Signal Transduction of Physiological Concentrations of Vasopressin in A7r5 Vascular Smooth Muscle Cells

A ROLE FOR PYK2 AND TYROSINE PHOSPHORYLATION OF K+ CHANNELS IN THE STIMULATION OF Ca2+ SPIKING*

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The signal transduction pathway linking physiological concentrations of [Arg8]vasopressin (AVP) to an increase in frequency of Ca2+ spiking was examined in confluent cultures of A7r5 vascular smooth muscle cells. Immunoprecipitation/Western blot studies revealed a robust increase in tyrosine phosphorylation of the non-receptor tyrosine kinase, PYK2, in A7r5 cells treated with 4β-phorbol 12-myristate 13-acetate or ionomycin. 100 pm AVP also induced PYK2 tyrosine phosphorylation, and this effect was inhibited by protein kinase C inhibitor Ro-31-8220 (1–10 μM) or chelerythrine chloride (1–20 μM). In fura-2-loaded A7r5 cells, the stimulation of Ca2+ spiking by 100 pm AVP or 1 nm 4β-phorbol 12-myristate 13-acetate was completely blocked by PP2 (10 μM, a Src family kinase inhibitor). Salicylate (20 mM, recently identified as a PYK2 inhibitor) and the tyrosine kinase inhibitor, tyrphostin A47 (50 μM), but not its inactive analog, tyrphostin A63, also blocked AVP-stimulated Ca2+ spiking. PYK2 phosphorylation was inhibited by both PP2 and salicylate, whereas tyrosphostin A47 failed to inhibit PYK2 tyrosine phosphorylation. ERK1/2 kinases did not appear to be involved because 1) 100 pm AVP did not appreciably increase ERK1/2 phosphorylation and U-0126 (2.5 μM) did not inhibit AVP-stimulated Ca2+ spiking; and 2) epidermal growth factor (10 nm) robustly stimulated ERK1/2 phosphorylation but did not induce Ca2+ spiking. Delayed rectifier K+ channels may mediate the PYK2 activity because Kv1.2 channel protein co-immunoprecipitated with PYK2 and tyrosine phosphorylation of Kv1.2 was stimulated by AVP and inhibited by Ro-31-8220, PP2, and salicylate but not tyrphostin A47. Our findings are consistent with a role for PYK2 and phosphorylation of K+ channels in the stimulation of Ca2+ spiking by physiological concentrations of AVP.

Periodic or oscillatory increases in cytosolic free [Ca2+] ([Ca2+]i) in vascular smooth muscle cells are believed to underlie arterial vasomotion. These rhythmic contractions of resistance arteries and arterioles are important for local perfusion of tissues (1) as well as a determinant of blood pressure and peripheral resistance (2). Vasomotion correlates with action potentials in the smooth muscle cells of the artery wall (3–7). This activity depends on activation of L-type voltage-sensitive Ca2+ channels (5, 7) and may be triggered or enhanced by vasoconstrictor hormones (3, 8–12). The mechanisms whereby vasoconstrictor hormones stimulate Ca2+-dependent action potentials in vascular smooth muscle cells have not been elucidated.

AVP is a potent vasoconstrictor peptide. It binds to heptahedral V1a vasopressin receptors on vascular smooth muscle cells, leading to G protein-dependent activation of phospholipase C (PLC) and the consequent release of Ca2+ from intracellular stores. This signal transduction pathway is activated independently of L-type voltage-sensitive Ca2+ channels (13) and requires nanomolar concentrations of AVP for half-maximal activation (14, 15). We have identified previously a novel signal transduction pathway in A7r5 vascular smooth muscle cells that is activated by physiological concentrations of AVP (between 10 and 100 pm) and leads to oscillations of [Ca2+]i that increase in frequency with [AVP] (15, 16). This effect of low [AVP] is dependent on L-type voltage-sensitive Ca2+ channels (15) and correlates with action potential generation (16), suggesting that it may represent an effect equivalent to stimulation of arterial vasomotion in vivo. We have recently shown that AVP-stimulated Ca2+ spiking in A7r5 cells involves phospholipase D (17) and activation of one or more protein kinase C (PKC) isoforms (18). It remains to be elucidated how activation of PKC ultimately produces Ca2+ spiking. One possibility is that PKC activation leads to membrane depolarization and consequently to activation of L-type voltage-sensitive Ca2+ channels. We have preliminary data that suggest that inhibition of delayed rectifier K+ channels (Kc channels) may provide the trigger for L-type Ca2+ channel activation (16). The present study examines the possibility that one or more tyrosine kinases may serve as intermediary links in this signaling cascade. In particular, we focus on the non-receptor tyrosine kinase PYK2 (proline-rich tyrosine kinase 2, also known as RAFTK or CADTK), a member of the focal adhesion kinase family (p125FAK). This paper is available online at http://www.jbc.org/
vated by stimuli that increase [Ca$^{2+}$], or activate PKC in cultured rat aortic smooth muscle cells (19–21). PYK2 has also been linked with inhibition of delayed rectifier K$^{+}$ channels in non-muscle cells (22, 23).

