses, the tissues were lifted by their holders, dipped in ice-cold Ca²⁺-free Krebs’ solution, containing 1 mM SOV and 5 mM EDTA, and immediately frozen in liquid nitrogen. The frozen tissues were crushed to powder using a Cryo-Press device (Microtec, Chiba, Japan) chilled in liquid nitrogen. The powder from each tissue was transferred in ice-cold sucrose buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM SOV, 10 mM NaF, and 0.5% Triton X-100) and centrifuged at 14,000g for 10 min at 4°C. Supernatants were collected and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Samples containing equal amounts of protein were solubilized in equal volume of SDS-Laemmli

5-hydroxytryptamine (Lanlyou et al., 1994; Jinsi et al., 1996; Watts et al., 1996). It should be emphasized that most of the studies linking protein tyrosine phosphorylation with smooth muscle contractility are indirect and in most part based on use of inhibitors of PTK. The specific PTK, protein tyrosine phosphatases (PTP) and their substrates involved in smooth muscle contraction, are currently unknown. Here, we provide evidence suggesting for the first time that protein tyrosine phosphorylation may be involved in the regulation of the amplitude of the smooth muscle contraction evoked by ATP.
buffer and boiled for 5 min at 90°C. The proteins (20 μg) were separated by electrophoresis on 10% Tris-SDS polyacrylamide gels (Ready-Gel; Bio-Rad, Hercules, CA) and electrically transferred onto Hybond nitrocellulose membranes (Amersham Biosciences Inc., Piscataway, NJ). The membranes were blocked using the Odyssey blocking buffer (Li-COR, Lincoln, NE) and incubated for 2 h with the mouse anti-phosphotyrosine antibody (PY-99; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as well as the rabbit anti-ERK1/2 polyclonal antibody (Promega, San Obispo, CA). The membranes were further incubated for 1 h with the goat anti-mouse antibody coupled to Alexa-Flour 680 (Molecular Probes, Eugene, OR) as well as the goat anti-rabbit antibody coupled to IRDye 800 (Rockland, Gilbertsville, PA) diluted at 1:100,000. During laser scanning of the membranes, direct fluorescence from labeled proteins was simultaneously detected by two separate infrared detection channels of the Imaging System Odyssey (LI-COR). Proteins phosphorylated on tyrosine (Alexa 680) were detected in red, and total ERK protein was detected in green (IRDye 800). Estimation of molecular size of protein bands was made by extrapolation from standard curve of broad-range prestained SDS-polyacrylamide gel electrophoresis standards (Bio-Rad) loaded on each gel. To eliminate any variability of the amount of the loaded total protein, we normalized the phosphotyrosine signal intensities in each lane against ERK, and the relative amount of the loaded total protein, we normalized the phosphotyrosine signal intensities in each lane against ERK, and the relative intensity ratios (in arbitrary units) have been plotted in Fig. 6B.

**Results**

Stimulation of the sympathetic nerves of the guinea pig vas deferens by a single electrical impulse (duration of 0.1 ms) evokes a contractile response with low amplitude, which is mediated primarily by neurally released ATP (Sneddon et al., 1982; Sneddon and Westfall, 1984). Accordingly, this contractile response is mimicked by exogenously applied ATP (Fig. 1). Pretreatment of the tissue with SOV caused a robust increase of the amplitude of the contractile responses evoked by both a single stimulus and a bolus injection of exogenous ATP (Fig. 1). The facilitation of the purinergic contraction evoked by SOV was time-dependent. Thirty seconds after the introduction of SOV, the amplitude of the contractile response of the guinea pig vas deferens to a single impulse was tripled (data not shown). Full facilitation of the contractile response was maintained for at least 60 min (longer exposure to SOV has not been tested). The facilitation evoked by SOV was also reversible and reproducible. The control level of the amplitude of the purinergic contraction was restored within 30 min after washout of the drug. A new application of SOV produced virtually the same increase of the amplitude of contractions evoked by either EFS or ATP, and this cycle has been repeated up to five times without loss of effect.

