

# Modulation of Kv1.5 Currents by Protein Kinase A, Tyrosine Kinase, and Protein Tyrosine Phosphatase Requires an Intact Cytoskeleton

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## ABSTRACT

The regulation of cardiac delayed rectifier potassium (Kv) currents by cAMP-dependent protein kinase (PKA) contributes to the control of blood pressure and heart rate. We investigated the modulation by PKA and protein phosphatases of cloned Kv1.5 channels expressed in *Xenopus laevis* oocytes. Exposure of oocytes to activators of PKA (100 nM forskolin, 1 mM 8-bromo-cAMP, or 1 mM 3-isobutyl-1-methylxanthine) had no effect on the amplitude of Kv1.5 currents. Inhibition of PKA by injection of protein kinase A inhibitor peptide or exposure to myristoylated protein kinase A inhibitor peptide (M-PKI; 100 nM) reduced currents mediated by Kv1.5. M-PKI also reduced the amplitude of currents mediated by mutated Kv1.5 channels in which the COOH terminal PKA phosphorylation sites and PSD-95, Disc-large, and ZO-1-binding domain were removed. The reduction of Kv1.5 currents by M-PKI was attenuated by inhi-

bition of actin polymerization by 1  $\mu$ M cytochalasins B and D, but was not affected by 10  $\mu$ M phalloidin (stabilizes actin filaments) or 50  $\mu$ M colchicine (disrupts microtubules). Treatment of oocytes with antisense oligonucleotides against  $\alpha$ -actinin-2 abolished the reduction in Kv1.5 current by M-PKI. These observations suggest that Kv1.5 currents are activated by endogenous PKA in "resting" oocytes and that inhibition of PKA activity reveals the action of endogenous phosphatases. Indeed, injection of alkaline phosphatase reduced currents mediated by Kv1.5. Further preincubation of oocytes with 1 mM sodium orthovanadate (a protein tyrosine phosphatase inhibitor) abolished the reduction in Kv1.5 currents by M-PKI. We conclude that currents encoded by Kv1.5 are regulated by PKA and protein tyrosine phosphatase and that this regulation requires an intact actin cytoskeleton and  $\alpha$ -actinin-2.

In cardiac myocytes, delayed rectifier potassium (Kv) currents mediate  $I_{K_r}$ , important in the repolarization of action potentials of ventricular and atrial myocytes (Firek and Giles, 1995). Different components of  $I_{K_r}$  have been identified in cardiac myocytes and, in some cases, components of  $I_{K_r}$  have been attributed to specific Kv channels (Barry and Nerbonne, 1996). For example, many cardiac myocytes have rapidly activating, slowly inactivating components of  $I_{K_r}$  referred to as  $I_{K_{ur}}$  (ultra-rapid). This current is distinguished by its rapid activation kinetics and relatively high sensitivity to 4-AP (Nattel et al., 1999). The kinetic and pharmacological properties of  $I_{K_{ur}}$  in human and rat atrial myocytes resemble those of cloned Kv1.5 channels (Paulmichl et al., 1991; Fedida et al., 1993; Overturf et al., 1994). In support of a role for Kv1.5 currents in atrial myocytes, Kv1.5 was cloned from human and rat atria (Paulmichl et al., 1991; Fedida et al., 1993) and mRNA encoding this channel and Kv1.5 protein

have been detected in rat atria (Dixon and McKinnon, 1994; Mays et al., 1995). Furthermore, treatment of cultured rat and human atrial myocytes with antisense oligonucleotides designed to reduce expression of Kv1.5 channels attenuated the magnitude of  $I_{K_{ur}}$  (Feng et al., 1997; Bou-Abboud and Nerbonne, 1999). Thus, a variety of functional and molecular data suggest that Kv1.5  $\alpha$  subunits mediate  $I_{K_{ur}}$  in human and rat atrial myocytes.

$I_{K_{ur}}$  is characteristically increased by the activity of PKA as part of the response to  $\beta$ -adrenergic activation (Li et al., 1996; Yue et al., 1999). To elucidate the molecular mechanisms underlying the increase in  $I_{K_{ur}}$  after PKA stimulation, responses of cloned Kv channels to activation and inhibition of protein kinases have been studied in heterologous expression systems. However, it has been difficult to relate the behavior of heterologously expressed channels with that of native channels.  $\beta$ -Adrenergic activation of PKA increased Kv1.2 currents expressed in *Xenopus laevis* oocytes (Huang et al., 1994) but acute activation of PKA had no effect on full-length Kv1.1 or Kv2.1 currents (Wilson et al., 1994;

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**ABBREVIATIONS:** Kv, delayed rectifier potassium currents; PKA, cAMP-dependent protein kinase; ANOVA, analysis of variance; IBMX, 3-isobutyl-1-methylxanthine; PKI, protein kinase A inhibitor peptide; M-PKI, myristoylated PKA inhibitor peptide 14-22 amide; PDZ, PSD-95, Disc-large, and ZO-1; PTP, protein tyrosine phosphatase; HEK, human embryonic kidney.

Levin et al., 1995). Interestingly, the varied responses to the activation of PKA in *X. laevis* oocytes are not limited to Kv channels. For example, activation of PKA increases heterologously expressed cystic fibrosis transmembrane conductance regulator currents (Levesque et al., 1992) but not L-type  $\text{Ca}^{2+}$  currents (Singer-Lahat et al., 1994).

Taken together, these findings suggest that the ability of a channel to respond to PKA may depend on the organization of the channels with respect to the endogenous protein kinases and phosphatases. As discussed in recent reviews (Johnson, 1999; Sheng and Pak, 2000), colocalization of kinases, phosphatases and their substrates provides for specificity of control and increases the speed of response. Thus, one expects that kinases and phosphatases will not be freely mobile, that they will be located near their substrates, and that this organization will influence function. However, the relationship between Kv channels, regulatory proteins, and the cytoskeleton is largely unknown. Previous work has focused on the PDZ-binding domains of Kv1 family members that localize these channels at synapses (Tejedor et al., 1997; Tiffany et al., 2000) and the binding of Kv1.5 channels to  $\alpha$ -actinin-2 (Maruoka et al., 2000).  $\alpha$ -Actinin-2 is a dimer of two antiparallel peptides each containing two EF-hand domains and an actin-binding domain separated by a 30-nm spacer of four spectrin repeat motifs (Beggs et al., 1992). The actin-binding domains enable  $\alpha$ -actinin-2 to bundle actin microfilaments, whereas the spectrin repeat domain binds to human Kv1.5 (Maruoka et al., 2000; Cukovic et al., 2001) and to NMDA receptors (Wyszynski et al., 1997). The combination of channel-binding and actin-binding domains gives  $\alpha$ -actinin-2 the ability to link channels to the actin cytoskeleton, thereby localizing them near regulatory kinases and phosphatases.

In this study, we examined the regulation of Kv1.5 channels by PKA and protein phosphatases using the *X. laevis* oocyte expression system. We find that there is a basal activation of Kv1.5 channel currents by endogenous PKA and that the regulation of these channels involves protein tyrosine phosphatase and an intact cytoskeleton. Some of these results have been presented in abstract form (Mason et al., 2001).

