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Functional Coupling of Human L-Type Ca^{2+} Channels and Angiotensin AT_{1A} Receptors Coexpressed in *Xenopus laevis* Oocytes: Involvement of the Carboxyl-Terminal Ca^{2+} Sensors

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ABSTRACT

A human recombinant L-type Ca^{2+} channel ($\alpha_{1C,77}$) was coexpressed with the rat angiotensin AT_{1A} receptor in *Xenopus laevis* oocytes. In oocytes expressing only $\alpha_{1C,77}$ channels, application of human angiotensin II (1–10 μM) did not affect the amplitude or kinetics of Ba^{2+} currents (I_{Ba}). In sharp contrast, in oocytes coexpressing $\alpha_{1C,77}$ channels and AT_{1A} receptors, application of 1 nM to 1 μM angiotensin gradually and reversibly inhibited I_{Ba}, without significantly changing its kinetics. The inhibitory effect of angiotensin on I_{Ba} was abolished in oocytes that had been preincubated with losartan (an AT_{1A} receptor antagonist) or thapsigargin or injected with 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate, pertussis toxin, guanosine-5′-*O*-(2-thio)diphosphate, or heparin, suggesting that the recombinant α_{1C} channels were regulated by angiotensin through G protein-coupled AT_{1A} receptors via activation of the inositol trisphosphate-dependent intracellular Ca^{2+} release pathway. Consistent with this hypothesis, no cross-signaling occurred between the AT_{1A} receptor and a splice variant of α_{1C} lacking Ca^{2+} sensors ($\alpha_{1C,86}$). The data suggest that the regulation of recombinant L-type Ca^{2+} channels by angiotensin is mediated by inositol trisphosphate-induced intracellular Ca^{2+} release and occurs at the molecular motif responsible for the Ca^{2+}-induced inactivation of the channels.

Voltage-gated Ca^{2+} channels are a major route for Ca^{2+} entry into cells in response to stimulation by hormones, neurotransmitters, or drugs. The resulting rise in cytoplasmic free Ca^{2+} triggers a cascade of intracellular signaling events, which underlie a variety of cellular responses, ranging from contraction and secretion to growth and mitogenesis. Therefore, identification of the molecular basis for functional coupling between Ca^{2+} channels and hormone or neurotransmitter receptors may provide critical information on cellular signaling mechanisms.

The cardiac L-type Ca^{2+} channel is composed of the pore-forming α_{1C} and auxiliary β and α_2/δ subunits (Catterall, 1995). In an artificial expression system, the $\alpha_{1C}\beta\alpha_2/\delta$ complex is sufficient to give rise to Ca^{2+} channels exhibiting all of the major electrophysiological properties observed *in vivo*. However, functional regulation of the recombinant Ca^{2+} channel remains largely unknown. For example, in cardiac or vascular cells, the α_{1C} channel is modulated by protein kinase A- and protein kinase C-dependent phosphorylation (McDonald *et al.*, 1994). However, when all three recombinant subunits of the channel are coexpressed in *Xenopus laevis* oocytes or in eukaryotic systems (Chinese hamster ovary or human embryonic kidney cells), their modulation through phosphorylation is either strongly reduced or essentially absent (Bouron *et al.*, 1995; Zong *et al.*, 1995; Shuba *et al.*, 1997), even though the expressed channels display the same voltage dependence, gating kinetics, unitary conductance, and pharmacological properties as the native α_{1C} L-type Ca^{2+} channels. These findings demonstrate the complexity of molecular signaling involving the α_{1C} Ca^{2+} channels; this complexity extends to the largely unexplored area of "cross-talk" between recombinant α_{1C} channels and hormone receptors that are coexpressed in *X. laevis* oocytes.

The coupling of α_{1C} Ca^{2+} channels with angiotensin II AT_1 receptors has attracted much attention. For example, the L-type Ca^{2+} channel blockers verapamil, diltiazem, and nifedipine have been shown to block angiotensin II-mediated vascular contraction *in vivo* in humans (Andrawis *et al.*, 1992). Activation of AT_1 receptors seems to be associated with both immediate contractile and long term growth responses in vascular smooth muscle and cardiac myocytes (Baker *et al.*, 1992; Sadoshima and Izumo, 1993; Miyata and Haneda, 1994). Supporting the possibility of interactions between the G protein-coupled AT_1 receptors (Anand-Srivastava, 1983; Ohya and Spereliakis, 1991) and voltage-activated Ca^{2+} channels is the regulation of neuronal (Scott and

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ABBREVIATIONS: PTX, pertussis toxin; IP_3, inositol trisphosphate; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; $I_{Cl(Ca)}$, Ca^{2+}-activated Cl^- current; I_{Ba}, Ba^{2+} current; I_{Cl}, Cl^- current; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Dolphin, 1987) and cardiac (Yatani *et al.*, 1987) L-type Ca^{2+} channels by PTX-sensitive or -insensitive G proteins. Similar interactions have been suggested for angiotensin II activation of L-type Ca^{2+} currents in rat portal vein myocytes (Macrez-Lepretre *et al.*, 1996) and T-type Ca^{2+} currents in adrenal zona glomerulosa cells (Lu *et al.*, 1996).

