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ATP-INDUCED Ca^{2+} -SIGNALING ENHANCES RAT GASTRIC MICROVASCULAR ENDOTHELIAL CELL MIGRATION

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The effects of exogenous ATP on Ca^{2+} signaling and wound healing were investigated in rat gastric microvascular endothelial cells (RGMEC). ATP (10 μ M) triggered a significant rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from 46 ± 2 nM at baseline to peak values averaging 283 ± 31 nM ($n = 5$ experiments, 132 cells). Return to the basal $[Ca^{2+}]_i$ was delayed by slowly declining plateau phase that persisted for 200 ± 30 s. Removal of extracellular Ca^{2+} did not significantly affect the peak rise in $[Ca^{2+}]_i$, but reduced the plateau. ATP (10 μ M) also significantly increased the migration of RGMEC in a wounded monolayer. Addition of the non-subtype selective purinergic receptor antagonist, suramin, abrogated the effects of ATP on $[Ca^{2+}]_i$ and migration. We conclude that local elevation of ATP acting through purinergic receptors induce Ca^{2+} signals in RGMEC and may contribute to endothelial cell migration.

Key words: *gastric endothelial cells, ATP, calcium signaling, suramin wound repair, angiogenesis*

INTRODUCTION

Angiogenesis, leading to restoration of the microvasculature, is required for tissue wound and ulcer healing (1, 2). The microvascular network of capillaries, and collecting venules is essential for supplying the regenerating tissue with oxygen and nutrients as well as for the removal of toxic metabolic products (3). In the stomach, inhibition of the angiogenic response to ethanol-induced injury (4) by cyclooxygenase inhibitors (5) or antagonists of endothelial growth factors (4, 6, 7) delays ulcer healing and promotes the formation of gastric mucosal erosions. Overall, angiogenesis is controlled by a balance between stimulatory and inhibitory factors and by the interaction of the endothelial cells with their microenvironment (8–10). The autocrine and paracrine actions of angiogenic

growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and the angiopoietins are now well established (11–13). In response to these factors endothelial cells migrate, proliferate, and form the endothelial tubes and capillary structures that undergo transformation into capillary vessels leading to the restoration of the microvascular network. Hence, migration of endothelial cells is crucial to angiogenesis and endothelial wound repair. Yet, the full range of molecules that induce migration remains to be explored. The release of intracellular ATP through cytolysis, exocytotic secretion or membrane transport elevates the concentration of extracellular ATP at wound sites to the range of 50–100 μM (14). ATP can act as a potent chemotactic agent and extracellular signaling molecule through an interaction with cell membrane receptors, which are designated as purinergic receptors and expressed by many cell types including endothelial cells. The nucleotide receptors, designated P₂, comprise a large multi-gene family, which can be divided into two subgroups: 1) P₂X receptors, ATP-regulated ion channels, and 2) P₂Y receptors, G protein-coupled receptors (15–17). Recent studies have demonstrated that suramin, a potent inhibitor of both types of P₂ receptors, is an anti-angiogenic agent (18–20). While this action of suramin has been attributed to inhibition of VEGF and bFGF binding to their receptors (21–25), the possibility that it inhibits ATP-induced purinergic stimulated endothelial cell migration has not been examined. Purinergic receptor isotypes are heterogeneously expressed throughout the vascular system (26, 27). However, their presence in the gastric microvasculature has not been extensively studied. Therefore, to ascertain direct relevance to the gastric mucosa, we examined the effects of ATP on gastric microvascular endothelial cells. In this work, we have characterized the actions of ATP on Ca²⁺ signaling in rat gastric microvascular endothelial cells (RGMEC) and determined the effects of purinergic receptor blockade by suramin on Ca²⁺ signaling and RGMEC migration.

MATERIALS AND METHODS

Chemicals and Solutions

Basic fibroblast growth factor (bFGF) was obtained from Life Technologies, (Grand Island, NY). Ionomycin was obtained from Calbiochem (San Diego, CA). Fura-2 acetoxy-methoxy ester and thapsigargin (TG) were obtained from Molecular Probes (Eugene, OR). All other salts and reagents were obtained from Sigma-Aldrich Inc. (St. Louis, MO).