Src family kinases (SKFs) and epidermal growth factor receptors (EGFR) are tyrosine kinases that have been implicated as activators and/or downstream mediators of PYK2 in other systems (19, 21, 24–26). Activation of PYK2 is associated with its autophosphorylation on tyrosine 402. This phosphotyrosine moiety may then serve as a docking site for the SH2 domain of Src (22, 26). Another tyrosine residue in PYK2 (Tyr-881) may also be phosphorylated and serve as a docking site for Grb2, leading to activation of ERK1/2, members of the family of mitogen-activated protein kinases (MAPKs) (22, 26). The roles of SKFs and transactivation of EGFR or MAPKs in AVP-stimulated Ca$^{2+}$ spiking have not been examined previously. The results of the present study are consistent with roles for SKFs and PYK2 activation leading to tyrosine phosphorylation of delayed rectifier K$^{+}$ channels in this novel signal transduction pathway. However, activation of EGFR or ERK1/2 does not appear to be either necessary or sufficient to induce Ca$^{2+}$ spiking in A7r5 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media were from Invitrogen or MediaTech (Herndon, VA). Fura-2-AM, fura-2 pentapotassium salt, fluo-3-AM, and Pluronic P127 were from Molecular Probes, Inc. (Eugene, OR). Monoclonal anti-PKC and anti-PYK2 antibodies and polyclonal anti-phosphotyrosine antibodies were from Transduction Laboratories (San Diego, CA). Monoclonal anti-KV1.2 and anti-phosphophorysine (clone 4G10) and polyclonal anti-PYK2 were from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-KV1.2 channel antibodies were from Chemicon (Temecula, CA). Monoclonal anti-Src antibodies were from Oncogene Research Products (San Diego, CA). Anti-phospho-ERK antibodies were from Promega (Madison, WI). AVP, epidermal growth factor, salicylate, and ionomycin were from Sigma. 4j-Phorbol 12-myristate 13-acetate, chelerythrine chloride, tyrophostin A47 and A63, and Ro-31-8220 were from Calbiochem. U-0126 was from Biomol (Plymouth Meeting, PA). Pefabloc SC was from Roche Molecular Biochemicals.

**Cell Culture**—A7r5 cells were cultured as described previously (13). Cells were subcultured onto rectangular (9 × 22-mm, number 15) glass coverslips or plastic tissue culture dishes (Corning Glass). Confluent cell monolayers were used 2–5 days after plating. The standard curve (using SigmaPlot® software, SPSS Inc., Chicago, IL) and used to convert ratios (r) into [Ca$^{2+}$], In situ calibration of fura-2 fluorescence by direct determination of minimum and maximum ratios ($R_{min}$ and $R_{max}$, respectively (29)) from within cells yields similar calibrated values. Traces shown are representative of at least three similar experiments.

**Immunoprecipitation and Western Blotting**—A7r5 cells were grown to confluence on 100-mm tissue culture dishes (Corning Glass). Cells were washed and incubated in control medium (see above) for 3 h at room temperature, followed by treatment for the indicated time in control medium ± agonist. The medium was aspirated, and 0.8 ml of ice-cold lysis buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 1.5 mM MgCl$_2$, 10% glycerol, 150 mM NaCl, 10 mM MgCl$_2$, 150 mM Na$^{3+}$, 10 mM leupeptin, 10 mM aprotinin, 1 mM Na$_3$VO$_4$, 50 mM HEPES, pH 7.4) was added to the dish on ice for 10 min. Cell lysates were collected, sonicated for 15 s, and centrifuged at 16,000 × g for 20 min at 4 °C. The protein concentration in the supernatant was determined using a bicinchoninic acid protein assay (Pierce), and 600 μg of protein from each sample was incubated with 3 μl of polyclonal anti-phosphotyrosine antibody overnight at 4 °C with rocking. 40 μl of packed protein A-Sepharose beads (Sigma) were then added to each sample and incubated with rocking for 60 min at 4 °C. The beads were then pelleted by centrifugation at 14,000 × g and washed three times in 500 μl of lysis buffer.