In this study, we have used the *X. laevis* oocyte expression system to study the functional coupling between recombinant rat AT_{1A} receptors and splice variants of recombinant human α_{1C} Ca^{2+} channels with or without the molecular motif responsible for Ca^{2+}-dependent inactivation of the channel. We show that heterogeneously expressed Ca^{2+} channels and AT_{1A} receptors are functionally coupled via the G protein/IP_3-mediated Ca^{2+} signaling cascade. Additionally, we report that the molecular locus for the angiotensin-induced modulation of the α_{1C} Ca^{2+} channel is independent of permeation of Ca^{2+} through the pore and is confined to the carboxyl-terminal cytoplasmic motif (positions 1572–1651), which contains multiple Ca^{2+} sensors of the channel.

Materials and Methods

Preparation of mRNAs. Template $\alpha_{1C,77}$ (Soldatov *et al.*, 1995) and $\alpha_{1C,86}$ (Soldatov *et al.*, 1997) cDNAs were linearized by digestion with *Bam*HI. Capped transcripts were synthesized *in vitro* with T7 RNA polymerase, using the mRNA cap kit (Stratagene, La Jolla, CA). mRNAs were dissolved in water (0.5 μg/μl). Rat angiotensin AT_{1A} receptor (Murphy *et al.*, 1991) transcripts were kindly provided by Kathryn Sandberg (Georgetown University).

Oocyte preparation and injection. Mature female *X. laevis* frogs were purchased from Xenopus I (Ann Arbor, MI). Clusters of oocytes were defolliculated by shaking for 2 hr at room temperature in 25 ml of medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5 (adjusted with NaOH), and 0.2% collagenase A (Boehringer Mannheim, Indianapolis, IN). Oocytes were injected with 50–100 nl of $\alpha_{1C,77}$ or $\alpha_{1C,86}$ mRNA premixed with mRNAs coding for auxiliary β_1 (Ruth *et al.*, 1989) and $\alpha_2\delta$ subunits (Singer *et al.*, 1991) and the AT_{1A} receptor (in a 1:1:1:0.05 molar ratio). Injected oocytes were incubated at 18° in sterile Barth's medium supplemented with 10,000 units/liter penicillin, 10 mg/liter streptomycin, 50 mg/liter gentamicin, and 0.5 mM theophylline (all from Sigma Chemical Co., St. Louis, MO).

Electrophysiological measurements. Whole-cell ion currents were recorded at room temperature (20–22°) by a two-electrode, voltage-clamp method, as previously described (Soldatov *et al.*, 1998). Current traces were elicited at 30-sec intervals by 1-sec (current-voltage relationships) or 250-msec test pulses to +20 mV, from a holding potential of −90 mV. The Ba^{2+} extracellular (bath) solution contained 50 mM NaOH, 1 mM KOH, 10 mM HEPES, and 40 mM $Ba(OH)_2$ (pH adjusted to 7.4 with methanesulfonic acid). Voltage-clamped oocytes were continuously perfused with control experimental solutions at the rate of ~10 ml/min (bath volume, ~150 μl). Human angiotensin II (Sigma) was applied extracellularly. In some experiments, oocytes were injected with 50 nl of PTX (5 μg/ml), 10 mM GDPβS, 94 mM Cs_4BAPTA (pH 7.4), or 10 μM heparin (molecular weight, ~3000; Sigma) approximately 1 hr before the experiment. In other experiments, oocytes were incubated at 18° overnight in Ca^{2+}-free Barth's solution containing 10 nM thapsigargin (RBI, Natick, MA), to deplete their intracellular Ca^{2+} stores. Results are shown as mean ± standard error. I_{Ba}, determined in the presence of 5 μM (±)-PN200–110 to block the L-type current, did not exceed 3–5% of the total current.

Results

Coexpression of the $\alpha_{1C,77}$ channel with the AT_{1A} receptor allows regulation of Ca^{2+} channels by angiotensin. Coinjection into *X. laevis* oocytes of cRNAs coding for the conventional $\alpha_{1C,77}$ channel and auxiliary β_1 and $\alpha_2\delta$ subunits gave rise to the expression of well defined, slowly inactivating currents through Ca^{2+} channels 2–3 days after the injection of cRNAs (Soldatov *et al.*, 1995). With Ba^{2+} as a charge carrier, step depolarization to +20 mV from a holding potential of −90 mV activated a slowly inactivating, L-type