Cell Culture

Rat gastric microvascular endothelial cells (RGMEC) were isolated as previously described (28). In brief, the gastric mucosa from the stomachs of normal rats was sequentially digested. First, mucosal fragments were digested in sterile Hank's buffered saline solution (HBSS) containing 1 mg/ml type II collagenase (Collaborative Biochemicals, Two Oak Park, MA) and 0.1% BSA (Sigma Chemicals, St. Louis, MO) for 1 hour at 37°C. Second, the fragments were placed in HBSS containing 1 mM EDTA and 0.5% trypsin for 15 min at 37°C. After removal of the enzyme solution cells were harvested by centrifugation and sieving through polypropylene meshes (Spectra, Laguna Hills, CA). Endothelial cells were isolated via magnetic separation utilizing rat specific anti-PECAM-1 antibody (Chemicon, Temecula, CA) bound to magnetic Dymabeads M-450 (Dynal Inc, Lake Success, NY) as originally described in (29). The RGMEC demonstrated positive immunostaining for rat endothelial cell-specific antigens P-selectin and Tie-2. Cells were maintained in culture in Endothelial Serum Free Medium (Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 µg/ml heparin (Collaborative Biochemicals, Two Oak Park, MA) and antibiotic/antimycotic (Life Technologies, Grand Island, NY).

Calcium imaging

Twenty four hours before imaging, RGMEC were subcultured onto glass coverslips coated with rat tail collagen type V (Collaborative Biochemicals, Two Oak Park, MA) and allowed to grow to 50–70% confluence. RGMEC cells were loaded with the calcium sensitive dye fura-2 by placing them in media containing 3 µM of fura-2/AM ester and 2.5 mM probenecid for 30 minutes at 37°C in the incubator. After the initial incubation, the cells were washed 3 × with RGMEC growth media (described in *Cell Culture*) that also contained 2.5 mM probenecid and returned to the incubator for an additional 30 minutes to complete the hydrolysis of the fura-2 ester. After fura-2 loading, all cells were stored at room temperature in the dark until use. Before imaging, media were exchanged with mammalian Ringer containing (in mM): 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and Hepes (pH = 7.4; osmolality 290–310 mOsm/kg). In those experiments where Ca²⁺ free medium was used, CaCl₂ was omitted and 1 mM EGTA was added to the Ringer solution. The cells were transferred to a heated stage (Warner Instruments, Hamden, CT) on a Nikon TMD inverted microscope (Nikon USA, Melville, NY). For the imaging experiments, the cells maintained at 34 ± 2°C. Illumination for the imaging experiments was provided by a xenon arc-lamp (Sutter Instruments, Petaluma, CA) and transmitted through a filter wheel (Metaltech, Empix Imaging, Mississauga, ON, Canada) containing 340- and 380-nm excitation filters. A 400-nm dichroic mirror reflected the filtered light through a 40 X Nikon objective to illuminate the cells. Emitted light above 480 nm was received by a SIT camera (C2400, Hammamatsu Photonics, Bridgewater, NJ) and the video information was relayed to an image processing system (MetaFluor, Universal Imaging, West Chester, PA). Full field-of-view 8-bit images, averaged over 16 frames, were collected at 340- and 380-nm wavelengths. Digitally stored 340/380 ratios were constructed from background-corrected 340- and 380-nm images. Single-cell measurements of [Ca²⁺]_i were calculated from the 340/380 ratios using the equation of Grynkiewicz *et al.* (30) and a K_d of 245 nM for fura-2. The minimum 340/380 ratio was measured in single cells after incubation for 10 min in Ca²⁺-free Ringer containing 2 mM EGTA, 1 µM TG, and 10 µM ionomycin. Maximum ratio values were obtained after perfusion with Ringer containing 10 mM Ca²⁺, 1 µM TG, and 10 µM ionomycin.