The procedure for the Kv1.2 or PYK2 immunoprecipitation was similar except that a milder lysis buffer was used to preserve protein-protein interactions (100 mM NaCl, 1% Nonidet P-40 (IGEPAL CA-630), 0.25% sodium deoxycholate, 30 mM sodium pyrophosphate, 5 mM β-glycerocephosphate, 10 μg/ml leupeptin, 0.5 mM Pefabloc, 10 μg/ml aprotinin, 500 μg/ml Na$_3$VO$_4$, 20 mM HEPES, pH 7.4). 700 μg of cell lysates were incubated with 4 μl of monoclonal Kv1.2 antibodies or 5 μg of polyclonal anti-PYK2 antibodies overnight at 4 °C, and immune complexes were collected by incubation with 40 μl of packed protein G-agarose beads. For Western blotting, the immunoprecipitates were subjected to SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibody. After blotting, the membrane was washed and treated with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse or anti-rabbit IgG). The immunoreactive bands were visualized using enhanced chemiluminescence reagents (Amersham Biosciences) exposed to Hyperfilm (Amersham Biosciences) in the linear range of the film density. The films were scanned, and densitometric analysis was performed with NIH image software.

A variation of these methods was used to measure phosphorylation of ERK1/2. A7r5 cells grown on 100-mm plastic tissue culture dishes were equilibrated for 2 h in control medium at room temperature. The cells were then pretreated for 1 h with 2.5 μM U-0126 or vehicle or 30 min with 20 mM salicylate or vehicle, followed by treatment for up to 30 min with 100 μM AVP in the presence or absence of U-0126 or 20 mM salicylate. The cells were then lysed in 50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM Na$_3$VO$_4$, 0.01% Triton X-100, 10 μg ml$^{-1}$ aprotinin, 10 μg ml$^{-1}$ leupeptin, 0.5 mM Pefabloc, 10 mM HEPES, pH 7.4, scraped off the dish, and centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant (a volume containing 40 μg of protein) was subjected to SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and immunoblotted with polyclonal antibodies against phospho-ERK proteins (Promega, Madison,
**RESULTS**

**PYK2 in A7r5 Cells**—Stimuli that activate PKC or elevate [Ca$^{2+}$], have been found to activate the tyrosine kinase, PYK2, leading to its autophosphorylation on a tyrosine residue. The presence of PYK2 in A7r5 cells was confirmed by Western blot analysis that identified a band at ~112 kDa (Fig. 1) that did not cross-react with p125$^{src}$ antibodies (not shown). Immunoprecipitation using anti-phosphotyrosine antibodies revealed an increase in tyrosine-phosphorylated PYK2 in response to both PMA (1 nM) and ionomycin (1 μM), indicating that it can be activated by either PKC or increased [Ca$^{2+}$], in A7r5 cells (Fig. 2).

**Time-dependent Activation of PYK2 by AVP**—100 pM AVP was also found to stimulate PYK2 phosphorylation. The time course for stimulation of tyrosine phosphorylation of PYK2 by 100 pM AVP is shown in Fig. 3, A and B. A significant increase in tyrosine phosphorylation was detected after 2 min, followed by a further increase, which plateaued between 5 and 20 min and then declined at 30 min. The Ca$^{2+}$-spiking response to 100 pM AVP was typically delayed by several minutes (on average 4.2 ± 0.6 min, as reported previously (18)) but is sustained for as long as AVP is present, at least up to 1 h (18). Treatment of A7r5 cells with PLD (2.5 units/ml, 15 min), which has been shown previously to stimulate Ca$^{2+}$ spiking in A7r5 cells (17), also stimulated PYK2 tyrosine phosphorylation (not shown).

**PKC Dependence of PYK2 Activation**—The stimulation of PYK2 tyrosine phosphorylation by 100 pM AVP was inhibited in a concentration-dependent manner by the selective PKC inhibitor Ro-31-8220 (Fig. 4). This drug was shown previously to block AVP-stimulated Ca$^{2+}$ spiking (18). Similar results were obtained using another structurally unrelated PKC inhibitor, chelerythrine chloride (0.1–20 μM, not shown).