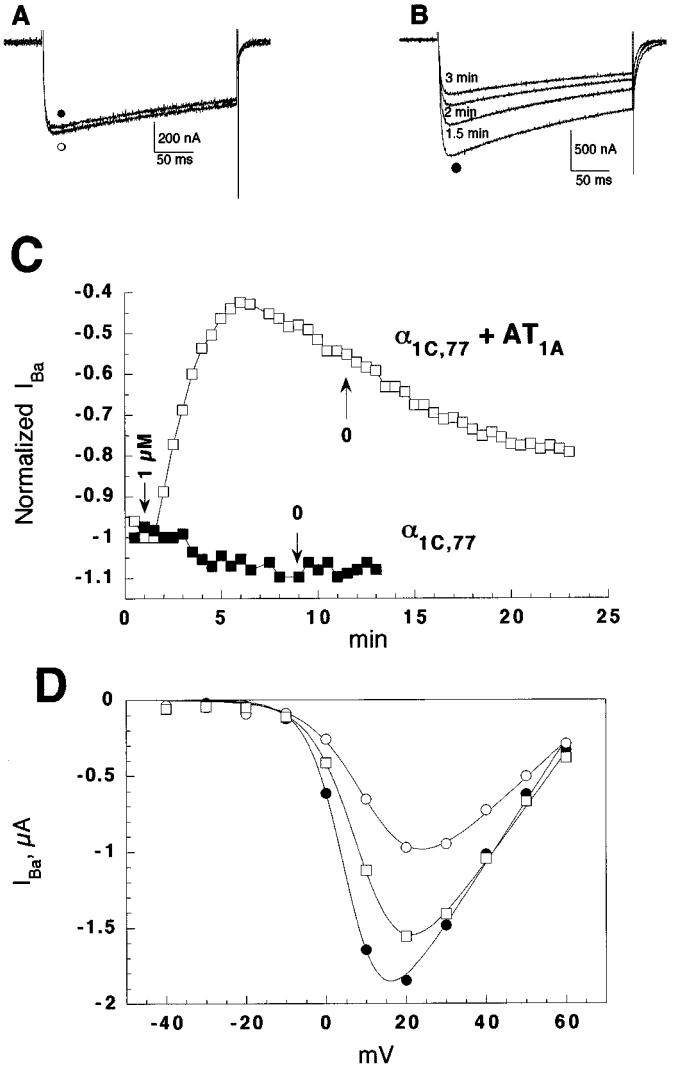

Fig. 1. Effect of angiotensin on Ca^{2+} currents. A, Representative traces of I_{Ba} through $\alpha_{1C,77}$ channels, elicited by stepwise depolarization to +20 mV from a holding potential of −90 mV, before (●) and 4 min after (○) application of 1 μM angiotensin. B, Traces of I_{Ba} through $\alpha_{1C,77}$ channels coexpressed with AT_{1A} receptors, recorded before (●) and 1.5, 2, and 3 min after application of 1 μM angiotensin. C, Time dependence and reversibility of the angiotensin effect on I_{Ba} through $\alpha_{1C,77}$ channels expressed alone (■) or coexpressed with AT_{1A} receptors (□). I_{Ba} amplitudes were measured in response to 250-msec test pulses to +20 mV, applied at 30-sec intervals, and were normalized to maximal I_{Ba} in the absence of angiotensin. *Arrows*, times of application of bath solutions containing the indicated concentrations of angiotensin. D, Current-voltage relationships for I_{Ba} through $\alpha_{1C,77}$ channels coexpressed with AT_{1A} receptors, before treatment (●), 5 min after treatment with 1 μM angiotensin (○), and after a 10-min perfusion with bath medium (□), obtained using the same oocyte as in B and C (□). Experiments were performed at room temperature (~21°).

I_{Ba} (mean amplitude, -1.64 ± 0.33 μA, $n = 9$). Application of 0.5–10 μM angiotensin to oocytes expressing only $\alpha_{1C,77}$ Ca^{2+} channels produced little or no change in the magnitude or the kinetics of the current, at all voltages examined (Fig. 1, A and C; Table 1).

In sharp contrast, in oocytes coexpressing the human $\alpha_{1C,77}$ channel and rat AT_{1A} receptor (Murphy *et al.*, 1991), angiotensin (0.1–1 μM) inhibited I_{Ba} by ~54% ($n = 12$), in a time- and concentration-dependent manner (Table 1). The suppressive effect of angiotensin developed within 3–4 min of the hormone exposure, but the effect slowly (20–30 min) reversed even in the presence of the hormone. Fig. 1C shows that 57.6% inhibition of I_{Ba} by 1 μM angiotensin reversed spontaneously and washout of the hormone did not accelerate the recovery of the current (Fig. 2). In the presence of angiotensin, I_{Ba} recovered by $90.2 \pm 4.0\%$ ($n = 7$) within 20–30 min. The voltage dependence of I_{Ba} at the peak of the hormone effect was often shifted by approximately +10 mV (Fig. 1D). These results suggest that the time course of the hormone effect is not critically dependent on the continued presence of the hormone.

Fig. 3A illustrates the concentration dependence of the angiotensin effect on $\alpha_{1C,77}$ channels. Under our experimental conditions, the maximal inhibitory effect (~60% suppression) was reached with 1 μM angiotensin. In none of the cells tested ($n = 12$) did the inhibitory effect on I_{Ba} exceed 60%. The estimated IC_{50} value for angiotensin was 33 ± 8 nM ($n = 4$), with a Hill coefficient of approximately 0.85.

Angiotensin failed to suppress the Ca^{2+} channels in the presence of the reversible AT_{1A} receptor antagonist losartan. Fig. 3B shows that 1 μM losartan had no effect by itself on I_{Ba} in an oocyte coexpressing AT_{1A} receptors and α_{1C} channels but completely blocked the angiotensin effect. Replacement of losartan-containing solution with one containing 1 μM angiotensin, however, produced up to 40% ($n = 3$) inhibition of I_{Ba}. The time course of the inhibition of I_{Ba} was slower than in control experiments (Figs. 1C, 4, and 5), which might have been partly caused by the slow dissociation of losartan from the AT_{1A} receptor sites. Taken together, these data suggest that the suppression of I_{Ba} through $\alpha_{1C,77}$ channels by angiotensin is mediated through the direct interaction of angiotensin with AT_{1A} receptors.