Cell migration assay

Twenty-four hours before wounding assay, RGMEC were subcultured onto 6-well plates at a density of 1.5×10^5 cells per well. The cells were then grown to confluence. Before wounding, the monolayer culture was washed with migration medium (Endothelial-SFM containing L-glutamine, 0.1% BSA, and 100 $\mu\text{g}/\text{ml}$ heparin). The intact monolayers of RGMEC were wounded with an 8-mm wide longitudinal cut by a razor blade, washed again with fresh migration media and returned to the incubator for a 15 min equilibrium. Subsequently, cells were treated with either vehicle or 10 μM ATP or 10 ng/ml bFGF in the presence or absence of 100 μM suramin. In the experiments using suramin, the inhibitor was added to the cells 15 min prior to wounding and maintained in the media for the length of the assay. RGMEC migration in the wounded monolayers was evaluated 48 hrs after treatment. Briefly, after fixation in methanol and staining with hematoxylin and eosin, migration was determined by measuring the area of cells that migrated into the wound using a video imaging system. Phase contrast digital images of a low power field were acquired with the $4 \times$ objective using a Nikon inverted microscope. Migration area was measured using MetaMorph software (Universal Imaging, West Chester, PA). The values were expressed as a mean \pm SEM from three independent experiments.

Data analysis

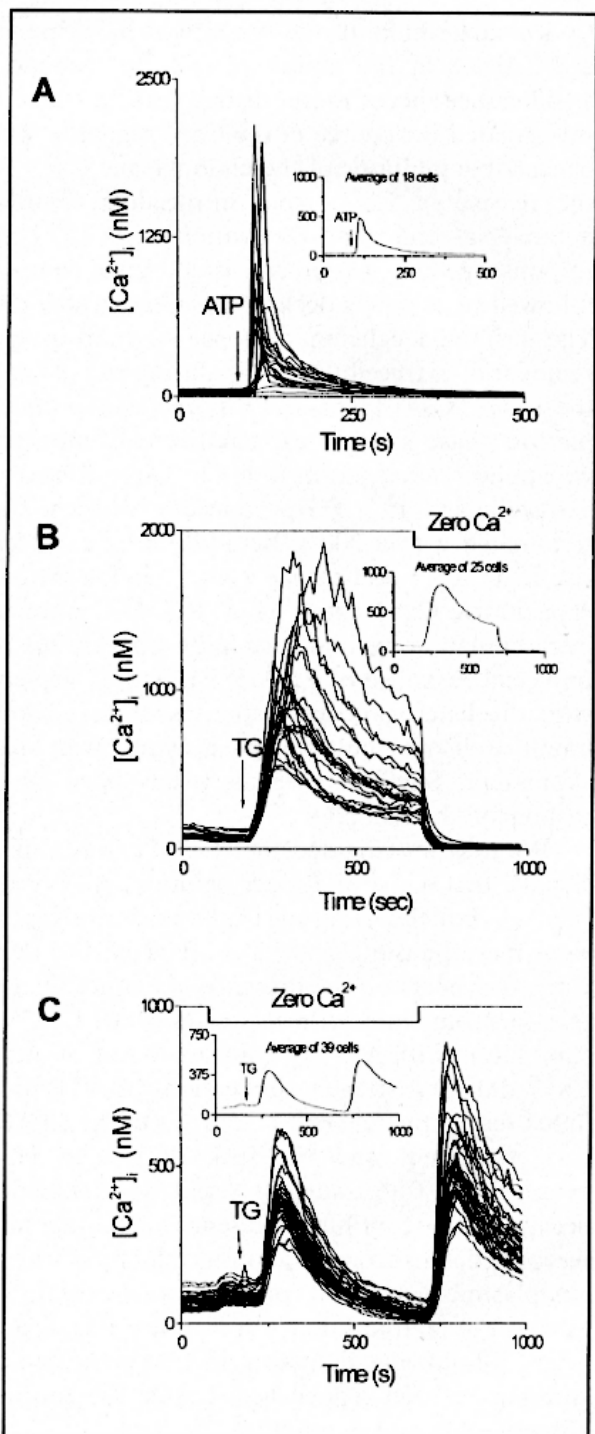
Numerical values for single-cell $[\text{Ca}^{2+}]_i$ tracings were analyzed using the data analysis and technical graphics program, Origin (Microcal, Northampton, MA). Statistical analysis was performed on data sets using Excel 5.0 (Microsoft, Redmond, WA). Data are reported as mean \pm standard deviation. Multi-sample hypotheses were tested by single-factor analysis of variance (ANOVA) and performing Newman-Kuels multiple range tests to determine the significance of difference between pairs of means. Data were considered statistically different if p was < 0.05 .