## Materials and Methods

**Creation of Kv1.5 $\Delta$ C59 Mutant.** Kv1.5 cloned from the canine colon (Overturf et al., 1994) possesses two potential PKA phosphorylation sites at Ser<sup>538</sup> and Ser<sup>545</sup>, and a PDZ-binding domain at the COOH terminus. We deleted the terminal 59 residues of canine Kv1.5 COOH terminus to remove these sites (Kv1.5 $\Delta$ C59). To confirm the mutation, the Kv1.5 $\Delta$ C59 construct was sequenced using the ABI Prism sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on a Genetic Analyzer 310 (ABI). Plasmid DNA was linearized with *Nde*I, and capped transcripts were synthesized in vitro with T7 RNA polymerase with the T7 mMessage mMachine transcription kit (Ambion, Austin, TX).

**$\alpha$ -Actinin-2 Antisense Experiments.** The sequence of  $\alpha$ -actinin-2 antisense and sense oligonucleotides were as described by Maruoka et al. (2000). Phosphorothioate oligonucleotides were synthesized by Biosource International (Foster City, CA) and diluted in molecular biology grade water to 1  $\mu\text{g}/\mu\text{l}$ .

**Isolation and Two-Microelectrode Voltage-Clamp of Oocytes.** *X. laevis* oocytes were isolated as described previously (Overturf et al., 1994). Stage V and VI oocytes were injected with 50 nl of cRNA encoding either Kv1.2 (1.9  $\mu\text{g}/\mu\text{l}$ ; GenBank accession number

L19740), Kv1.5 wild-type (0.035  $\mu\text{g}/\mu\text{l}$ ; GenBank accession number U08596), Kv1.5 $\Delta$ C59 (3.8  $\mu\text{g}/\mu\text{l}$ ), Kv2.2 (1.9  $\mu\text{g}/\mu\text{l}$ ; GenBank accession number U69962), or Kv3.1 (Y07521, 0.06  $\mu\text{g}/\mu\text{l}$ ) using a Drummond Nanoject microinjector (Drummond Scientific Co, Broomall, PA). Whole-cell currents were recorded using the two-microelectrode voltage-clamp technique. Glass microelectrodes were filled with 3 M K-aspartate and had resistances of 1 to 3 M $\Omega$ . Oocytes were superfused with a low chloride  $\text{Ca}^{2+}$ -free solution designed to minimize the endogenous  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current. This solution contained 96 mM Na isethionate, 2 mM KCl, 2.8 mM  $\text{MgCl}_2$ , 5 mM HEPES, and 0.05 mM niflumic acid, pH 7.4. Reagents were either applied to the bath (volume, 0.5 ml) via a gravity-fed perfusion system or injected into the oocyte. The mixing time to exchange bath solutions was approximately 30 s. Each experiment was performed at room temperature (24–28°C) on oocytes collected from two or more frogs. Voltage protocols were applied using pCLAMP 6.0 software (Axon Instruments, Union City, CA). In short, 400-ms voltage steps were applied from a holding potential of  $-80$  mV to test potentials ranging from  $-50$  to  $+50$  mV in 10 mV increments. Linear leak and capacitance currents were removed using a P/5 protocol.

We characterized the rate of activation of the Kv1.5 conductance by measuring the time to half-activation of the peak current at 0 mV ( $t_{0.5}$ ). The voltage-dependence of activation was determined by normalizing the initial currents upon repolarization to  $-40$  mV, plotting the currents as a function of the activating potential and fitting to the Boltzmann equation to obtain the half-activation voltage ( $V_{\text{Act}}$ ) and slope factor ( $s$ ). We tested the possibility that the effects of the drugs tested were caused by internalization of the Kv1.5 channels by monitoring the capacitance of the oocyte membrane. This was measured as the area under the current elicited by a voltage step from  $-80$  to  $-70$  mV. None of the treatments had a significant effect on membrane capacitance.

**Data and Statistical Analysis.** Data were analyzed using pCLAMP 6.0 (Axon Instruments) and Origin software (Microcal, Northampton, MA). Values are expressed as the mean  $\pm$  S.E.M of  $n$  oocytes. Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA). Results were analyzed in two ways: the difference from control with respect to time was analyzed using one-way ANOVA, and the difference between groups of oocytes subjected to different treatments was analyzed using two-way ANOVA. When differences over time or between treatments were found by ANOVA, the Bonferroni post hoc test was used, with  $p$  values  $< 0.05$  regarded as significant.

**Solutions and Drugs.** Forskolin and 8-bromo-cAMP were obtained from Sigma (St. Louis, MO). Calf intestine alkaline phosphatase was obtained from Roche Molecular Diagnostics (Indianapolis, IN). All other compounds were obtained from Calbiochem (La Jolla, CA).

## Results

**Activators of Protein Kinase A Do Not Affect Kv1.5 Current.** *X. laevis* oocytes injected with cRNA encoding wild-type Kv1.5 displayed outward current during step depolarizations to potentials positive to  $-20$  mV. These currents had the properties of Kv1.5, as characterized previously (Overturf et al., 1994), and were not seen in uninjected oocytes. We examined the effect of agents that stimulate PKA activity on wild-type Kv1.5 current with the results summarized in Fig. 1. Superfusion of oocytes with either forskolin (100 nM, A), 8-bromo-cAMP (1 mM, B) or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100  $\mu\text{M}$ , C) over a 15-min period had no effect on the magnitude of Kv1.5 currents. To ensure that PKA was maximally activated, we also examined the response of wild-type Kv1.5 current to a PKA cocktail that activates cystic fibrosis trans-

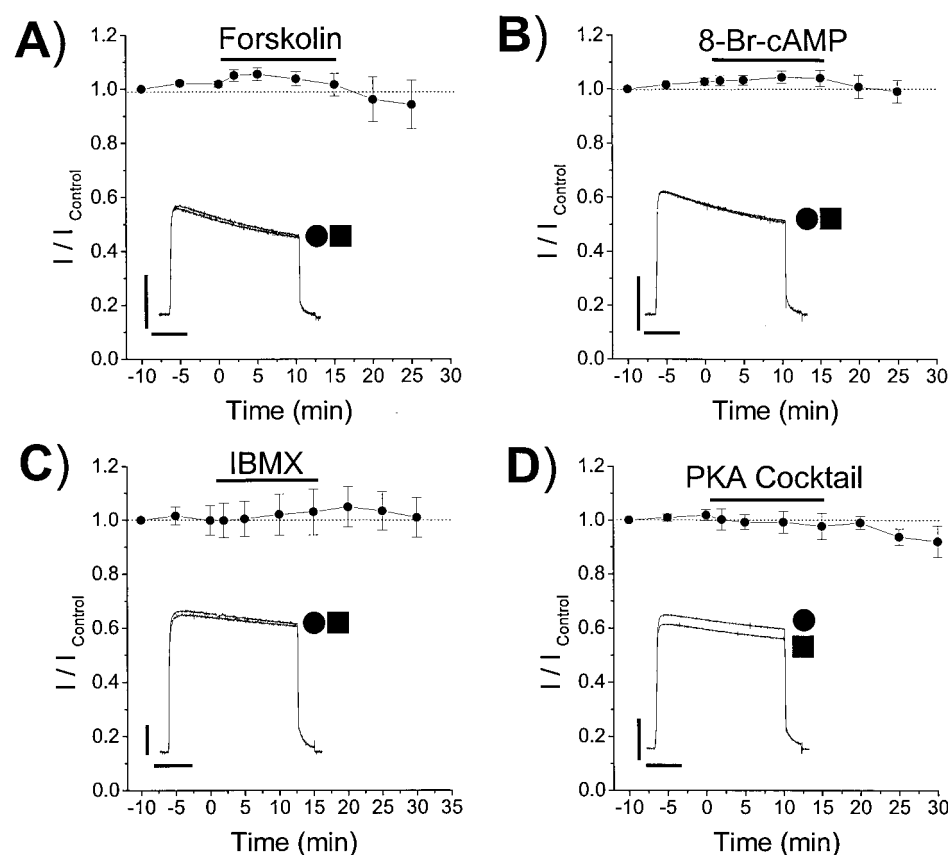
membrane conductance regulator currents expressed in *X. laevis* oocytes (Levesque et al., 1992). D shows that the superfusion of the PKA cocktail consisting of forskolin (10  $\mu$ M), 8-bromo-cAMP (1 mM) and IBMX (100  $\mu$ M) had no effect on Kv1.5 currents. Thus, agents that increase intracellular cAMP levels have no effect on Kv1.5 currents in *X. laevis* oocytes. We then considered the possibility that Kv1.5 channels were already fully activated by the endogenous PKA activity in *X. laevis* oocytes and examined the effect of inhibiting PKA activity on wild-type Kv1.5 currents.