Angiotensin activates a transient I_{Cl}. The rapid application of the hormone in Cl^-–free solutions was often but not always accompanied by activation of a large, transient, inward current lasting ~2 min. The activation of this inward holding current, measured at −90 mV in Cl^-–free extracellular solution (Fig. 4, *lower*), preceded the decrease in I_{Ba}. This current had properties similar to those previously identified (Hartzell, 1996; Gomez-Hernandez *et al.*, 1997) for $I_{Cl(Ca)}$. During the activation of I_{Cl}, I_{Ba} often exhibited decreased inactivation kinetics, producing large, slowly deactivating, tail currents (Fig. 4, *upper*, *traces 2* and *3*). Interestingly, the angiotensin-induced, transient suppression of I_{Ba} outlasted the activation of $I_{Cl(Ca)}$ by 2–3 min (Fig. 4), suggesting either different affinities of Ca^{2+} channels and Ca^{2+}-activated Cl^- channels for Ca^{2+} or differences in the spatial distribution of the two channels with respect to the intracellular Ca^{2+} pools. Lower affinity of $I_{Cl(Ca)}$ for activation by Ca^{2+}, compared with Ca^{2+}-induced inactivation of Ca^{2+} channels, and variations in the Ca^{2+} contents of intracellular Ca^{2+} pools of the oocytes might be partly responsible for the variations in the magnitude of I_{Cl} in different oocytes.

The IP_3/Ca^{2+} signaling pathway is involved in channel regulation by angiotensin. Ca^{2+} stores in *X. laevis* oocytes are known to be regulated through the activation of IP_3-sensitive Ca^{2+} release channels (Berridge and Irvine, 1989; Putney *et al.*, 1989). These channels are thought to be involved in receptor-mediated Ca^{2+} signaling, and their activation is known to evoke $I_{Cl(Ca)}$ in oocytes (Yao and Parker, 1993; Hartzell, 1996). Consistent with this idea, in oocytes bathed in Barth's solution and expressing only AT_{1A} receptors, a transient (2–3-min) I_{Cl} was activated upon rapid application of angiotensin (data not shown). To further characterize the steps in the regulation of recombinant α_{1C} channels by AT_{1A} receptors, when coexpressed in oocytes, we probed the various steps of the IP_3-mediated Ca^{2+} signaling cascade by inhibiting the G proteins, blocking the IP_3 receptor, and interfering with the rise in intracellular Ca^{2+} levels.

Release of intracellular Ca^{2+} mediates the angiotensin-induced effects. The depletion of intracellular Ca^{2+} stores by overnight incubation of oocytes with 10 nM thapsigargin (Thastrup *et al.*, 1990) completely abolished the effect of 1 μM angiotensin on I_{Ba} (Fig. 6, A and B). No significant difference in the amplitude of I_{Ba} in control and thapsigargin-incubated oocytes was observed (Table 1). Similarly, oocytes injected with Ca^{2+} buffers failed to respond to angiotensin. Fig. 6, C and D, shows data recorded from an oocyte that was injected with 50 nl of 94 mM Cs_4BAPTA solution 30 min before measurements of I_{Ba}. The data ($n = 4$) showed

TABLE 1

Inhibition of I_{Ba} in *X. laevis* oocytes by angiotensin depends on the expression of the α_{1C} subunit of Ca^{2+} channel and the AT_{1A} receptor

Oocytes were injected with mRNAs coding for auxiliary β_1 and $\alpha_2\delta$ subunits of the Ca^{2+} channel, mixed with the indicated mRNAs. I_{Ba} traces were elicited by 250-msec test pulses to +20 mV from a holding voltage of −90 mV. Average amplitudes of I_{Ba} were measured before (control) and 5 min after application of 0.5–10 μM angiotensin. In some experiments, oocytes were injected 30–60 min before measurements with 50 nl of 5 μg/ml PTX, 10 mM GDPβS, 10 μM heparin, or 94 mM Cs_4BAPTA or were incubated overnight with 10 nM thapsigargin.

mRNA	Drug	Average amplitude of I_{Ba}		Inhibition of I_{Ba}	n^a
		Control	Angiotensin		
		μA		%	
$\alpha_{1C,77}$	None	-1.64 ± 0.33	-1.57 ± 0.30	3 ± 3	9
$\alpha_{1C,77}$ + AT_{1A}	None	-1.57 ± 0.29	-0.72 ± 0.15	54 ± 4	12
$\alpha_{1C,86}$ + AT_{1A}	None	-0.43 ± 0.19	-0.42 ± 0.19	0	4
$\alpha_{1C,77}$ + AT_{1A}	PTX	-1.24 ± 0.29	-1.23 ± 0.29	0	4
$\alpha_{1C,77}$ + AT_{1A}	GDPβS	-1.01 ± 0.31	-1.03 ± 0.33	0	4
$\alpha_{1C,77}$ + AT_{1A}	Thapsigargin	-2.57 ± 0.40	-2.60 ± 0.42	0	3
$\alpha_{1C,77}$ + AT_{1A}	BAPTA	-0.54 ± 0.09	-0.55 ± 0.09	0	5
$\alpha_{1C,77}$ + AT_{1A}	Heparin	-1.18 ± 0.24	-1.12 ± 0.22	4 ± 2	6

[a] n, number of tested oocytes.

that signaling between $\alpha_{1C,77}$ channels and AT_{1A} receptors in response to 0.1–1 μM angiotensin was completely suppressed.