RESULTS

Purinergic stimulation induces Ca^{2+} signaling in RGMEC

ATP (10 μM) induced an increase in the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in rat gastric microvascular endothelial cells (RGMEC) within 30 seconds of application (*Figure 1 A*). Return to basal Ca^{2+} levels was delayed by a slowly declining plateau phase that persisted for 200 ± 30 s. Single-cell analysis of the Ca^{2+} signals from RGMEC revealed a significant variability in the amplitude of the peak Ca^{2+} response induced by ATP. Some cells attained a $[\text{Ca}^{2+}]_i$ greater than 1 μM and others had no measurable response resulting in a population response that was significantly less than that of the highest responders (*Fig. 1 A, inset*). The average basal $[\text{Ca}^{2+}]_i$ for all cells studied was 46 ± 2 nM. ATP triggered a rise in $[\text{Ca}^{2+}]_i$ to peak values averaging 283 ± 31 nM ($n = 132$ cells, 5 experiments).

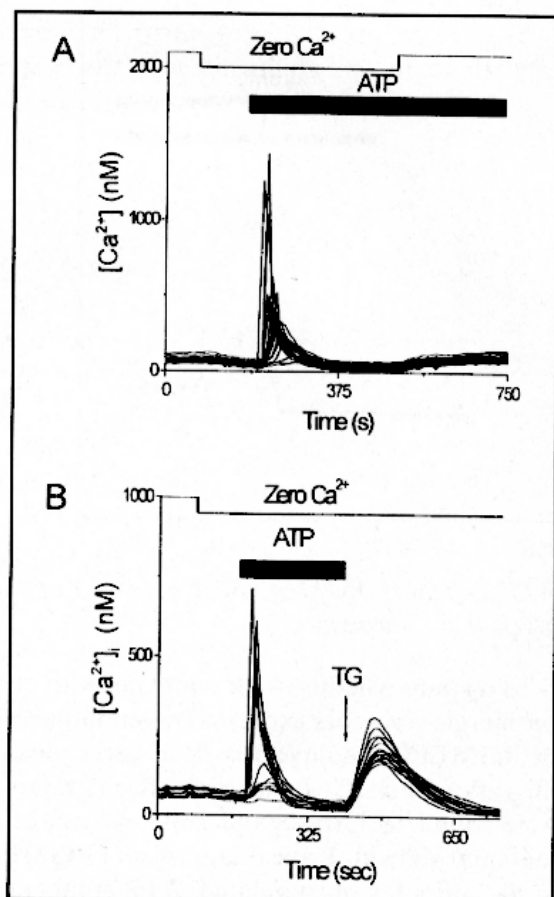
Fig. 1. Application of ATP or thapsigargin induces elevations in $[Ca^{2+}]_i$ in RGMEC. $[Ca^{2+}]_i$ was measured as described in Materials and Methods under "Calcium imaging". (A) Representative recordings of $[Ca^{2+}]_i$, before and following the application of ATP (arrow). Basal $[Ca^{2+}]_i$ was determined in mammalian Ringer. At 100 s, 10 μ M ATP was added to the Ringer (arrow). The amplitude and duration of ATP-evoked calcium signal varies significantly among the cells in the field as illustrated by single cell traces and the average response of the population (*inset*). (B) Thapsigargin (TG)-stimulated mobilization of $[Ca^{2+}]_i$ was measured as described for ATP. Representative tracings are shown and average data from all cells on the field are presented in the inset. After basal $[Ca^{2+}]_i$ was determined in mammalian Ringer, TG (1 μ M) was applied to the bath (arrow). The dependence of the sustained rise in $[Ca^{2+}]_i$ on extracellular Ca^{2+} was tested by replacing the mammalian Ringer solution with Ca^{2+} -free Ringer containing 1 mM EGTA (Zero Ca^{2+} , *application bar*). (C) Following the determination of the basal $[Ca^{2+}]_i$, RGMEC were perfused with Ca^{2+} -free Ringer (*application bar*), TG was added (arrow) in the absence of extracellular Ca^{2+} to measure internal Ca^{2+} release (first increase in fluorescence). Ca^{2+} was then re-added to measure the influx component (*application bar*, second increase in fluorescence). For clarity, the Ca^{2+} application bars were omitted from the insets.