The effect of SOV on the purinergic contractile response was also concentration-dependent. SOV (0.1–1000 μM) had no effect on the resting tone of the guinea pig vas deferens but increased the response evoked by EFS (one impulse) from 0.62 ± 0.69 to 31.06 ± 1.1 g/s, which represents a 50.2 ± 3.2-fold increase. Facilitation was observed at approximately 3 μM SOV, and the half-maximal effect was achieved at 245 ± 23 μM (Fig. 2, A and B). However, when applied in concentrations greater than 1 mM, SOV produced spontaneous contractions of the guinea pig vas deferens. To avoid unspecific effects of SOV, concentrations higher than 1 mM were not used in this study.

The contractile response of the guinea pig vas deferens evoked by exogenously applied ATP (100 μM) was increased

![Fig. 1. Effects of SOV on the contractile responses of the guinea pig vas deferens to neurogenic stimulation and exogenously applied ATP. The waveforms (top) show the contractile responses of the prostatic portion of the guinea pig vas deferens to a single impulse of EFS or bolus injection of ATP (100 μM). After recording three control contractile responses in 20-min intervals, the tissue preparations were treated with SOV (300 μM) for 10 min and stimulated again. The average area (mean ± S.E.M.) under the waveforms (g s⁻¹) from five experiments is presented (bottom). The tissues stimulated with EFS and ATP in each of these experiments were dissected from the same animal. Note the robust, approximately 36-fold increase of the responses evoked by EFS and approximately 6-fold increase of responses evoked by exogenously applied ATP.](image-url)
from 4.3 ± 0.33 to 23.7 ± 1.2 g/s in the presence of SOV (0 to 1 mM) (Fig. 3). This change represents approximately 6-fold increase over the amplitude of the control contraction. The half-maximal facilitation, derived from nonlinear regression curves was achieved with 122 ± 42 μM SOV.

We have tested the effect of SOV on the contractile response of the guinea pig vas deferens evoked by α,β mATP, a slowly degradable analog of ATP, which is also a more potent agonist of the P2X, receptors. Accordingly, application of α,β mATP at a concentration of 3 μM produced smooth muscle contraction with an amplitude of 4.6 ± 0.49 g/s, which is similar to that produced by 100 μM ATP (Fig. 3). The contractile response evoked by α,β mATP in presence of SOV was also increased in a concentration-dependent manner to a maximum of 24.5 ± 1.3 g/s (a 6-fold increase). The half-maximal facilitation was achieved with 100 ± 39 μM SOV.

The comparison of the effects of SOV on the contractions evoked by ATP and α,β mATP presented in Fig. 3 demonstrates that both agonists produce contractile responses that are similarly enhanced in the presence of increasing concentrations of SOV.

If an inhibition of a PTP was involved in the action of SOV, then pretreatment of the tissue preparations with an appropriate inhibitor of the tyrosine kinase responsible for the phosphorylation of the target protein(s) might prevent the SOV-evoked facilitation of the purinergic contractile response of the guinea pig vas deferens. To test this hypothesis, we have carried out experiments with a number of protein tyrosine kinase inhibitors. Two structurally unrelated antagonists of tyrosine kinase activity, genistein and 2,5-dihydroxycinnamic acid methyl ester, produced a dose-dependent inhibition of the SOV-evoked facilitation of the contractile
tyrosine phosphorylation in at least five bands containing proteins with estimated molecular masses of 180, 123, 97, 77, and 42 kDa (compare lane 1 with lane 3). Pretreatment with genistein inhibited the increase in tyrosine phosphorylation evoked by SOV in all those bands (Fig. 6A, lane 2). The level of tyrosine phosphorylation of proteins with molecular mass of 180 and 123 kDa observed under control conditions (lane 3) was also significantly decreased by genistein (Fig. 6B).