PTX-sensitive G proteins and IP_3 receptors mediate the angiotensin-induced effects. AT_{1A} receptors in mammalian cells are known to be coupled to G proteins (Lu *et al.*, 1996). In *X. laevis* oocytes coexpressing AT_{1A} receptors and Ca^{2+} channels, we probed the functional manifestation of G protein coupling. In oocytes that had been preinjected with 50–100 nl of GDPβS (10 mM), angiotensin (1 μM) failed to produce significant inhibitory effects on I_{Ba} (Fig. 6, C and D; Table 1). Because the effect of angiotensin was also blocked in parallel experiments with microinjection of 50–100 nl of PTX (5 μg/ml) (Fig. 6, C and D; Table 1), the coupling between the recombinant AT_{1A} receptors and $\alpha_{1C,77}$ Ca^{2+} channels seemed to be mediated through the activation of endogenous G proteins of the G_i type.

To directly examine the involvement of IP_3-sensitive Ca^{2+} release channels, oocytes coexpressing α_{1C} channels and AT_{1A} receptors were injected with 50 nl of 10 μM heparin (known to block IP_3 receptors) (Guillemette *et al.*, 1989) 30–60 min before the experiment. Fig. 6, E and F, shows that, in heparin-injected oocytes, angiotensin failed to produce its Ca^{2+} channel-suppressive effect on either the current-voltage relationship (Fig. 6E; Table 1) or the time course of I_{Ba} (Fig. 6F).

Therefore, it seems that the release of Ca^{2+} via the IP_3 signaling pathway mediates the angiotensin-induced suppressive effect on I_{Ba}. We examined whether the effect of the hormone was directly related to the content of intracellular Ca^{2+} stores. Fig. 5 shows data from an experiment in which, after inhibition and recovery of I_{Ba} in the presence of 1 μM angiotensin and a 10-min washout in Ba^{2+}-containing Ringer's solution, the reapplication of 1 μM angiotensin failed to produce any effect on I_{Ba}. However, after 5 min of incubation of the oocyte in normal Ca^{2+}-containing Barth's solution, the response of Ca^{2+} channels to 1 μM angiotensin partially

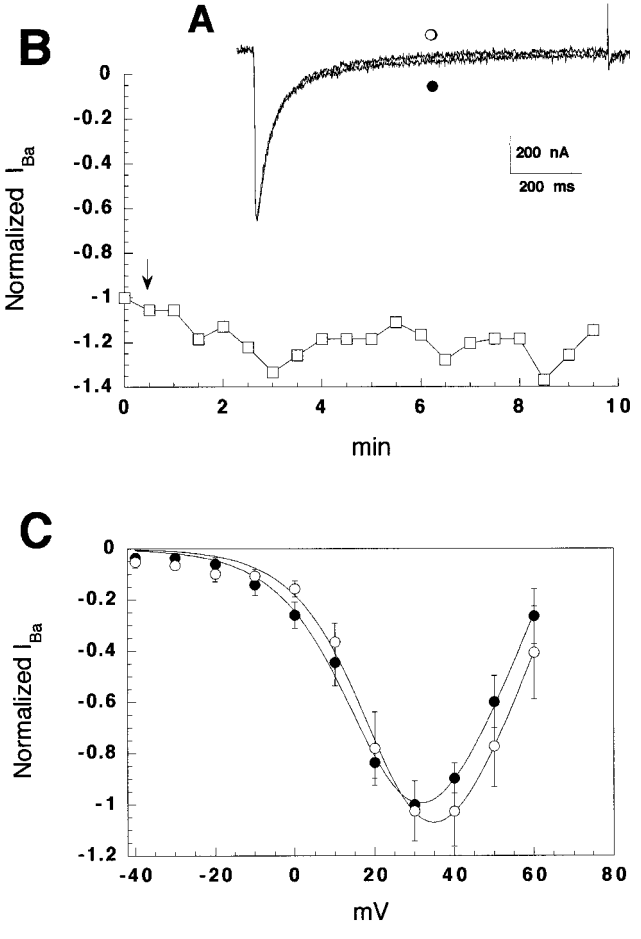

Fig. 2. Failure of angiotensin to suppress I_{Ba} through $\alpha_{1C,86}$ channels coexpressed with AT_{1A} receptors. A, Representative traces of I_{Ba} elicited by stepwise depolarization to +20 mV, from a holding potential of −90 mV, recorded before (●) and 5 min after (○) application of 1 μM angiotensin. B, Time dependence of the effect of 1 μM angiotensin added to the external Ba^{2+} solution (*arrow*) on I_{Ba} through the $\alpha_{1C,86}$ channels. C, Averaged current-voltage relationships (n = 3) determined before (●) and 5 min after (○) application of 1 μM angiotensin. In B and C, the amplitudes of I_{Ba} were normalized to maximal I_{Ba} in the absence of angiotensin.