To determine if the variation in response among RGMEC reflected a difference in the ability of cells to respond to purinergic stimulation or a difference in a more distal part of their Ca^{2+} -signaling pathway, we investigated the source of the Ca^{2+} signal in RGMEC. Using thapsigargin, an irreversible inhibitor of the endoplasmic Ca^{2+} ATPase, we directly stimulated the release of Ca^{2+} from intracellular stores. This treatment resulted in a universal and robust elevation of $[\text{Ca}^{2+}]_i$ in RGMEC (Figure 1B). The response to thapsigargin consisted of two components: an initial peak response followed by a slowly declining plateau, which lasted for more than 10 minutes. The sustained calcium response to thapsigargin required Ca^{2+} -influx since removal of extracellular Ca^{2+} during the plateau resulted in a rapid return to the basal $[\text{Ca}^{2+}]_i$ (Figure 1B, application bar). This result suggests that the plateau phase is due to capacitative calcium entry (31). To assess this possibility, we applied thapsigargin in a Ca^{2+} -free Ringer (Figure 1C). In the absence of extracellular Ca^{2+} , TG produced a transient Ca^{2+} response in RGMEC that terminated within 300 s. Re-addition of extracellular Ca^{2+} caused a vigorous rise in $[\text{Ca}^{2+}]_i$ indicating a Ca^{2+} influx pathway as would be expected for capacitative Ca^{2+} entry. Thus, RGMEC were capable of releasing Ca^{2+} from intracellular stores and sustaining Ca^{2+} influx following stores depletion. The universal response of RGMEC to stores depletion by thapsigargin compared with the heterogeneous responses observed following stimulation with ATP might indicate multiple phenotypes with respect to purinergic receptor expression. Such heterogeneity has been observed in the cerebrovascular system of the rat (26).

We next investigated the role of extracellular Ca^{2+} in ATP-induced Ca^{2+} signals. In Ca^{2+} -free Ringer solution, ATP was able to evoke an increase in $[\text{Ca}^{2+}]_i$, but the duration of the response was abbreviated and the transients were monophasic (Figure 2A). The partial dependence of the ATP-induced Ca^{2+} transients on the presence of extracellular Ca^{2+} suggests that both Ca^{2+} release from intracellular stores and Ca^{2+} influx are produced during stimulation with ATP. In contrast to our results with thapsigargin, repletion of Ca^{2+} during sustained applications of ATP did not evoke a secondary Ca^{2+} influx (compare Figure 1C and 2A). The short duration of the ATP-induced Ca^{2+} transient and the lack of rise in $[\text{Ca}^{2+}]_i$ upon the repletion of extracellular Ca^{2+} suggest rapid desensitization of the purinergic receptor occurs in the continued presence of the agonist. To test the possibility that receptor desensitization allowed refilling of the endoplasmic Ca^{2+} stores from cytoplasmic Ca^{2+} , we probed store content after ATP-stimulation in the absence of extracellular Ca^{2+} with thapsigargin (Figure 2B). The rise in $[\text{Ca}^{2+}]_i$ following thapsigargin treatment demonstrates that the intracellular stores have been replenished despite the continued presence of ATP and the absence of extracellular Ca^{2+} .

Fig. 2. ATP releases Ca^{2+} from intracellular stores. (A) RGMEC were stimulated with $10 \mu\text{M}$ ATP (application bar) in the absence of extracellular calcium resulting in a Ca^{2+} transient. During continuous application of ATP, repletion of extracellular Ca^{2+} did not result in Ca^{2+} influx (application bar). (B) Similar to (A), except that the cells were challenged with $1 \mu\text{M}$ TG in Ca^{2+} -free solution following the completion of the ATP-induced Ca^{2+} transient.