Evidence in the literature demonstrates that the vasoconstriction evoked by NE or selective α2-adrenoceptor agonists is inhibited by genistein (Abebe and Agrawal, 1995; Jinsi et al., 1996). These authors have suggested that tyrosine kinases may play an important role in the adrenergic contraction of vascular smooth muscle mediated via α2-adrenoceptors. We have also tested the effects of genistein on the adrenergic contraction, which in the guinea pig vas deferens is mediated via α1-adrenoceptors. Prolonged neurogenic electrical stimulation (20 s at 8 Hz) evokes a biphasic contractile response of the guinea pig vas deferens (Fig. 7). The first twitch contraction is mediated predominantly by ATP, whereas the second, tonic contraction is mediated exclusively by NE. Consistent with the evidence presented above, the purinergic twitch contraction was significantly increased in the presence of SOV (300 μM). Pretreatment with genistein (100 μM) prevented this facilitation and further reduced the purinergic twitch. The adrenergic tonic component of the contractile response, however, was affected to a much lesser extent (Fig. 7). Similar results were obtained in experiments with exogenously applied NE. There was approximately 50% increase of the amplitude of the NE-evoked contraction in the presence of SOV. The amplitude of this effect seems to be relatively modest in comparison with the 8-fold increase of the contractile response evoked by exogenously applied ATP. A moderate decrease of contractions evoked by exogenously applied NE was also observed in the presence of genistein (data not shown).

Although widely used as an antagonist of PTP, when applied in higher concentrations SOV can also inhibit other enzymes, including the Na+/K+-ATPase. To test whether effects other than inhibition of PTP might be involved in the observed facilitation of the purinergic contractile responses by SOV, we have compared the effects of SOV with that of ouabain, a selective inhibitor of the Na+/K+-ATPase. The results presented in Fig. 8 show that there are significant differences between the modes of action of the two drugs. Application of ouabain (300 μM) produced an increase of the amplitude of the neurogenic contractile response of the guinea pig vas deferens evoked by a single impulse of EFS (Fig. 8A). Approximately 15 min after the exposure to ouabain a spontaneous contraction developed, and the tissue preparations stopped responding to nerve stimulation. Application of SOV (300 μM) produced a greater increase of the amplitude of the neurogenic contractions than that produced by ouabain (Fig. 8, compare A with B). Unlike ouabain, SOV did not produce a spontaneous contraction, and its facilitatory effect continued after the tissues treated with ouabain stopped responding to neurogenic stimulation (Fig. 8A and B). Addition of genistein (100 μM) to the superfusing solution already containing SOV quickly inhibited the facilitation of the neurogenic contractile responses evoked by SOV (Fig. 8C).

It can be argued that if SOV can inhibit enzymes such as phosphatases and the Na+/K+-ATPase, it might also inhibit...
the breakdown of extracellular ATP and thus enhance its contractile action. To test this hypothesis, we studied the breakdown of extracellular ATP by the ecto-enzymes expressed in the guinea pig vas deferens. The amount of e-ATP present in the sample taken after 10 min of incubation with the tissue preparations was subtracted from the amount of e-ATP present in the buffer that has not been in contact with a tissue. The difference, measured in picomoles, represents the amount of e-ATP that has been metabolized by ecto-enzymes and reflects the sum total of the ecto-ATP-metabolizing activity of the guinea pig vas deferens. The results presented in Fig. 9 show that the plot of substrate (e-ATP) concentrations versus the ATP-metabolizing activity had a hyperbolic fit with a $K_D$ value of $414 \pm 68 \mu M$. In presence of the phosphatase inhibitor cocktail 2, which contains sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole, the observed $K_D$ value was increased to $673 \pm 170 \mu M$, whereas the $V_{max}$ value was increased from $1768 \pm 103$ to $2075 \pm 236 \text{ pmol/10 min}$. A comparison of the best fit values by $t$ test (GraphPad Prism) revealed that there were no significant differences between the ATP-metabolizing activity of the vas deferens tissue preparations under control conditions and in the presence of the phosphatase inhibitor cocktail 2 ($P < 0.05$). The results demonstrate that ATP is not significantly metabolized by the guinea pig vas deferens tissue preparations. From the initially applied $100 \mu M$ e-ATP, only $350 \text{ pM}$ was metabolized within 10 min. The evidence also shows that neither SOV nor the other phosphatase inhibitors have any significant effect on the metabolism of extracellular ATP.