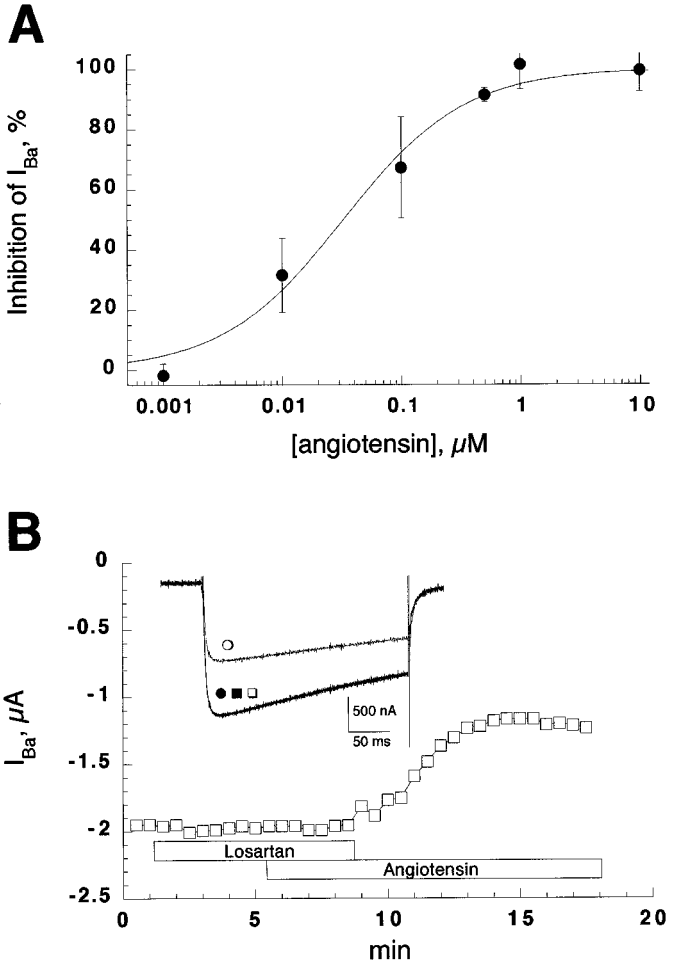

Fig. 3. A, Concentration-response relationship for the angiotensin effect. Angiotensin, at the indicated concentrations, was applied to an oocyte coexpressing $\alpha_{1C,77}$ channels and AT_{1A} receptors. I_{Ba} was measured at +20 mV, after 5 min of equilibration. The averaged concentration dependence clearly shows saturation of the effect at 1 μM angiotensin. The curve was normalized to the maximal effect and then fit by the function $I = 1/\{1 + (IC_{50}/[Ang])^n\}$, where I is the normalized I_{Ba} amplitude, IC_{50} is the concentration of angiotensin producing 50% inhibition of I_{Ba}, [Ang] is the concentration of angiotensin in the bath solution, and n is the Hill coefficient. The regression coefficient was 0.992. Values are means ± standard errors of four oocytes. B, Time course of the effect of 1 μM losartan and/or 1 μM angiotensin on peak I_{Ba} evoked by stepwise depolarization to +20 mV from a holding potential of −90 mV, in an oocyte expressing $\alpha_{1C,77}$ channels and AT_{1A} receptors. *Horizontal bars*, times at which losartan and/or angiotensin was applied to the oocyte. *Inset*, traces of I_{Ba} recorded at 1 (●), 4 (□), 8 (■), and 15 (○) min in this experiment.

recovered. This finding is consistent with the idea that, during incubation in Barth's solution, IP_3-sensitive intracellular Ca^{2+} stores are replenished with Ca^{2+} by entry of Ca^{2+} through depletion-activated Ca^{2+} channels (Zweifach and Lewis, 1995; Lepple-Wienhues and Cahalan, 1996; Parekh and Penner, 1997) or $\alpha_{1C,77}$ Ca^{2+} channels, making it possible for angiotensin to induce Ca^{2+} release.

A Ca^{2+}-insensitive $\alpha_{1C,86}$ Ca^{2+} channel coexpressed with the AT_{1A} receptor is not modulated by angiotensin. To examine a molecular motif possibly involved in angiotensin-mediated modulation of Ca^{2+} channels, a recently described Ca^{2+} channel isoform ($\alpha_{1C,86}$) lacking the Ca^{2+} sensors responsible for Ca^{2+}-induced modulation (Soldatov *et al.*, 1997) was coexpressed with AT_{1A} receptors in *X. laevis* oocytes. In contrast to the effect of angiotensin on the $\alpha_{1C,77}$ channel (Figs. 1, 3, and 4), the $\alpha_{1C,86}$ channel was insensitive to modulation by angiotensin (Table 1). Fig. 2A demonstrates that neither the amplitude nor the kinetics of I_{Ba} were significantly changed in the presence of angiotensin. There was often a 5–15% increase in the amplitude of I_{Ba} (Fig. 2B), which resembled the small increase of I_{Ba} observed in oocytes expressing $\alpha_{1C,77}$ without the AT_{1A} receptor (Fig. 1C). Interestingly, the voltage dependence of I_{Ba} through $\alpha_{1C,86}$ channels was also reversibly shifted to more positive potentials in the presence of 1 μM angiotensin (Fig. 2C), in a manner similar to that observed for $\alpha_{1C,77}$ (Fig. 1D). This shift might be the result of additional screening effects of the released Ca^{2+} on the plasma membrane cation-binding sites. The absence of angiotensin effects in oocytes coexpressing $\alpha_{1C,86}$ with AT_{1A} receptors suggests that the Ca^{2+} sensors of the Ca^{2+} channel are critical in mediating the suppressive effect of angiotensin on the channel.