Effects of suramin on the ATP-induced intracellular Ca^{2+} rise in RGMEC

Suramin, a potent and non-subtype selective purinergic receptor antagonist, inhibited ATP-induced stimulation. The sensitivity of the ATP-induced Ca^{2+} response to suramin was investigated in RGMEC in 5 independent experiments. Because of the slow onset kinetics of P2 receptor blockade by suramin (15), the antagonist was allowed to equilibrate with the cells before application of ATP (Figure 3, application bars). As illustrated in Figure 3, the rise in $[\text{Ca}^{2+}]_i$ evoked by ATP in the presence of suramin was reduced by 94%. In the presence of $100 \mu\text{M}$ suramin, the baseline $[\text{Ca}^{2+}]_i$ was 66 ± 5 nM and $10 \mu\text{M}$ ATP produced a rise in $[\text{Ca}^{2+}]_i$ to only 78 ± 7 nM ($n = 86$) in comparison with an increase to 283 ± 31 nM ($n = 121$) when ATP was applied in the absence of suramin. Importantly, subsequent application of $1 \mu\text{M}$ TG produced a strong rise in $[\text{Ca}^{2+}]_i$, indicating that suramin blocked the effects of ATP at the receptor level and did not affect the downstream Ca^{2+} signaling pathway in RGMEC.

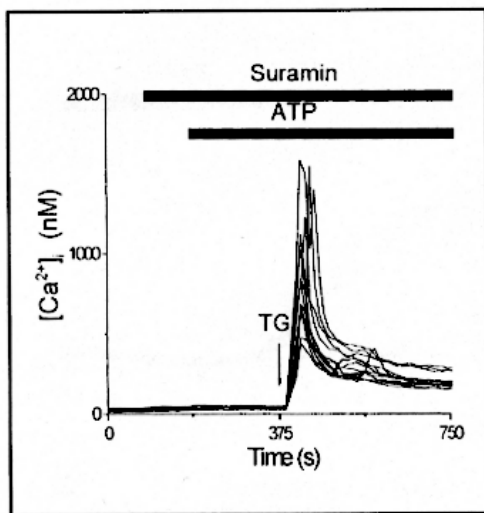


Fig. 3. Suramin blocks $[Ca^{2+}]_i$ signaling elicited by ATP. $[Ca^{2+}]_i$ was measured as described in Materials and Methods under "Calcium imaging". After measuring the basal $[Ca^{2+}]_i$ concentration, suramin (100 μ M) was added at 100 s (application bar) and had no significant effect on $[Ca^{2+}]_i$. ATP (10 μ M) failed to induce a Ca^{2+} transient when applied after suramin (application bar). Application of TG (1 μ M) to RGMEC in the continued presence of suramin demonstrates that the cells were capable of releasing Ca^{2+} from intracellular stores and producing capacitative calcium influx.

ATP enhances RGMEC migration in response to wounding, an effect that is blocked by suramin

To study whether ATP contributes to endothelial wound injury repair via purinergic receptors expressed on endothelial cells, we created an 8 mm wound in RGMEC monolayers with a sterile razor blade and monitored RGMEC migration into the wound area. After 12 hours in culture, cells at the wound edge were found to form cytoplasmic extensions protruding into the wound area (data not shown). Typical examples of RGMEC migration into the wound areas at 48 hours for unstimulated, ATP-stimulated and bFGF-stimulated cells are shown in Figure 4 A. After 48 hours, RGMEC had migrated a distance of several cell diameters into the wound area. Inclusion of 10 μ M ATP in the media after the wounding markedly enhanced the ability of RGMEC to migrate into wounds. The extent of migration induced by ATP was nearly equivalent to that produced by 10 nM bFGF suggesting that ATP could be a potent angiogenic agent. Pre-equilibrating the wounded culture with 100 μ M suramin abrogated this effect of ATP. In agreement with previous studies (21–24), 100 μ M suramin also inhibited the stimulatory effect of bFGF. Suramin had no effect on the RGMEC migration into the wound area under the non-stimulated conditions. These observations were confirmed by measuring the area covered by the migrating RGMEC at 48 hrs (Figure 4 B). In untreated monolayers, RGMEC covered $0.11 \pm .02$ mm²/mm wound ($n = 18$). Treatment with ATP more than doubled the area covered by migrating RGMEC to $0.24 \pm .01$ mm²/mm wound ($n = 25$, $p < .001$). Similarly, bFGF stimulated the migration of RGMEC resulting in migration area of $0.27 \pm .02$ mm²/mm wound ($n = 15$, $p < .001$). Suramin significantly inhibited the migration induced by either ATP