**Inhibition of PYK2 Phosphorylation by Salicylate and PP2**—Salicylate has been reported recently (30) to inhibit selectively PYK2 tyrosine phosphorylation in angiotensin II-stimulated cardiac fibroblasts. Salicylate (20 mM) in-
hibited AVP-stimulated PYK2 tyrosine phosphorylation by 82% \( (p < 0.01, n = 3; \text{Fig. 5A}) \) and completely abolished AVP-stimulated \( \text{Ca}^{2+} \) spiking (Fig. 5B) in A7r5 cells. In three independent paired experiments, the mean frequency of \( \text{Ca}^{2+} \) spiking in cells treated with 100 \( \mu \)M AVP alone was 7.8 \( \pm \) 1.1 min\(^{-1}\), whereas no spiking was observed in cells treated with 100 \( \mu \)M AVP.
AVP in the presence of 20 mM salicylate. This concentration of salicylate did not prevent 100 nM AVP-stimulated release of Ca^{2+} from intracellular stores, the [Ca^{2+}]_{i} response to a high external [K^{+}] solution (not shown), or EGF-stimulated ERK1/2 phosphorylation (see below).

PP2 is a relatively selective inhibitor of SFKs (31). 10 μM PP2 completely inhibited AVP-stimulated Ca^{2+} spiking, whereas its inactive analog, PP3, had no effect (Fig. 5C). PP2 also abolished PMA-stimulated Ca^{2+} spiking (not shown). AVP-stimulated tyrosine phosphorylation of PYK2 was significantly inhibited (by 65 ± 3%, n = 3, p < 0.05) by PP2 (Fig. 5D). SFKs have been found to associate with active PYK2 by binding to its phosphorylated tyrosine (Tyr-402; see Refs. 22 and 26). We examined the possibility that Src and PYK2 might become associated following AVP treatment. Co-immunoprecipitation results are shown in Fig. 6. A7r5 cells were treated for varying times with 100 pM AVP followed by immunoprecipitation of PYK2. Although PYK2 was readily detected in the immunoprecipitates (and depleted from the supernatants), Src was not detectable in the immunoprecipitates at any time point (but was readily detected in the supernatants). Similar results were obtained by immunoprecipitating Src and probing for PYK2 (not shown).

Tyrosphostin Inhibition of Ca^{2+} Spiking—The effects of another tyrosine kinase inhibitor, tyrophostin A47 (TyrA47), on the Ca^{2+}-spiking responses to 100 pM AVP or 1 nM PMA are shown in Fig. 7 (A–F). TyrA47 (50 μM) completely abolished the Ca^{2+} -spiking response to both agents, whereas the inactive analog, TyrA63 (50 μM), did not affect the responses. However, in contrast to salicylate or PP2, neither TyrA47 nor TyrA63 prevented AVP- or PMA-stimulated tyrosine phosphorylation of PYK2 (Fig. 7G).

Potential Downstream Effectors of PYK2—Transactivation of EGF receptors (EGFR) and activation of ERK1 and ERK2 MAPKs have been implicated as downstream effectors in other PYK2-mediated cell responses (9–22, 24, 25, 32). Activation of ERK1/2 requires dual threonine and tyrosine phosphorylation, both catalyzed by another highly specific kinase, MEK. EGF at concentrations ranging from 1 pM to 100 nM failed to stimulate Ca^{2+} spiking in A7r5 cells, whereas 50 pM AVP elicited a robust Ca^{2+} -spiking response in the same cells (not shown). Despite its inability to stimulate Ca^{2+} spiking, EGF (10 nM) robustly activated ERK1/2 (Fig. 8A). Salicylate (20 μM) did not prevent EGF-stimulated ERK1/2 phosphorylation (Fig. 8A), whereas U-0126 (2.5 μM, a selective MEK inhibitor) completely abolished this effect (Fig. 8B). Phosphorylation of ERK1/2 in response to 100 pM AVP was undetectable in 6 of 9 experiments (Fig. 8A) and U-0126 did not inhibit AVP-stimulated Ca^{2+} spiking (Fig. 8C; frequency of Ca^{2+} spiking in response to 100 μM AVP was 3.8 ± 0.5 min^{-1} in the absence of U-0126 and 5.3 ± 0.9 min^{-1} in the presence of U-0126, p > 0.1, n = 4). These results suggest that activation of EGF receptors or ERK1/2 MAPKs is neither necessary nor sufficient to elicit the Ca^{2+} -spiking effect.