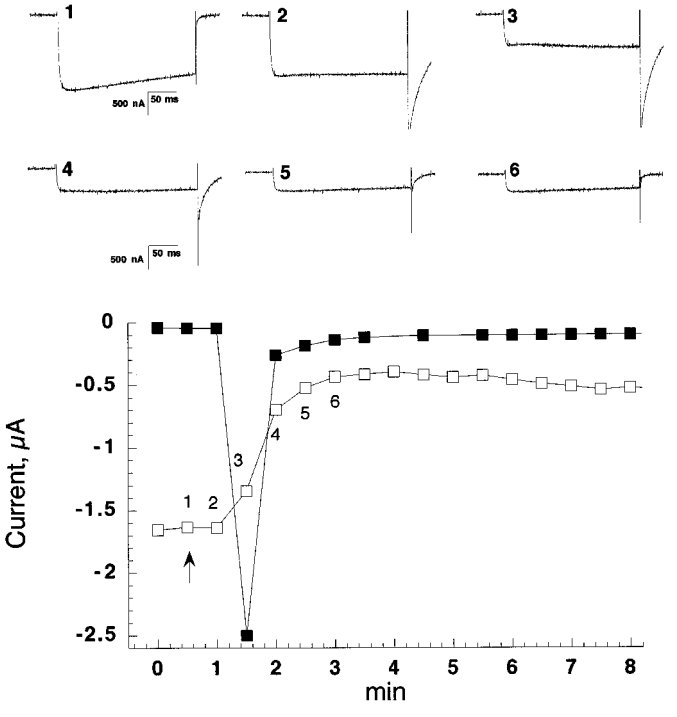

Fig. 4. Time course of the development of angiotensin effects on I_{Ba} and $I_{Cl(Ca)}$ in oocytes coexpressing AT_{1A} receptors and $\alpha_{1C,77}$ channels. *Lower*, time dependence of the effect of angiotensin on the holding current, $I_{Cl(Ca)}$ (■), measured at −90 mV and on I_{Ba} (□) measured at +20 mV. *Arrow*, time when 1 μM angiotensin was applied to the oocyte. *Upper*, traces of I_{Ba} recorded at the times indicated (*numbers in lower*).

Discussion

Our results show conclusively that, in the oocyte expression system, human recombinant α_{1C} Ca^{2+} channels can be modulated by angiotensin through AT_{1A} receptors via the G protein-dependent, IP_3-activated Ca^{2+} release system. Inhibition of any of the key steps in the IP_3-dependent Ca^{2+} signaling pathway, including blockade of AT_{1A} receptors (by losartan), G proteins (by GDPβS or PTX), or IP_3 receptors (by heparin) and depletion of intracellular Ca^{2+} stores (by thapsigargin or BAPTA), eliminated the suppressive effect of angiotensin on the Ca^{2+} channels. The hormone-induced transient increase of the intracellular Ca^{2+} concentration also activated Ca^{2+}-dependent Cl^- channels (Hartzell, 1996; Gomez-Hernandez *et al.*, 1997), which was monitored in our experiments as the transient increase in the holding current at −90 mV (Fig. 4). The Ca^{2+}-dependent outward Cl^- flux (inward I_{Cl}) seems to produce sufficient increases in membrane conductance to cause slowing of the inactivation kinetics of I_{Ba} and development of slowly deactivating "tails" (Fig. 4).

It is intriguing to note that, although the hormone suppressed the amplitude of I_{Ba} by releasing intracellular Ca^{2+}, the kinetics of the current was not significantly accelerated (Fig. 1B), as might have been expected from a comparison of Ca^{2+} and Ba^{2+} current traces recorded in the oocyte expression system (e.g., Fig. 2A in the report by Soldatov *et al.*, 1998). One possible explanation for this result is that pore-permeating Ca^{2+} and intracellularly released Ca^{2+} may regulate the α_{1C} channel activity by targeting different molecular sites (Ca^{2+} sensors) associated with the channel. Similar dual modulation of Ca^{2+} channel kinetics by intracellular Ca^{2+} was first observed in dorsal root ganglion neurons (Morad *et al.*, 1988). In that case, photorelease of caged Ca^{2+}

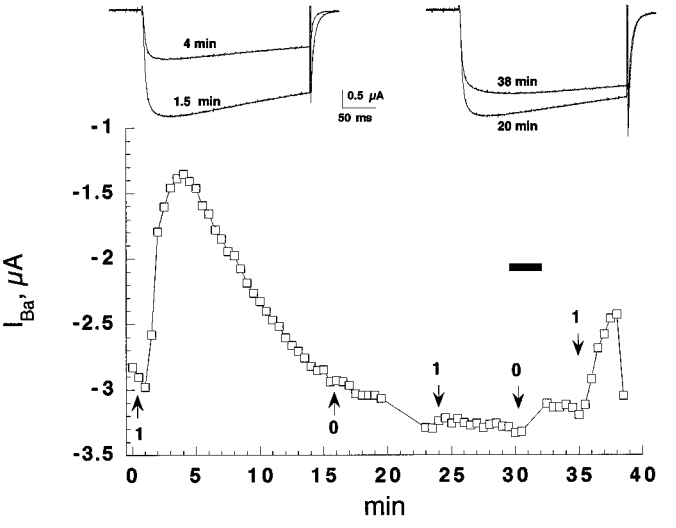

Fig. 5. Dependence of the reversibility of the angiotensin effect on the loading of intracellular Ca^{2+} stores. *Lower*, time course of the effect of 1 μM angiotensin applied (*arrows 1*) to an oocyte bathed in 40 mM Ba^{2+}-containing solution. Test pulses to +20 mV were applied every 30 sec. *Arrows 0*, washout of angiotensin. The suppressive effect of angiotensin was fully reversed in the oocyte, but washout for 10 min in Ba^{2+}-containing solution did not restore the angiotensin response. *Horizontal bar*, incubation of the oocyte in normal Barth's solution containing 2 mM Ca^{2+}. Reapplication of angiotensin even after 5 min of incubation in Ca^{2+}-containing Barth's solution restored the angiotensin effect. *Upper*, traces of I_{Ba} recorded at the indicated times.