**PKA Inhibitor Peptides Reduce Kv1.5 Current.** Protein kinase A inhibitor peptide (5-24) (PKI 5-24) is a synthetic peptide that specifically binds to the catalytic subunit of PKA and reduces PKA activity (Cheng et al., 1986). Control Kv1.5 currents were recorded for 10 min before oocytes were injected using a third microelectrode with 50 nl of PKI 5-24 (200  $\mu$ M), to give a final estimated intraoocyte concentration of 10  $\mu$ M or deionized water, and the currents monitored for a further 30 min. As shown in Fig. 2A, injection of PKI 5-24 caused a time-dependent reduction in the magnitude of wild-type Kv1.5 current. PKI 5-24 reduced peak Kv1.5 current over a 30-min period (to  $0.61 \pm 0.04$  of control 30 min postinjection,  $p < 0.001$ ,  $n = 7$ ). In contrast, injections of water did not suppress Kv1.5 current over a 30-min time course (to  $0.93 \pm 0.03$  of control 30 min after injection,  $n = 4$ ). The reduction in wild-type Kv1.5 current in oocytes injected with PKI 5-24 was significantly different from water-injected oocytes from 15 min after injection onwards ( $p < 0.01$ , two-way ANOVA).

We also examined the effect of a membrane permeant (myristoylated) protein kinase A inhibitor 14-22 amide (M-

PKI), that inhibits PKA activity in the same manner as PKI 5-24 (Harris et al., 1997). Figure 2B shows that superfusion of M-PKI (100 nM) induced a slowly developing inhibition of wild-type Kv1.5 current. Peak Kv1.5 current was reduced to  $0.87 \pm 0.11$  of control after 15-min exposure to M-PKI ( $n = 6$ ) and continued to decrease to  $0.58 \pm 0.10$  of control after 10-min wash ( $p < 0.001$ ,  $n = 6$ ). To ensure that the effect of M-PKI on Kv1.5 currents was not caused by the presence of the myristoyl group or a nonspecific effect of the myristoylated peptide, we used a myristoylated PKC inhibitor 19-27 peptide amide (Ward and O'Brian, 1993). As shown in Fig. 2B, myristoylated PKC inhibitor 19-27 peptide amide (100 nM) had no significant effect on peak Kv1.5 current ( $1.15 \pm 0.06$  of control after 15 min and  $1.09 \pm 0.07$  of control after 10-min wash,  $n = 5$ ). The reduction in wild-type Kv1.5 current in oocytes superfused with M-PKI was significantly different from those treated with myristoylated PKC inhibitor 19-27 peptide amide from 15 min on ( $p < 0.01$ , two-way ANOVA).

The reduction in wild-type Kv1.5 current induced by PKI 5-24 and M-PKI was caused by a voltage-independent reduction in Kv1.5-mediated conductance. Tail currents in control and after a 10-min wash were normalized to the maximum conductance under control conditions. M-PKI significantly reduced Kv1.5 conductance at potentials greater than  $-10$  mV (data not shown). Both PKI 5-24 and M-PKI caused a slight hyperpolarizing shift in the voltage required to half-activate the current. For M-PKI,  $V_{Act}$  was  $-10.9 \pm 0.4$  mV in control, which shifted to  $-15.0 \pm 0.7$  mV after 10-min wash ( $n = 6$ ). M-PKI had no effect on the slope of the activation curve ( $11.2 \pm 0.4$  mV in control versus  $11.8 \pm 0.6$  mV after



**Fig. 1.** Activators of PKA do not affect wild-type Kv1.5 current. Oocytes expressing wild-type Kv1.5 were superfused with agents to stimulate PKA activity. A, effect of forskolin (100 nM) on peak Kv1.5 current over time. B, effect of 8-bromo-cAMP (1 mM) on peak Kv1.5 current over time. C, effect of IBMX (100  $\mu$ M) on peak Kv1.5 current over time. D, effect of a PKA cocktail consisting of forskolin (10  $\mu$ M), 8-bromo-cAMP (1 mM), and IBMX (100  $\mu$ M) on peak Kv1.5 current over time. The insets show illustrative traces recorded at a test potential of +50 mV during control (●) and after 15-min exposure to the test agent (■). The vertical scale bar is 2  $\mu$ A and the horizontal scale bar is 100 ms.



10-min wash,  $n = 6$ ) or  $t_{0.5}$  ( $15.3 \pm 5.8$  ms in control versus  $11.3 \text{ ms} \pm 3.5$  after 10-min wash,  $n = 6$ ). The reduction in the Kv1.5 current was not caused by a shift in the reversal potential ( $E_{\text{Rev}} = -85.7 \pm 1.5$  mV control,  $E_{\text{Rev}} = -87.3 \pm 1.1$  mV after 15 min M-PKI, and  $E_{\text{Rev}} = -83.4 \pm 10.0$  mV after 10-min wash,  $n = 4$ ). Thus, inhibition of PKA activity by specific PKA inhibitory peptides caused a significant reduction in wild-type Kv1.5 conductance.

In the following experiments, we used the membrane-permeant PKA inhibitor M-PKI to minimize the experimental difficulties encountered after the insertion of a third microelectrode during recording. We saw variability in the reduction in Kv1.5 currents in response to M-PKI from 25% (e.g., Figure 3C) to 60% (e.g., Figure 2). This is similar to the suggestion by Ivanina et al. (1994) that the extent of basal PKA activity is variable between oocytes from different frog donors.

**The Effect of PKA Activators and Inhibitory Peptides on Other Kv Channels.** To examine whether differences in the primary structure of Kv channels determined their regulation by PKA we examined the effects of forskolin, 8-bromo-cAMP, and M-PKI on other Kv family members (Kv1.2, Kv1.5, Kv2.2, and Kv3.1); results are summarized in Fig. 3. As observed for Kv1.5, superfusion for 15 min with forskolin (100 nM, Fig. 3A) or 8-bromo-cAMP (1 mM, Fig. 3B) had no effect on the magnitude of any of the Kv currents examined. These data imply that like Kv1.5, channels encoded by Kv1.2, Kv2.2, and Kv3.1 do not respond to agents that increase intracellular cAMP levels when expressed in *X. laevis* oocytes.