We next determined whether Kv1.2-delayed rectifier K^{+} channels might be a potential effector for PYK2 in the stimulation of Ca^{2+} spiking. Kv1.2 channels have been shown to be tyrosine-phosphorylated by PYK2, leading to an inhibition of outward K^{+} currents in Xenopus oocytes (22). We found that, in A7r5 cells, treatment of the cells with 100 pM AVP significantly increased tyrosine phosphorylation of the Kv1.2 channel protein (Fig. 9A) and that Kv1.2 channel protein co-immunoprecipitated with PYK2 (Fig. 9B). The amounts of Kv1.2 detected in the PYK2 immunoprecipitates from untreated cells were similar to those from cells treated with 100 pM AVP in five independent experiments (Fig. 9B and results not shown). AVP-stimulated tyrosine phosphorylation of Kv1.2 was significantly inhibited by PP2, Ro-31-8220, and salicylate, but not by tyrophostin A47 (% inhibition = 100, 36.9, 31.3, and 18.1, respectively; Fig. 9C).

**DISCUSSION**

We have identified previously (15) a novel signal transduction pathway activated by physiological concentrations of AVP that lead to stimulation of Ca^{2+} spiking in vascular smooth muscle cells. The Ca^{2+} spikes are due to action potentials and are dependent on L-type voltage-sensitive Ca^{2+} channels (15, 16, 33, 34). The frequency of action potential firing/Ca^{2+} spiking increases with increasing AVP concentration (15, 16). This frequency-modulated response provides a potential mechanism for fine-tuning of arterial constriction that may be important for determining regional tissue blood supply as well as peripheral vascular resistance.
We have recently postulated (18) an essential role for one or more PKC isoforms in the AVP-stimulated Ca$_{2+}$/H$_{11001}$-spiking response. Considering the ultimate involvement of voltage-sensitive Ca$_{2+}$/H$_{11001}$ channels, a link between PKC and membrane potential may be proposed. Our preliminary studies have suggested that inhibition of voltage-gated K$\mathrm{v}$ channels may provide the membrane depolarization necessary to trigger Ca$_{2+}$/H$_{11001}$ spiking in response to AVP (16). PKC-mediated inhibition of delayed rectifier K$\mathrm{v}$ channels has been proposed to explain angiotensin II-induced constrictor responses in rabbit portal vein smooth muscle cells (35, 36). Salter et al. (37) have reported that endothelin-1 causes vasoconstriction and inhibition of delayed rectifier K$\mathrm{v}$ currents in rat pulmonary artery myocytes.

It is not clear whether these vasoconstrictor actions involve direct serine or threonine phosphorylation of K$\mathrm{v}$ channels by PKC or an indirect effect involving other signaling intermediates. An indirect effect of PKC on K$\mathrm{v}$ channels has been suggested by Huang et al. (38) who found that, in Xenopus oocytes, activation of G$_{q}$-coupled receptors or treatment with PMA inhibited K$\mathrm{v}$ currents by a mechanism that was dependent on tyrosine phosphorylation of the channels. PYK2, a tyrosine kinase that may be activated by PKC, was subsequently proposed to mediate the tyrosine phosphorylation and inhibition of delayed rectifier K$\mathrm{v}$ channels (22, 23) in Xenopus or mammalian expression systems.

We have found that PYK2 is expressed in vascular smooth muscle cells and is tyrosine-phosphorylated by stimuli that activate PKC or increase [Ca$_{2+}$/H$_{11001}$], including AVP, which both activates PKC and increases [Ca$_{2+}$/H$_{11001}$]. AVP-stimulated PKC activation is apparently required for PYK2 tyrosine phosphorylation.
or pretreated for 1 h with 2.5–3 nM EGF or 100 pM AVP for 30 min in the presence or absence of 20 mM salicylate (following a 30-min pretreatment with salicylate alone). ERK1/2 phosphorylation was detected by Western blot analysis using phospho-ERK antibodies (see Experimental Procedures). The same blots were re-probed for total ERK (lower panels). All results shown are representative of at least 3–6 similar experiments.