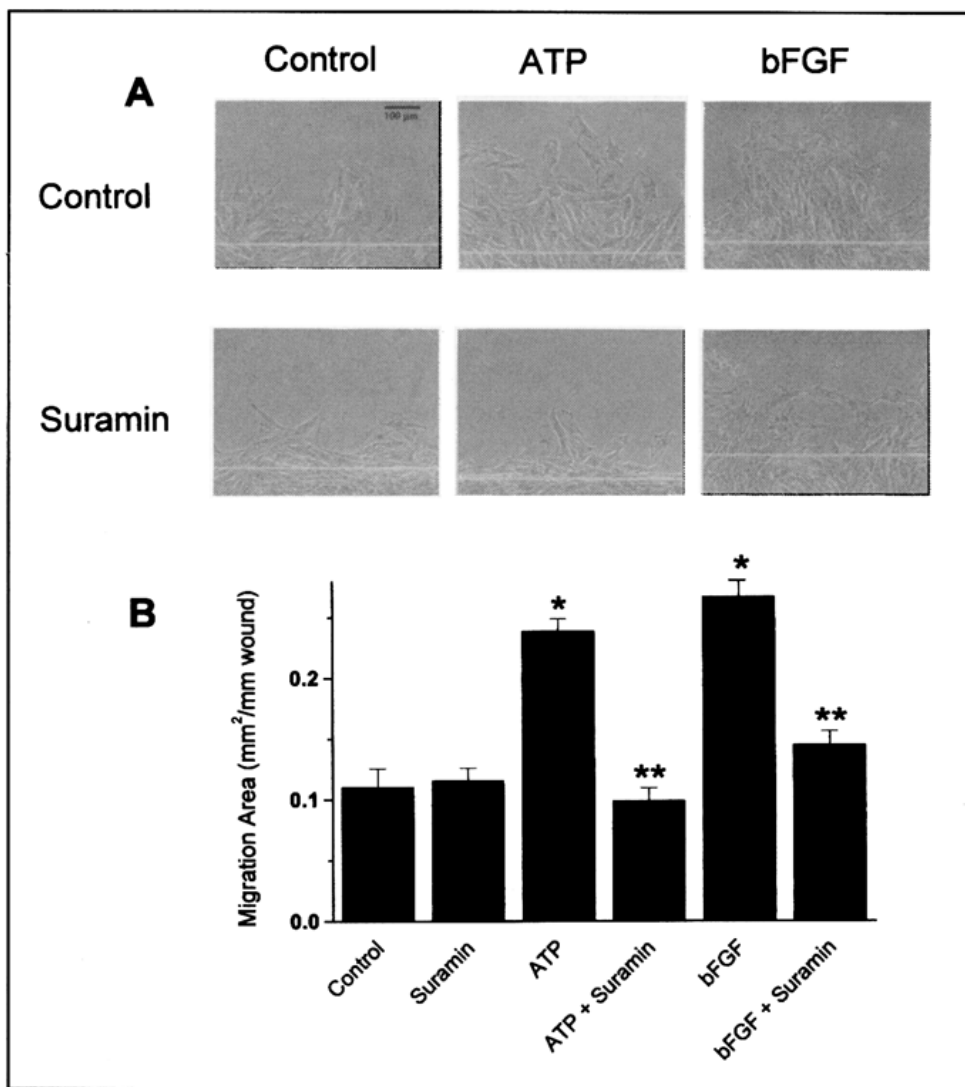


Fig. 4. Purinergic stimulation of RGMEC migration. (A) Effect of ATP, bFGF and suramin on RGMEC monolayer wound repair. RGMEC monolayer cultures were wounded with razor blade and the media was exchanged with migration media as described in Materials and Methods under *Migration Studies*. ATP (10 μ M), bFGF (10 ng/ml) and suramin (100 μ M) were added to the culture alone or in the combinations indicated. When the effect of suramin on ATP- or bFGF-induced RGMEC migration was tested, suramin was added 15 minutes prior to the other agents. Representative images are shown from each group at 48 hrs after treatment. (B) Inhibition of ATP- or bFGF-stimulated RGMEC migration by suramin. The RGMEC migration was quantified as described in Materials and Methods under "Migration Studies". Concentrations of all agents are as described in (A) and the columns represent the quantification of the area of cell migration into the wound, normalized for the width of the wound. Data are presented as average from three independent experiments with 3 replicates in each experiment and the error bars represent standard error of the mean. Overall differences were compared by the ANOVA and differences between individual means were compared by a Newman-Keuls multiple range test; (*) significant difference with respect to control, $p < 0.001$; (**) significant decrease in migration from that induced by ATP or bFGF in the absence of suramin, $p < 0.001$).