We also investigated the sensitivity of these Kv channels to M-PKI. As shown in Fig. 3C, Kv1.2 currents were slowly reduced by M-PKI (100 nM,  $0.63 \pm 0.08$  of control after 15 min 100 nM M-PKI,  $p < 0.05$ ) that decreased further on washout (to  $0.51 \pm 0.09$ ,  $p < 0.01$ ,  $n = 5$ ). In contrast, Kv2.2

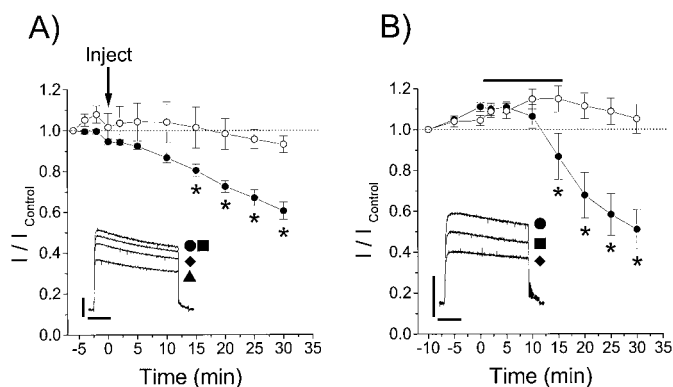
and Kv3.1 currents were unaffected by superfusion of M-PKI. Thus, inhibition of PKA by PKA inhibitor peptides suppressed Kv1.5 and Kv1.2 currents in oocytes, but not Kv2.2 or Kv3.1 currents.

### Expression and Modulation of Mutant Kv1.5ΔC59.

We examined the amino acid sequence of each of the Kv channels for differences that could account for the variability in the response to M-PKI shown in Fig. 3. All the Kv channels have potential PKA phosphorylation sites on the intracellular termini; wild-type Kv1.5 cloned from the canine colon (Overturf et al., 1994) possesses only two consensus PKA phosphorylation sites (R/K-R/K-X-S/T), both on the COOH terminus at Ser<sup>538</sup> and Ser<sup>545</sup>. A notable difference between the Kv1 family and the Kv2 and Kv3 families is the presence of a PDZ sequence at the COOH terminus of Kv1 family members. This sequence motif mediates the interaction between Kv1.1 and the cytoskeleton (Jing et al., 1997). Furthermore, these workers suggested that PKA regulated the extent of this interaction. We created a COOH-terminal deletion that removed the terminal 59 amino acid residues from canine Kv1.5 deleting both the potential PKA phosphorylation sites and the PDZ-binding domain (E-D-S-V). We injected oocytes with sufficient cRNA encoding Kv1.5ΔC59 (100× higher concentration) to give currents of a similar magnitude as wild-type Kv1.5 and examined the response to M-PKI (100 nM) with the results summarized in Fig. 4. Kv1.5ΔC59 currents were  $0.89 \pm 0.03$  of control after 15 min M-PKI, and reduced further to  $0.76 \pm 0.04$  of control after 10-min wash at a test potential of +50 mV ( $n = 13$ ). This was not significantly different from wild-type currents ( $0.90 \pm 0.03$  of control after 15 min M-PKI,  $0.78 \pm 0.04$  of control after 10-min wash,  $n = 9$ ). Therefore, the modulation of Kv1.5 by PKA does not require phosphorylation of the PKA phosphorylation sites on the COOH terminus, nor an interaction between the PDZ domain and the cytoskeleton.

### Modulation of Kv1.5 Currents by PKA Requires an Intact Cytoskeleton.

We investigated further whether the cytoskeleton was involved in the PKA modulation of wild-type Kv1.5 by preincubating oocytes for at least 4 h with cytoskeletal modifying agents and examining the response to M-PKI. The results of these experiments are summarized in Fig. 5. As shown in Fig. 5A, exposure to M-PKI had no effect on the amplitude of wild-type Kv1.5 currents in oocytes preincubated in cytochalasin B (1 μM), a compound that inhibits the addition of actin monomers to actin filaments ( $0.95 \pm 0.04$  of control after 15 min,  $0.87 \pm 0.04$  of control after 10-min wash,  $n = 7$ ). In contrast, Kv1.5 currents were reduced in control oocytes ( $0.80 \pm 0.05$  of control after 15 min,  $0.59 \pm 0.11$  of control after 10-min wash,  $p < 0.05$ ,  $n = 5$ ). The difference between the cytochalasin B-treated oocytes and control oocytes was significant ( $p < 0.01$  by two-way ANOVA). Similar results were obtained with oocytes preincubated in cytochalasin D ( $1.0 \pm 0.04$  of control after 15 min,  $0.97 \pm 0.06$  of control after 10-min wash,  $n = 4$ ,  $p < 0.01$  by two-way ANOVA, data not shown). In contrast, pretreatment of oocytes with either phalloidin, a stabilizer of actin microfilaments (10 μM, data not shown), or colchicine (50 μM), an agent that disrupts microtubules, had no effect on the inhibition of wild-type Kv1.5 currents by M-PKI ( $0.83 \pm 0.12$  of control after 15 min,  $0.67 \pm 0.19$  of control after 10-min wash,  $n = 5$ ). Thus, the normal turnover of actin filaments is necessary for the full effect of the inhibition of PKA on Kv1.5



**Fig. 2.** PKA inhibitor peptides reduce wild-type Kv1.5 current. Oocytes expressing wild-type Kv1.5 were exposed to PKA inhibitor peptides. A, injection of 200 μM PKI 5–25 (to give a final estimated intraoocyte concentration of 10 μM) reduced the magnitude of Kv1.5 current over the 30 min time course (●). The inset shows representative currents recorded at a test potential of +50 mV under control (●),  $t = 0$  min after injection (■),  $t = 15$  min after injection (◆), and  $t = 30$  min after injection (▲). Injection of water had no effect on the magnitude of Kv1.5 current (○). B, superfusion of the membrane-permeable PKI inhibitor peptide 14-22 (M-PKI, 100 nM) over a 15-min period caused a slowly developing reduction in Kv1.5 current (●). The inset shows representative currents recorded at a test potential of +50 mV under control (●), after 15-min M-PKI (■), and after 10-min wash (◆). Perfusion of the myristoylated PKC inhibitor peptide 19-27 had no effect on Kv1.5 current (○). The vertical scale bar is 2 μA and the horizontal scale bar is 100 ms. \*, Significantly different from control  $P < 0.05$  (two-way ANOVA).

current, suggesting that the actin cytoskeleton is an important component of PKA signaling to Kv1.5 channels.