![Image](https://example.com/image.png)

**FIG. 8.** ERK activation by EGF but not AVP. A, representative Western blot showing phosphorylation of ERK1 and ERK2 in A7r5 cells treated with 10 nM EGF or 100 nM AVP for 30 min in the presence or absence of 20 mM salicylate (following a 30-min pretreatment with salicylate alone). ERK1/2 phosphorylation was detected by Western blot analysis using phospho-ERK antibodies (see "Experimental Procedures"). The same blots were re-probed for total ERK (lower panels) to verify uniform loading. B, representative Western blot showing phosphorylation of ERK1 and ERK2 in A7r5 cells pretreated for 1 h with vehicle or 2.5 μM U-0126 followed by treatment for 30 min with 10 nM EGF. ERK1/2 phosphorylation was detected by Western blot analysis using phospho-ERK antibodies. C, fura-2-loaded A7r5 cells were pretreated with vehicle followed by treatment with 100 pM AVP (top panel) or pretreated for 1 h with 2.5 μM U-0126 followed by treatment with 100 pM AVP (lower panel). All results shown are representative of at least 3–6 similar experiments.

Inhibition of AVP-stimulated Ca\(^{2+}\) spiking by different tyrosine kinase inhibitors (PP2, salicylate, and tyrphostin A47) suggests that tyrosine phosphorylation is essential for this effect. Salicylate prevents AVP-stimulated PYK2 phosphorylation and Ca\(^{2+}\) spiking but not EGF-stimulated ERK1/2 activation. A similarly selective effect of salicylate was reported recently by Wang and Brecher (30), who found that 20 mM salicylate abolished angiotensin II- or platelet-derived growth factor-stimulated tyrosine phosphorylation of PYK2 without inhibiting platelet-derived growth factor-stimulated phosphorylation of PLC-γ or angiotensin II-stimulated phosphorylation of EGFR in cardiac fibroblasts.

SFKs have been implicated in PYK2 signaling (21, 24–26, 32, 39) as well as in direct tyrosine phosphorylation of \(K_\alpha\) channels (40). A role of SFKs in AVP signal transduction is indicated by the inhibition of Ca\(^{2+}\) spiking by PP2 (Fig. 5C). PP2 also inhibited PMA-stimulated Ca\(^{2+}\) spiking, suggesting that Src is downstream of PKC in the signal transduction cascade. Our findings are consistent with studies in other cell systems, which have suggested that activation of Src or one of its family members is necessary for PYK2 activation (26, 32, 39, 41, 42). Once activated, PYK2 is believed to autophosphorylate on tyrosine 402, which then acts as a scaffold for binding of Src via its SH2 domain (22, 26). Src may then phosphorylate other tyrosines on PYK2 such as Tyr-881 to allow Grb2 binding and propagation of the signal to MAPKs (22, 26). At least two recent studies (32, 41) have indicated that Src itself does not associate with PYK2, but rather another member of the family, Yes, binds to PYK2 and is necessary for PYK2 signaling. Our results indicate that in A7r5 cells Src and PYK2 do not co-immunoprecipitate (Fig. 6). Additional studies will be required to determine whether another SFK is responsible for activation of PYK2 and perhaps also for phosphorylation of Kv1.2 channels. SFKs have been found to act directly on L-type Ca\(^{2+}\) channels in other cell systems (43), but we do not find any effect of 100 pM AVP on L-type Ca\(^{2+}\) currents under voltage clamp conditions in A7r5 cells, suggesting that L-type channels are not a direct target of Src family kinases at concentrations of AVP that stimulate Ca\(^{2+}\) spiking.

We found that tyrphostin A47 inhibits AVP-stimulated Ca\(^{2+}\) spiking but not tyrosine phosphorylation of PYK2 or of Kv1.2. This finding leads us to speculate that the target of TyrA47 may be another signaling event that is not dependent on PYK2 activation but is nonetheless required for stimulation of Ca\(^{2+}\) spiking. One possibility may be activation of non-selective cat-

\(^2\) L. I. Brueggemann and K. L. Byron, unpublished observations.
A7r5 cells were treated with 100 pM AVP for 10 min and co-immunoprecipitation of Kv1.2 with PYK2.

Tyrosine phosphorylation of Kv1.2 was assessed by immunoprecipitation (IP) with monoclonal anti-PYK2 antibodies and immunoblotting with monoclonal anti-phosphotyrosine (pTyr) antibodies. A representative Western blot is shown; similar results were obtained in five independent experiments.