**Discussion**

Before a definitive conclusion about a role of tyrosine phosphorylation in the purinergic contraction can be reached, one has to consider any additional or “side effects” of the drugs used in this study. It is well known that both SOV and genistein have multiple sites of action. For example, the
action of SOV on smooth muscle contractile response to ATP could theoretically be due to an inhibition of enzymes involved in the metabolic clearance of ATP. The extracellular metabolism of ATP has been attributed to the actions of membrane bound ecto-ATPases (Zimmermann, 2000) as well as soluble nucleotidases that are released during nerve activation (Todorov et al., 1997). It has been shown that SOV has no effect on the activity of either group of enzymes (Mihaylova-Todorova et al., 2001, 2002). However, the possibility that the breakdown of extracellular ATP by the ecto-enzymes expressed in the guinea pig vas deferens tissue preparations may involve not only ecto-ATPases but also ecto-phosphatases has not been addressed. Our results demonstrate that not only SOV but also other phosphatase inhibitors such as sodium molybdate, sodium tartrate, and imidazole, all of which are included in the phosphatase inhibitor cocktail 2 (Sigma-Aldrich) failed to affect the breakdown of ATP by the guinea pig vas deferens tissue preparations. The changes of both $K_D$ and $V_{max}$ of the enzymatic breakdown of ATP after pretreatment of the tissue preparations with the phosphatase inhibitor cocktail 2 were not statistically significant (Fig. 9). The results from this study have also demonstrated that only a minimal amount of the exogenously applied ATP has been metabolized by the guinea pig vas deferens tissue preparations even though the reaction was let to continue for 10 min. Moreover, the results shown in Fig. 3 show that SOV increases the contractile effects not only of ATP but also of its metabolically stable analog α,β mATP. Together, this evidence argues against the possibility that SOV facilitates the ATP-evoked contractile response of the vas deferens by preventing its clearance.

Another side effect of SOV might be related to its ability to block the Na⁺/K⁺-ATPase, i.e., it may produce an "ouabain-like" effect. Indeed, an inhibition of the electrogenic Na⁺/K⁺ pump could cause cell membrane depolarization and thereby an increase of the amplitude of the contractile response to ATP (Urquilla et al., 1978). However, an inhibition of the Na⁺/K⁺ pump should also produce a spontaneous contractile activity within a short period of time, after which the tissue preparation should stop responding to stimulation due to equilibration of the concentration gradients of Na⁺ and K⁺.

The results presented in Fig. 8A demonstrate that ouabain produces all these expected effects. However, the evidence from our studies argues against the possibility that an ouabain-like or another side effect such as inhibition of Ca²⁺-ATPases is involved in the facilitation evoked by SOV. First, the dose-response curve presented in Fig. 2 demonstrate that SOV facilitates the purinergic contractile response of the guinea pig vas deferens with threshold concentration in the low micromolar range and with EC₅₀ value of approximately 100 μM. SOV acts as a selective antagonist of protein tyrosine phosphatases when used in this range of concentrations (Taylor, 2003). Second, even when applied at the concentration of 300 μM, which produces near maximal facilitation effect, SOV does not trigger spontaneous contractile activity (Fig. 8B). A contractile effect should accompany any significant increase of intracellular Ca²⁺ due to increased influx of Ca²⁺ (i.e., ouabain-like effect) or inhibition of Ca²⁺ extrusion due to an inhibition of the Na⁺/K⁺ pump. Third, longer exposure to SOV (up to 60 min) did not cause a spontaneous contraction and did not abolish the response of the tissue to neurogenic stimulation or application of exogenous ATP. Fourth, genistein and 2,5-dihydroxycinnamic acid methyl ester, two structurally unrelated inhibitors of tyrosine kinase activity, abolished the facilitation of the purinergic contractions evoked by SOV. Fifth, the changes in the level of protein tyrosine phosphorylation evoked by SOV and genistein are consistent with their functional effects.