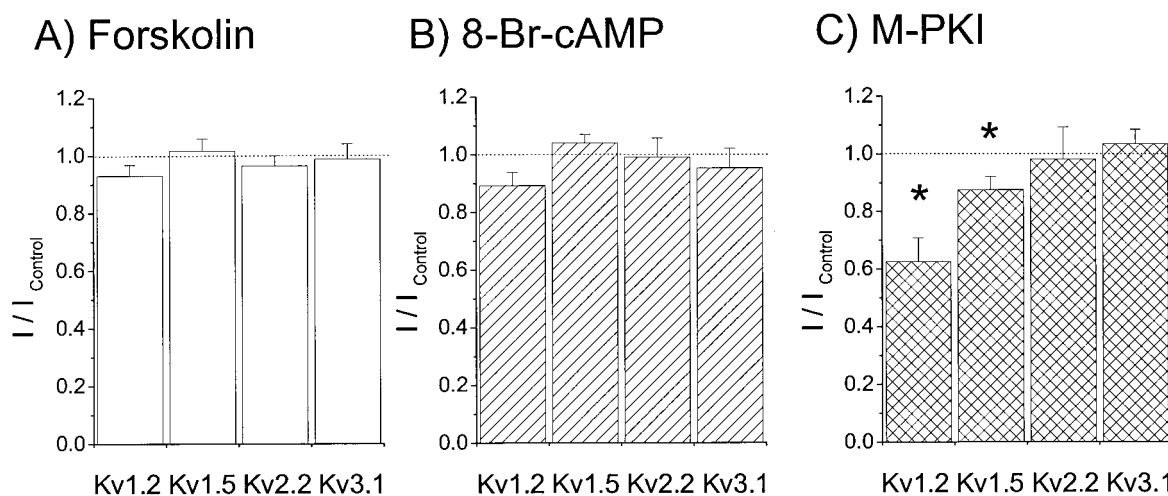
**Role of  $\alpha$ -Actinin-2 in the Modulation of Kv1.5 Currents by PKA.** Recently, Fedida and coworkers (Maruoka et al., 2000; Cukovic et al., 2001) showed that  $\alpha$ -actinin-2, an actin-binding protein, interacted with the NH<sub>2</sub> terminus of the Kv1.5  $\alpha$  subunit. We investigated whether  $\alpha$ -actinin-2 influenced the regulation of wild-type Kv1.5 currents by PKA, with results summarized in Fig. 6. Two days before electrophysiological assay, *X. laevis* oocytes were injected with either 50 ng of antisense or sense oligonucleotides against  $\alpha$ -actinin-2 or molecular biology-grade water. The next day, oocytes were injected with cRNA encoding wild-type Kv1.5. As shown in A,  $\alpha$ -actinin-2 antisense significantly reduced the magnitude of Kv1.5 current compared with oocytes injected with either  $\alpha$ -actinin-2 sense oligonucleotide or water. Peak Kv1.5 current recorded at a potential of +50 mV was  $1.0 \pm 0.2$   $\mu$ A for antisense-treated oocytes ( $n = 24$ ), compared with  $2.0 \pm 0.4$   $\mu$ A for sense-treated oocytes ( $n = 21$ ) and  $2.2 \pm 0.3$   $\mu$ A for water-injected oocytes ( $n = 25$ ). To determine whether the reduction in Kv1.5 currents was specific for Kv1.5 channels, we examined the effect of  $\alpha$ -actinin-2 oligonucleotides on channels encoded by Kv2.2. The magnitude of Kv2.2 currents was unaffected by  $\alpha$ -actinin-2 antisense oligonucleotide. Kv2.2 current was  $6.9 \pm 2.1$   $\mu$ A for antisense-treated oocytes ( $n = 7$ ),  $5.2 \pm 1.5$   $\mu$ A for sense-treated oocytes ( $n = 5$ ) and  $4.3 \pm 1.1$   $\mu$ A for water-injected oocytes ( $n = 7$ , data not shown). These data imply that  $\alpha$ -actinin-2 antisense treatment specifically reduces Kv1.5 currents.

The majority of oocytes treated with  $\alpha$ -actinin-2 antisense oligonucleotide had Kv1.5 currents ranging from 0 to 1  $\mu$ A at a potential of +50 mV. To characterize the electrophysiological properties of Kv1.5 currents in  $\alpha$ -actinin antisense-treated oocytes and their response to PKA inhibition, we selected oocytes that expressed currents greater than 1  $\mu$ A. The electrophysiological properties of Kv1.5 currents in these oocytes treated with  $\alpha$ -actinin-2 antisense oligonucleotide did not differ from control oocytes. That is, Kv1.5 currents in oocytes treated with  $\alpha$ -actinin-2 antisense activated at potentials greater than  $-20$  mV.  $V_{\text{Act}}$  and  $s$  values were  $-13.8 \pm$

0.2 mV and  $6.9 \pm 0.2$  mV for  $\alpha$ -actinin-2 antisense-treated oocytes ( $n = 9$ ), and  $-15.6 \pm 0.2$  mV and  $s = 7.1 \pm 0.1$  mV for  $\alpha$ -actinin-2 sense-treated oocytes ( $n = 8$ , data not shown). The  $t_{0.5}$  values were  $9.4 \pm 1.9$  ms for  $\alpha$ -actinin-2 antisense-treated oocytes and  $10.4 \pm 2.1$  ms for  $\alpha$ -actinin-2 sense-treated oocytes.

Fig. 6B shows the response of wild-type Kv1.5 currents to M-PKI in oocytes injected with either  $\alpha$ -actinin-2 antisense or sense oligonucleotides. M-PKI reduced Kv1.5 currents in oocytes injected with actinin-2 sense oligonucleotide ( $0.91 \pm 0.02$  of control after 15 min M-PKI, and  $0.77 \pm 0.04$  of control after 10-min wash ( $n = 6$ ,  $p < 0.001$ ). In contrast, M-PKI had no effect on Kv1.5 currents in oocytes injected with  $\alpha$ -actinin-2 antisense oligonucleotide ( $1.04 \pm 0.04$  of control after 15 min M-PKI,  $1.01 \pm 0.06$  of control after 10-min wash,  $n = 6$ ). Thus, the results with cytochalasins B and D, and  $\alpha$ -actinin-2 antisense suggest that the reduction in Kv1.5 currents observed after the inhibition of basal PKA activity requires an intact actin cytoskeleton and  $\alpha$ -actinin-2.

**Phosphatase Modulation of Kv1.5 current.** The data above suggest that Kv1.5 currents are fully activated by basal PKA activity in *X. laevis* oocytes and that inhibition of PKA activity by PKA inhibitor peptides reveals the action of endogenous phosphatases. To investigate whether the suppression of wild-type Kv1.5 current by PKA inhibitor peptides was consistent with the action of endogenous phosphatases we examined the effect of exogenous alkaline phosphatase on wild-type Kv1.5 current, with the data summarized in Fig. 7. Control Kv1.5 currents were recorded for 10 min before oocytes were injected using a third microelectrode with alkaline phosphatase (50 nl of 1 U/ $\mu$ l alkaline phosphatase to give a final estimated intraoocyte concentration of 0.05 U), and the currents monitored for a further 30 min. Control oocytes were injected with boiled alkaline phosphatase (heated to 100°C for 3 h and allowed to cool to room temperature before injection). Fig. 7A shows that injection of alkaline phosphatase caused a slowly developing inhibition of peak Kv1.5 current over 30 min after injection. Peak Kv1.5 current was  $0.97 \pm 0.02$  of control at 0 min after injection, and decreased to  $0.79 \pm 0.03$  of control after 15 min, and  $0.58 \pm 0.06$  of control 30 min after injection ( $p < 0.001$ ,  $n =$



**Fig. 3.** Effect of PKA activators and M-PKI on Kv channels. Oocytes expressing Kv1.2, Kv1.5, Kv2.2, or Kv3.1 were superfused with forskolin (100 nM), 8-bromo-cAMP (1 mM), or M-PKI (100 nM). Bar represents normalized current at a test potential of +50 mV after 15 min perfusion of agent. \*, Significantly different from control  $P < 0.05$  (one-way ANOVA).