Tyrosine phosphorylation of Kv1.2 in A7r5 cells treated for 10 min with 100 pM AVP in the presence or absence of 20 mM salicylate (30 min pretreatment), 10 μM Ro-31-8220 (30 min pretreatment), 50 μM tyrphostin A47 (3 h pretreatment), or 10 μM PP2 (30 min pretreatment). A representative immunoblot (top) and cumulative data from 3 to 5 experiments (bottom) are presented. *, p < 0.01 versus control. †, p < 0.05 versus AVP. ‡, p < 0.01 versus AVP.

**FIG. 9.** Tyrosine phosphorylation of Kv1.2-delayed rectifier K⁺ channel and co-immunoprecipitation of Kv1.2 with PYK2. A, A7r5 cells were treated with 100 pM AVP for 10–20 min. Tyrosine phosphorylation of Kv1.2 was assessed by immunoprecipitation (IP) with monoclonal anti-Kv1.2 antibodies and immunoblotting with monoclonal anti-phosphotyrosine (pTyr) antibodies. A representative Western blot is shown in the top left panel and cumulative results (mean ± S.E.) from densitometric analysis of 6–8 blots are shown in the lower panel. Data are presented as fold increase above control (time = 0), which was set to 1. * denotes significant difference from control (p < 0.05). B, association between PYK2 and Kv1.2 was assessed by immunoprecipitation with monoclonal anti-PYK2 antibodies followed by immunoblotting with polyclonal Kv1.2 antibodies. A representative Western blot is shown; similar results were obtained in five independent experiments. C, tyrosine phosphorylation of Kv1.2 was evaluated as in A in A7r5 cells treated for 10 min with 100 pM AVP in the presence or absence of 20 mM salicylate (30 min pretreatment), 10 μM Ro-31-8220 (30 min pretreatment), 50 μM tyrphostin A47 (3 h pretreatment), or 10 μM PP2 (30 min pretreatment). A representative immunoblot (top) and cumulative data from 3 to 5 experiments (bottom) are presented. *, p < 0.01 versus control. †, p < 0.05 versus AVP. ‡, p < 0.01 versus AVP.

Potassium channels play an essential role in vascular smooth muscle cells in determining membrane potential and thereby regulating cell excitability. Numerous studies (47–50) have found that inhibition of delayed rectifier Kᵥ channels leads to vasorelaxation and/or arterial vasomotion. Tyrosine phosphorylation of Kᵥ channels has been linked to inhibition of Kᵥ currents (22, 23, 38, 40). We have found for the first time that Kv1.2 channels in vascular smooth muscle cells exist in a complex with PYK2 and are tyrosine-phosphorylated in response to physiological vasoconstrictor concentrations of AVP. Kv1.2 channels were recently found to be highly expressed in vascular smooth muscle of resistance arteries and to have increased expression levels in tissues from spontaneously hypertensive rats (51).

Arterial vasomotion is believed to originate within the smooth muscle cell layer of the artery wall because it can occur independently of endothelium (6, 8) or innervation (1, 3, 6, 52). Although AVP has been reported to stimulate vasomotion in several arterial preparations (3, 9, 10), the biochemical mechanisms underlying this effect have not been elucidated. What is known is that vasomotion is dependent on L-type voltage-sensitive Ca²⁺ channels and that it correlates with action potential firing in the vascular smooth muscle cells (6, 7). In addition, drugs that inhibit delayed rectifier Kᵥ channels have been shown to stimulate arterial vasomotion and/or action potential generation (8, 47–50). These observations along with the results of our present and previous studies (15–18) lead us to speculate that the signaling mechanisms coupling V₂₅₄, vasoressin receptors to firing of action potentials in arterial myocytes may proceed via a novel signal transduction pathway involving phospholipase D, protein kinase C, an Src family ion channels by AVP, for which we have previous evidence (44, 45). Such channels may also contribute to membrane depolarization in vascular smooth muscle (46) and may be regulated by tyrosine kinases (43). Additional studies will be required to determine whether AVP stimulates Ca²⁺ spiking by a combination of Kᵥ channel inhibition (mediated by PYK2) and activation of an additional tyrphostin A47-sensitive pathways, perhaps leading to activation of non-selective cation channels.