or bFGF to 0.1 ± 0.01 mm²/mm wound ($n = 24$, $p < .001$) and 0.15 ± 0.01 mm²/mm wound ($n = 15$, $p < .001$), respectively. Thus, our data demonstrate that both ATP-induced RGMEC migration and Ca²⁺ signaling are potently inhibited by the purinergic antagonist, suramin.

DISCUSSION

Our study showed that ATP elicits Ca²⁺ signaling in RGMEC. The [Ca²⁺]_i rise triggered by ATP is composed of two components: an initial release of Ca²⁺ from intracellular stores and a subsequent Ca²⁺ influx. In addition, ATP induces RGMEC migration into a wounded monolayer and the extent of migration induced by ATP is equivalent to that produced by bFGF. Receptor blockade with the P2 receptor antagonist suramin inhibits both the Ca²⁺-signaling and endothelial cell migration produced by ATP.

At sites of tissue injury the free extracellular ATP concentration rises to a level of 20 μM within 3 to 7 minutes following the injury (14). Much of the initial rise can be accounted for by the release of adenine nucleotides following the cytolysis of injured endothelial and smooth muscle cells, which contain 5–10 mM ATP. Furthermore, significant quantities of ATP are released from platelets, basophilic leukocytes, and mast cells that are recruited to sites of injury (16). The number of platelets found at wound sites contains enough releasable ATP and ADP to raise serum ATP/ADP concentrations to 50 μM (32). Interestingly, stimulation of mast cells evokes the release of ATP in sufficient quantities to stimulate purinergic receptors on surrounding cells (33). These multiple sources of released ATP suggest significant amounts of ATP can accumulate within vascular and extravascular sites of tissue injury.

The role of purinergic stimulation in cell migration is complex. Recently, it has been demonstrated that purinergic stimulants act as chemoattractants for human neutrophils (34) and eosinophils (35). In rat mucosal mast cells, stimulation of P₂U (P₂Y₂) receptors as defined by nucleotide and pertussis toxin sensitivity elicits chemotaxis (36). However, in endothelial cells derived from guinea pig cardiac vasculature, ATP was ineffective in inducing chemotaxis (37). In addition to having direct effects on P2 receptors, adenine nucleotides can be converted to adenosine by the action of cell surface nucleotidases. The generated adenosine can then induce calcium signaling, DNA synthesis, cell proliferation and migration by binding to endothelial P1 receptors (38–43). Since suramin, which does not inhibit P1 receptors (16, 44), completely blocks the calcium signaling and migration in RGMEC, we conclude that the effects we observe are modulated by P2 receptors.

Endothelial cells display significant phenotypic heterogeneity (45, 46). In the rat, endothelial cells isolated from different regions of the vascular system

express different receptor purinergic subtypes (26). Microvascular and macrovascular endothelial cells from the same organ can express different receptor populations. These molecular differences may reflect differences in the physiological demands placed in cells at different anatomical sites. Furthermore, the complement of purinergic receptors expressed in a given cell type is a dynamic function of the state of cell differentiation. For example, in vascular smooth muscle cells a shift from a contractile to a proliferative phenotype was accompanied by a change in dominance from P2X to P2Y receptors (47). Thus it is crucial to examine the function of purinergic receptors in the endothelial cells of the tissue of interest. Our study represents the first description of the effect of purinergic receptor stimulation on calcium signaling and migration in gastric microvascular endothelial cells. The fact that ATP is released at wound sites in concentrations that evoked calcium signaling and migration of RGMEC suggests that ATP may participate in both the angiogenesis and the microvascular repair required for gastric wound healing. Our data also provide an alternative mechanism to explain the anti-angiogenic effects of suramin. In addition to inhibiting the binding of VEGF and bFGF to their receptors, suramin may act by inhibiting endothelial repair signals transduced by purinergic receptors.

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