7). In contrast, oocytes injected with boiled alkaline phosphatase did not show a significant inhibition of peak Kv1.5 current ( $0.88 \pm 0.09$  of control 30 min after injection,  $n = 6$ ). The reduction in Kv1.5 current in oocytes injected with alkaline phosphatase was significantly different from oocytes injected with boiled alkaline phosphatase ( $p < 0.01$ , two-way ANOVA). Injection of alkaline phosphatase did not affect the  $t_{0.5}$  of wild-type Kv1.5 current ( $4.8 \pm 0.9$  ms 0 min after injection versus  $4 \pm 0.7$  ms 30 min postinjection,  $n = 7$ ). Alkaline phosphatase caused a hyperpolarizing shift in  $V_{Act}$  from  $-20.1 \pm 0.2$  mV 0 min after injection to  $-25.4 \pm 0.5$  mV 30 min after injection ( $n = 7$ ) but with no change in the slope ( $s = 7.3 \pm 0.2$  mV versus  $6.3 \pm 0.4$  mV,  $n = 7$ ). Thus, injection of alkaline phosphatase suppressed wild-type Kv1.5 current in a similar manner to that after inhibition of PKA.

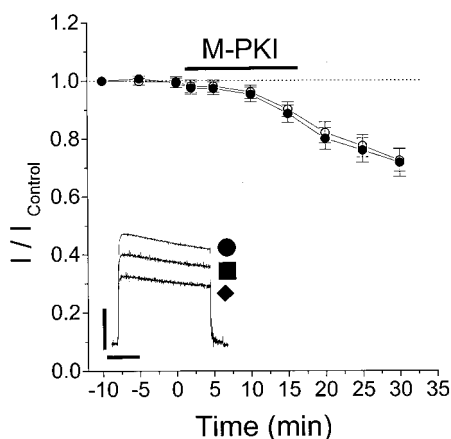
To identify the protein phosphatase that mediated the reduction in Kv1.5 current after inhibition of PKA activity we preincubated oocytes with specific protein phosphatase inhibitors. Preincubation of oocytes in inhibitors of serine/threonine protein phosphatases; okadaic acid (inhibits PP1 and PP2A, 100 nM), endothall (inhibits PP2A, 1  $\mu$ M), and delta-

methrin (inhibits PP2B, 100 nM) did not prevent the reduction in Kv1.5 currents in response to M-PKI (data not shown). In contrast, preincubation of oocytes in sodium orthovanadate (1 mM), a protein tyrosine phosphatase inhibitor (PTP), abolished the reduction in Kv1.5 current in response to M-PKI (100 nM, B). Kv1.5 currents were  $1.06 \pm 0.05$  of control after 15 min M-PKI,  $1.10 \pm 0.09$  of control after 10-min wash ( $n = 5$ ). This effect was significantly different from control oocytes; M-PKI reduced Kv1.5 currents by  $0.83 \pm 0.05$  of control after 15 min M-PKI and  $0.73 \pm 0.05$  of control after 10-min wash ( $n = 4$ ,  $p < 0.001$  by two-way ANOVA). Thus, an endogenous PTP mediates the reduction in Kv1.5 current after inhibition of PKA activity.

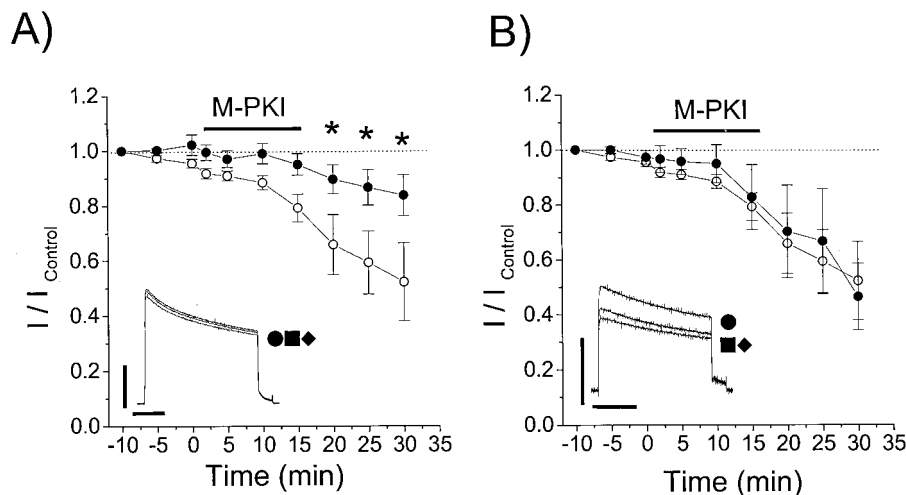
The requirement for PTP in the modulation of Kv1.5 currents by PKA suggested that Kv1.5 currents might also be modulated by tyrosine kinase. To investigate this possibility, we examined the response of Kv1.5 currents to the tyrosine kinase inhibitor genistein and its inactive analog daidzein. As shown in Fig. 7C, superfusion of genistein (100  $\mu$ M) for 15 min reduced the amplitude of Kv1.5 currents. Kv1.5 currents were  $0.74 \pm 0.04$  of control after 15 min genistein,  $0.67 \pm 0.06$  of control after 10-min wash ( $n = 5$ ). In contrast Kv1.5 currents were unaffected by superfusion of daidzein (100  $\mu$ M, data not shown). If genistein reduces Kv1.5 current by inhibition of a tyrosine kinase one expects this action to be occluded by inhibition of PTP. Accordingly, we examined the response of Kv1.5 currents to genistein in oocytes preincubated in 1 mM sodium orthovanadate. Fig. 7C shows that Kv1.5 currents in oocytes preincubated with sodium orthovanadate were unaffected by genistein. Thus, we find that Kv1.5 currents in resting oocytes are reduced by inhibitors of PKA or by an inhibitor of tyrosine kinases and that both responses require PTP.

## Discussion

We investigated the regulation by PKA of Kv1.5 currents expressed heterologously in *X. laevis* oocytes to develop information useful in understanding regulatory systems active in cardiac and vascular myocytes. This approach has an advantage in enabling the study of currents mediated by identified Kv channels. We found that treatments designed to increase levels of cAMP and PKA activity were without effect on Kv1.5 current amplitude, whereas either the inhi-



**Fig. 4.** Effect of M-PKI on Kv1.5 $\Delta$ C59. Oocytes expressing either Kv1.5 $\Delta$ C59 or wild-type Kv1.5 were superfused with M-PKI. Superfusion of M-PKI (100 nM) over a 15 min period caused a slowly developing reduction in Kv1.5 $\Delta$ C59 current (●) that continued to decline over a 15-min wash. The inset shows representative currents recorded at a test potential of +50 mV under control (●), after 15 min of M-PKI (■), and after 10-min wash (◆). This was similar to the effect of M-PKI on wild-type Kv1.5 currents (○). The vertical scale bar is 2  $\mu$ A and the horizontal scale bar is 100 ms.



**Fig. 5.** The role of the cytoskeleton in PKA regulation of Kv1.5. oocytes expressing Kv1.5 were preincubated agents to modify the cytoskeleton and subsequently superfused with M-PKI (100 nM). A, cytochalasin B (1  $\mu$ M) attenuated the reduction of Kv1.5 current by M-PKI (●) compared with untreated oocytes (○) superfused with M-PKI. B, colchicine (50  $\mu$ M) had no effect on the reduction of Kv1.5 current by M-PKI (●) compared with untreated oocytes (○). The insets show illustrative currents from preincubated oocytes under control (●), after 15 min of M-PKI (■), and after 10-min wash (◆). The vertical scale bar is 2  $\mu$ A and the horizontal scale bar is 100 ms. \*, Significantly different from control  $P < 0.05$  (two-way ANOVA).