Transactivation of EGFR has been implicated as a downstream mediator of PYK2 activation in angiotensin II-stimulated vascular smooth muscle cell hypertrophy (21, 24, 25, 32). However, we found that direct activation of EGFR by binding of EGF did not stimulate Ca²⁺ spiking despite activation of ERK1/2. We also found that salicylate did not affect EGF-stimulated ERK activation (Fig. 8A).

100 pM AVP did not detectably increase ERK1/2 phosphorylation, and the MEK inhibitor, U-0126, which completely abolished EGF-stimulated ERK1/2 phosphorylation, did not inhibit AVP-stimulated Ca²⁺ spiking. Although it did activate ERK1/2, EGF did not stimulate Ca²⁺ spiking. These findings lead us to conclude that ERK1/2 activation is neither necessary nor sufficient for the stimulation of Ca²⁺ spiking by AVP.

Potassium channels play an essential role in vascular smooth muscle cells in determining membrane potential and thereby regulating cell excitability. Numerous studies (47–50) have found that inhibition of delayed rectifier Kᵥ channels leads to vasorelaxation and/or arterial vasomotion. Tyrosine phosphorylation of Kᵥ channels has been linked to inhibition of Kᵥ currents (22, 23, 38, 40). We have found for the first time that Kv1.2 channels in vascular smooth muscle cells exist in a complex with PYK2 and are tyrosine-phosphorylated in response to physiological vasoconstrictor concentrations of AVP. Kv1.2 channels were recently found to be highly expressed in vascular smooth muscle of resistance arteries and to have increased expression levels in tissues from spontaneously hypertensive rats (51).

Arterial vasomotion is believed to originate within the smooth muscle cell layer of the artery wall because it can occur independently of endothelium (6, 8) or innervation (1, 3, 6, 52). Although AVP has been reported to stimulate vasomotion in several arterial preparations (3, 9, 10), the biochemical mechanisms underlying this effect have not been elucidated. What is known is that vasomotion is dependent on L-type voltage-sensitive Ca²⁺ channels and that it correlates with action potential firing in the vascular smooth muscle cells (6, 7). In addition, drugs that inhibit delayed rectifier Kᵥ channels have been shown to stimulate arterial vasomotion and/or action potential generation (8, 47–50). These observations along with the results of our present and previous studies (15–18) lead us to speculate that the signaling mechanisms coupling V₂₅₄, vasoressin receptors to firing of action potentials in arterial myocytes may proceed via a novel signal transduction pathway involving phospholipase D, protein kinase C, an Src family

S.E.) from densitometric analysis of 6–8 blots are shown in the lower panel. Data are presented as fold increase above control (time = 0), which was set to 1. * denotes significant difference from control (p < 0.05). B, association between PYK2 and Kv1.2 was assessed by immunoprecipitation with monoclonal anti-PYK2 antibodies followed by immunoblotting with polyclonal Kv1.2 antibodies. A representative Western blot is shown; similar results were obtained in five independent experiments. C, tyrosine phosphorylation of Kv1.2 was evaluated as in A in A7r5 cells treated for 10 min with 100 pM AVP in the presence or absence of 20 mM salicylate (30 min pretreatment), 10 μM Ro-31-8220 (30 min pretreatment), 50 μM tyrphostin A47 (3 h pretreatment), or 10 μM PP2 (30 min pretreatment). A representative immunoblot (top) and cumulative data from 3 to 5 experiments (bottom) are presented. *, p < 0.01 versus control. †, p < 0.05 versus AVP. ‡, p < 0.01 versus AVP.
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A schematic diagram is shown illustrating a hypothetical pathway whereby binding of physiological concentrations of AVP (10–100 pm) to V\(_{1a}\) vasopressin receptors activates a cascade of events including activation of PLD, PKC, an Src family kinase, and PYK2. PYK2 activation leads to tyrosine phosphorylation and the consequent inhibition of current through delayed rectifier K\(^+\) channels (K\(_{\text{r}}\)). This results in membrane depolarization and firing of action potentials involving Ca\(^{2+}\) influx via L-type voltage-sensitive Ca\(^{2+}\) channels (Ca\(_{\text{L}}\)). Such action potentials would be expected to produce rhythmic vasomotion in resistance arteries, leading to an increase in peripheral vascular resistance. Nanomolar concentrations of AVP activate PLC and the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR). The abbreviations used are: DAG, diacylglycerol; IP\(_{3}\), inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PIP\(_{2}\), phosphatidylinositol 4,5-bisphosphate; V\(_{1a}\), V\(_{1a}\) vasopressin receptors.

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