Genistein inhibits the activity of tyrosine kinases by interacting with their ATP-binding sites (Akiyama et al., 1987). In addition, it has been shown that genistein may also inhibit the function of L-VDCCs. The ATP-induced smooth muscle contraction is mediated via P2X₁ receptors, which when activated lead to the opening of L-VDCCs. The resulting influx of extracellular Ca²⁺ triggers action potentials and the twitch-like purinergic contraction (Sneddon and Westfall, 1984). It seems possible, therefore, that genistein could inhibit the purinergic contractile responses of the guinea pig...
and 42 KDa is enhanced in the presence of SOV and abolished by genistein. (Fig. 6B).

Fig. 6. Western blot analysis of protein tyrosine phosphorylation. Tyrosine-phosphorylated proteins were detected in lysates of guinea pig vas deferens tissues preparations stimulated for 1 min with ATP (100 μM) without (lane 3) or after pretreatment for 15 min with 300 μM SOV (lane 1) or 100 μM genistein + SOV (lane 2). A, phosphotyrosines were visualized in red with mouse anti-phosphotyrosine specific primary antibody (PY-99) and goat anti-mouse-Alexa Fluor 680 secondary antibody. Total ERK was visualized in green with rabbit anti-ERK1/2 primary antibody and goat anti-mouse-Alexa Fluor 680 secondary antibody. Direct fluorescence was detected by Odyssey infrared imaging system (LI-COR) equipped with dual channel simultaneous detection at 700- and 800-nm infrared emissions. The original red signal (700 nm) was converted to gray image (Fig. 6 A). The green signal from the total ERK detection is not shown. B, histogram of the integrated intensity ratios (arbitrary units of intensity of phospho-tyrosine signals normalized versus arbitrary units of intensity of total ERK2 signal). Tyrosine phosphorylation of proteins with approximate sizes of 180, 123, and 42 KDa is enhanced in the presence of SOV and abolished by genistein. (Fig. 6B).

Fig. 7. Effects of SOV and genistein on the biphasic contractile response of the guinea pig vas deferens evoked by EFS. The prostatic portions of the guinea pig vas deferens were set up for recording of contractions evoked by EFS under superfusion conditions. The tissues were stimulated for 20 s over 20-min intervals with a train of electrical impulses with 0.1-ms duration and supramaximal voltage at a frequency of 8 Hz. Top, initial twitch contraction is attributed to the postjunctional action of NE via \( \alpha \)-adrenoceptors. Both phases seem to be increased after application of SOV (300 μM) for 10 min. Addition of genistein (100 μM for 15 min) greatly reduces the purinergic twitch contraction but less so the adrenergic tonic contraction. Bottom, purinergic twitch contraction is abolished by pretreatment of genistein, which also prevents the facilitation by SOV. The adrenergic contraction, however, remains even though its amplitude is somewhat decreased.

vas deferens by blocking the P2X receptors and/or the L-VGCC directly. Several lines of evidence from studies of cardiac and vascular smooth muscle suggest that tyrosine phosphorylation may be involved in the regulation of the activity of L-VGCC in the smooth muscle of the guinea pig vas deferens as well.

Fig. 7. Effects of SOV and genistein on the biphasic contractile response of the guinea pig vas deferens evoked by EFS. The prostatic portions of the guinea pig vas deferens were set up for recording of contractions evoked by EFS under superfusion conditions. The tissues were stimulated for 20 s over 20-min intervals with a train of electrical impulses with 0.1-ms duration and supramaximal voltage at a frequency of 8 Hz. Top, initial twitch contraction is attributed to the postjunctional action of NE via \( \alpha \)-adrenoceptors. Both phases seem to be increased after application of SOV (300 μM) for 10 min. Addition of genistein (100 μM for 15 min) greatly reduces the purinergic twitch contraction but less so the adrenergic tonic contraction. Bottom, purinergic twitch contraction is abolished by pretreatment of genistein, which also prevents the facilitation by SOV. The adrenergic contraction, however, remains even though its amplitude is somewhat decreased.