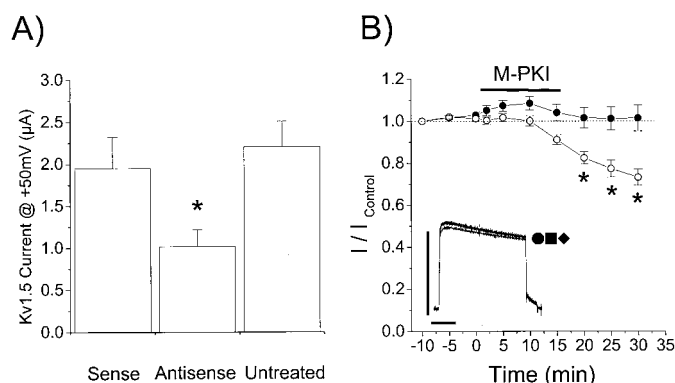
hibition of PKA by highly specific peptide inhibitors or the addition of exogenous alkaline phosphatase reduced Kv1.5 currents. Thus, the basal PKA activity in resting oocytes is maximally effective in increasing Kv1.5 currents.

We conclude that the amplitude of Kv1.5 current is increased by the phosphorylation of a protein by PKA. An obvious candidate substrate for PKA is the Kv1.5  $\alpha$  subunit itself. Indeed, this is the mechanism by which  $\beta$ -adrenergic activation of PKA increases Kv1.2 currents in *X. laevis* oocytes (Huang et al., 1994). With regard to Kv1.5, analysis of the amino acid coding sequence of canine Kv1.5  $\alpha$  subunit using DNASIS sequence analysis software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan) identified only two consensus sequences for PKA phosphorylation (R/K-R/K-X-S/

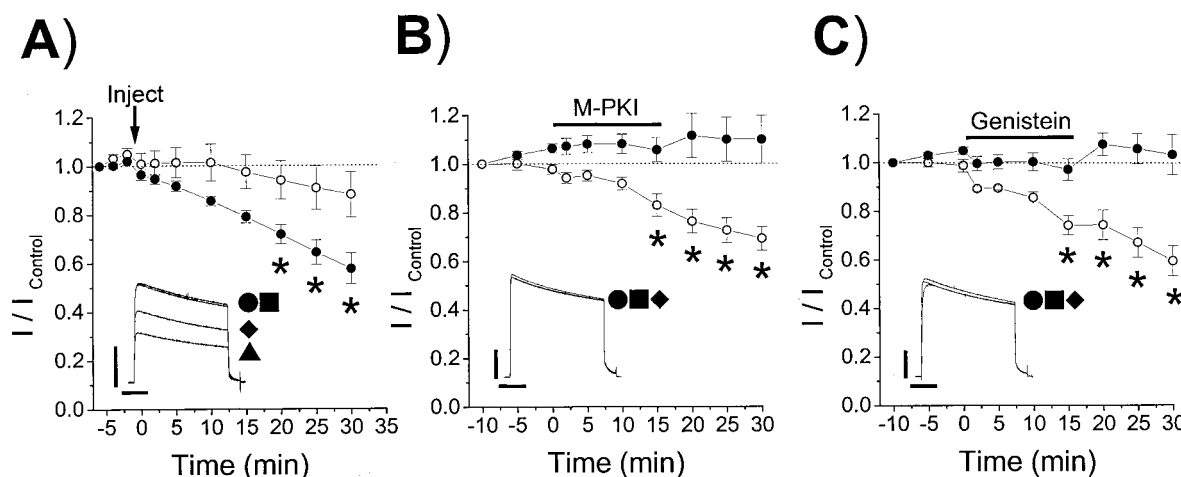
T), both of which are on the COOH terminal at Ser<sup>538</sup> and Ser<sup>545</sup>. Furthermore, the Kv1.5  $\alpha$  subunit is phosphorylated by PKA in vitro (Williams et al., 1998), although there are no data supporting a regulatory function for this phosphorylation. Indeed, our observation that the response of the Kv1.5 $\Delta$ C59 mutant channels (which lack both PKA phosphorylation sites) to inhibition of PKA was identical to that of wild-type channels implies that phosphorylation of these consensus sites is not necessary for regulation of Kv1.5 currents by PKA.

The reduction of Kv1.5 currents by the inhibition of PKA by M-PKI was attenuated in oocytes incubated in cytochalasins B and D, treatments that inhibit actin polymerization in oocytes (Theodoropoulos et al., 1994), and was abolished when oocytes were injected 2 days before recording with antisense cRNA designed to reduce synthesis of  $\alpha$ -actinin-2. These data imply that the actin cytoskeleton plays an essential role in the regulation of Kv1.5 currents by PKA. Two mechanisms have been proposed to link Kv1 family channels to the cytoskeleton: binding to PDZ proteins [all Kv1 channels, (Jing et al., 1997; Sheng and Pak, 2000)] and binding to  $\alpha$ -actinin-2 [Kv1.5 and Kv1.4, (Maruoka et al., 2000; Cukovic et al., 2001)]. We considered the PDZ-binding domain of Kv1.5 a likely source for an interaction of Kv1.5 channels with the cytoskeleton and PKA, and we investigated this by deleting this domain in our Kv1.5 $\Delta$ C59 mutant channel. However, the reduction of the Kv1.5 $\Delta$ C59 currents by inhibition of PKA was not different from the effect seen in wild-type currents, implying that the PDZ-binding domain is not required for the regulation of Kv1.5 currents by PKA.

An alternative mechanism for linking Kv1.5 channels to the cytoskeleton is by binding to  $\alpha$ -actinin-2 as described by Fedida and coworkers (Maruoka et al., 2000; Cukovic et al., 2001). These authors also showed that this interaction was functionally important. In particular, Kv1.5 currents in stably transfected HEK293 cells were increased by more than



**Fig. 6.** The effect of  $\alpha$ -actinin-2 on Kv currents. A, Kv1.5 currents were significantly smaller in magnitude in  $\alpha$ -actinin-2 antisense-treated oocytes compared with sense or water-injected oocytes. B, Kv1.5 current in oocytes injected with  $\alpha$ -actinin-2 antisense had an attenuated response to perfusion of M-PKI (100 nM,  $\bullet$ ) compared with sense-treated oocytes ( $\circ$ ). The inset shows illustrative currents from  $\alpha$ -actinin-2 antisense-injected oocytes under control ( $\bullet$ ), after 15 min of M-PKI ( $\blacksquare$ ), and after 10-min wash ( $\blacklozenge$ ). The vertical scale bar is 2  $\mu$ A and the horizontal scale bar is 100 ms. \*, Significantly different from control  $P < 0.05$  (two-way ANOVA).



**Fig. 7.** Protein tyrosine phosphatase mediates the reduction in wild-type Kv1.5 currents. A, oocytes expressing wild-type Kv1.5 were injected with 1 U/ $\mu$ l of alkaline phosphatase (to give a final estimated intraoocyte concentration of 0.05 U). Alkaline phosphatase reduced the magnitude of Kv1.5 current over the 30-min time course ( $\bullet$ ). The inset shows representative currents recorded at a test potential of +50 mV under control ( $\bullet$ ),  $t = 0$  min after injection ( $\blacksquare$ ),  $t = 15$  min after injection ( $\blacklozenge$ ), and  $t = 30$  min after injection ( $\blacktriangle$ ). Injection of boiled alkaline phosphatase had no effect on the magnitude of Kv1.5 current ( $\circ$ ). B, preincubation of oocytes in sodium orthovanadate (1 mM) abolished the reduction in Kv1.5 current in response to M-PKI (100 nM,  $\bullet$ ) compared with control oocytes ( $\circ$ ). The inset shows illustrative currents from sodium orthovanadate-treated oocytes under control ( $\bullet$ ), after 15 min M-PKI ( $\blacksquare$ ), and after 10-min wash ( $\blacklozenge$ ). C, preincubation of oocytes in sodium orthovanadate (1 mM) abolished the reduction in Kv1.5 current in response to genistein (100  $\mu$ M,  $\bullet$ ) compared with control oocytes ( $\circ$ ). The inset shows illustrative currents from sodium orthovanadate-treated oocytes under control ( $\bullet$ ), after 15 min genistein ( $\blacksquare$ ), and after 10-min wash ( $\blacklozenge$ ). The vertical scale bar is 2  $\mu$ A and the horizontal scale bar is 100 ms. \*, Significantly different from control  $P < 0.05$  (two-way ANOVA).

2-fold by exposure (2 to 24 h) to cytochalasin D or to antisense cRNA designed to reduce  $\alpha$ -actinin-2 synthesis. In contrast, we found that a similar exposure to cytochalasins had no effect on the magnitude of basal Kv1.5 currents and that treatment of oocytes with antisense specifically targeted against  $\alpha$ -actinin-2 reduced the amplitude of Kv1.5 current in *X. laevis* oocytes. These observations imply different regulatory mechanisms in the two expression systems. If similar differences are found in native cells (cardiac myocytes, smooth muscle myocytes, neurons) they could contribute to the varied responses of native Kv currents to PKA (Li et al., 1996; Ren et al., 1996; Aiello et al., 1998; Evans et al., 1999). In our study, exposure to cytochalasins or  $\alpha$ -actinin-2 antisense attenuated or abolished the reduction in Kv1.5 current by inhibition of PKA, respectively. These findings indicate that  $\alpha$ -actinin-2 is a component of a signaling system regulating the activity of Kv1.5 channels. This relationship between Kv1.5 channels and  $\alpha$ -actinin-2 is similar to that between  $\alpha$ -actinin-2 and NMDA receptors expressed in HEK293 cells (Wyszynski et al., 1997).

We have considered two mechanisms to explain how an intact actin cytoskeleton might contribute to the reduction of Kv1.5 currents by the inhibition of PKA. First, actin and  $\alpha$ -actinin-2 might localize PKA to its substrate such that cytochalasin and the antisense treatment would disrupt this localization and the phosphorylation of the substrate. This idea is attractive because it provides a ready explanation for the high basal activation of the Kv1.5 currents. This scheme predicts that disruption of the cytoskeleton would lead PKA to drift away from the vicinity of the channel, initiating dephosphorylation and reduction of Kv1.5 currents. However, our data show that the magnitude of Kv1.5 currents remains unchanged after the disruption of the cytoskeleton. Accordingly, although the cytoskeleton may target PKA to its substrate in *X. laevis* oocytes, that targeting is not essential in understanding our results. We have also considered the possibility that actin and  $\alpha$ -actinin-2 localize a protein phosphatase to a substrate important in the regulation of Kv1.5. We initially tested inhibitors of the serine/threonine family of protein phosphatases but found no effect on the reduction of Kv1.5 currents by M-PKI. In contrast, we showed that preincubation of oocytes in sodium orthovanadate, a PTP inhibitor, abolished the reduction of Kv1.5 currents by M-PKI, indicating that the action of tyrosine phosphatase is necessary for the reduction of Kv1.5 current by inhibition of PKA in *X. laevis* oocytes. Recently, Xu et al. (2001) showed that PTP associated with F-actin in a variety of cell lines, supporting the idea that the actin cytoskeleton may localize a PTP in the vicinity of the Kv1.5 channel.

PTP mediated the reduction of Kv1.5 currents after inhibition of PKA activity, suggesting that Kv1.5 currents were also modulated by a tyrosine kinase. Indeed we found that the tyrosine kinase inhibitor genistein reduced the magnitude of Kv1.5 currents in *X. laevis* oocytes, and that this reduction was abolished by sodium orthovanadate. We note that both sodium orthovanadate and genistein are relatively nonselective inhibitors of PTP and tyrosine kinase, respectively. However, the ability of sodium orthovanadate to abolish the inhibition of Kv1.5 currents by genistein indicates that sodium orthovanadate indeed inhibits the activity of an endogenous PTP in *X. laevis* oocytes. Recently, Cukovic et al. localized the  $\alpha$ -actinin-2 binding region on human Kv1.5 to

76 amino acids between residues 73 and 148 (Cukovic et al., 2001). This region encodes two proline-rich SH3-binding domains and the T1'A' multimerization domain (Scannevin and Trimmer, 1997). Interestingly, the SH3-binding domain of cloned human Kv1.5 (between residues 62 and 83) and native Kv1.5 in human myocardium directly associates with Src tyrosine kinase, and human Kv1.5 is tyrosine phosphorylated (Holmes et al., 1996). However the response of Kv1.5 currents to Src tyrosine kinase varies between cell types; Kv1.5 current is suppressed when coexpressed with v-Src in HEK293 cells (Holmes et al., 1996) whereas Kv1.5 currents are increased by Src in cultured spinal cord astrocytes (MacFarlane and Sontheimer, 2000).

In summary, the results presented here reveal that Kv1.5 currents are fully up-regulated by basal PKA and tyrosine kinase activity in *X. laevis* oocytes. However, our data with the mutant Kv1.5 $\Delta$ C59 channel indicate that regulation of Kv1.5 currents by PKA does not require the two phosphorylation sites on the COOH terminus of Kv1.5. If direct phosphorylation of the Kv1.5  $\alpha$  subunit is important, then the channel must be phosphorylated on one of the less favorable serine or threonine residues that remain on the mutated Kv1.5 $\Delta$ C59 channel (R-X-X-S/T or R-X-S/T), or on a tyrosine residue (X-E/D-Y-X). Analysis of amino acid coding sequence of canine Kv1.5  $\alpha$  subunit identifies four potential candidates that remain on the Kv1.5 $\Delta$ C59 channel: Tyr<sup>149</sup>, Ser<sup>157</sup>, and Tyr<sup>228</sup> on the NH<sub>2</sub> terminus and Thr<sup>537</sup> on the COOH terminus. Deletion analysis of these four sites is required to assess the role of these residues in the regulation of Kv1.5 by PKA. Inhibition of PKA activity by specific PKA inhibitor peptides reveals that the reduction in Kv1.5 currents is mediated by PTP. Furthermore, the modulation of Kv1.5 by PTP requires an intact actin cytoskeleton and the actin-binding protein  $\alpha$ -actinin-2. Therefore the Kv1.5/ $\alpha$ -actinin-2/actin link and tyrosine kinase may be important in mediating the increase in  $I_{Kur}$  currents in human and rat atrial myocytes and smooth muscle after  $\beta$ -adrenergic receptor stimulation.

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