Our results have demonstrated certain selectivity of the drugs used to modulate the activity of protein tyrosine kinases. From the wide spectrum of PTK inhibitors used, two, genistein and 2,5-dihydroxycinnamic acid methyl ester, were the most effective inhibitors of the SOV-evoked facilitation of the purinergic contractile response of the guinea pig vas deferens. The other three PTK antagonists, butein, levandustin C, and thyrphostin 23, were less effective in reducing the effects of SOV. This pharmacological profile of the PTK antagonists may prove valuable for the ultimate identification of the protein(s) involved in modulation the smooth muscle responsiveness to purinergic stimulation.

Interestingly, we have shown that both PTK and PTP inhibitors affected to a much greater extent the facilitation of the contractile responses produced by ATP than the contractile responses produced by the cotransmitter NE. This finding suggests that the second messenger system that mediates the smooth muscle contractile response to adrenergic stimu-
lation may be less dependent on protein tyrosine phosphorylation than is the response to purinergic stimulation. No further effort to clarify the role of protein tyrosine phosphorylation in the adrenergic contractile response of the guinea pig vas deferens has been made in this study.

Although not entirely selective at higher concentrations, the drugs used in this study seem to have only one common mechanism of action. They increase (SOV) or decrease (genistein and 2,5-dihydroxycinnamic acid methyl ester) the level of protein tyrosine phosphorylation. Therefore, the pharmacological evidence from our functional experiments, together with the evidence from our Western blot experi-

Fig. 8. Experimental demonstration of the difference between the facilitation of the neurogenic contractile responses of the guinea pig vas deferens evoked by ouabain and SOV. The prostatic portions of the guinea pig vasa deferentia were set up for recording of neurogenic contractile responses evoked by a single impulse of EFS, which was delivered at 2-min intervals. A, after recording three control responses (filled circles), the tissue preparation was superfused with ouabain (300 μM). Ouabain caused an initial increase of the amplitude of the neurogenic contraction followed by slowly developing contraction. Within 10 min, the ouabain-induced contraction reached a maximum and at that moment the tissue preparations stopped responding to nerve stimulation. B, application of SOV (300 μM) caused a greater and long-lasting increase of the amplitude of the neurogenic contraction. SOV did not produce a contractile response of its own nor did it inhibit the EFS-evoked contractions of the tissue preparation. C, addition of genistein (300 μM) to the superfusing solution already containing SOV quickly inhibited the contractile responses.

Fig. 9. Metabolism of e-ATP by the guinea pig vas deferens. The ecto-ATPase activity of the guinea pig vas deferens was determined from the difference between the initial amount of e-ATP added, and the amount of e-ATP remaining after 10-min incubation with the tissue preparations. Semilogarithmic plots of substrate concentrations (micromolar) versus ATPase activity (picromoles per 10 min) followed a hyperbolic fit with an observed $K_D$ value of 414 ± 68 μM. In presence of phosphatase inhibitor cocktail 2 (Sigma-Aldrich), which contains sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole, the observed $K_D$ value increased to 673 ± 170 μM, whereas the $V_{max}$ value showed little change (from 1768 ± 103 to 2075 ± 236 pmol/10 min). Comparison of best-fit values by $t$ test failed to reveal significant differences between control and the phosphatase inhibitor-treated curves.
ments seem to suggest that tyrosine phosphorylation of a protein(s), whose identity and signaling pathway are not known at this time, is involved in the regulation of the amplitude of the purinergic smooth muscle contraction. This novel element of the purinergic signaling in the smooth muscle may prove to be an appropriate pharmacological target for effective management of smooth muscle contractility.

References


Address correspondence to: Dr.Latchezar D. Todorov, Department of Pharmacology, University of Nevada School of Medicine, 1664 N Virginia St., Howard Medical Sciences Bldg. MS 318, Reno, NV 89557-0270. E-mail: toodorov@med.unr.edu