(10–50 μM) strongly suppressed the Na^+ current through the channel, without affecting the kinetics of its inactivation. In support of the idea of dual modulation, we recently reported that a segment (positions 1572–1651) of the cytoplasmic carboxyl-terminal tail of $\alpha_{1C,77}$ contains two separate Ca^{2+} sensors (molecular determinants for the Ca^{2+}-dependent inactivation of the channel) (Soldatov *et al.*, 1998). The identified Ca^{2+} sensors may differentially contribute to the Ca^{2+}-induced inactivation of the channel, because they may be selectively targeted by permeating versus cytoplasmic Ca^{2+} because of their specific locations with respect to the pore. Consistent with this idea, the $\alpha_{1C,86}$ channel, which lacks Ca^{2+} sensors in the carboxyl-terminal tail and does not show Ca^{2+}-dependent inactivation, conducts Ca^{2+} and Ba^{2+} with comparable kinetics (Soldatov *et al.*, 1997) and is insensitive to angiotensin-mediated increases in intracellular Ca^{2+} concentrations (Fig. 6; Table 1). Because segment 1572–1651 is the only molecular motif modified in the $\alpha_{1C,86}$ channel,

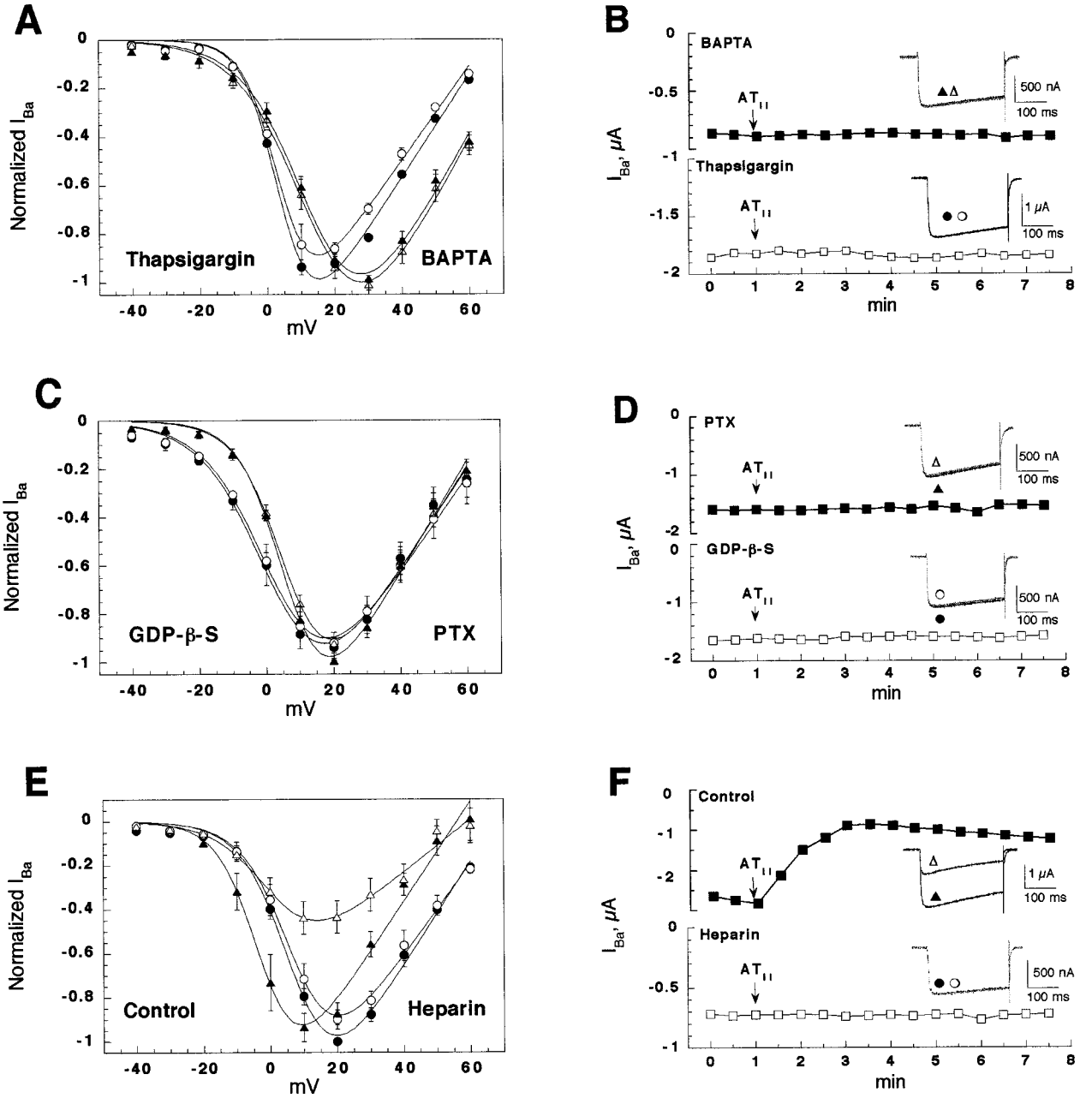

Fig. 6. Molecular steps in the modulation of I_{Ba} by angiotensin. Two sets of pairs of averaged current-voltage relationships (A, C, and E) and time-dependent relationships for the angiotensin effect (B, D, and F), as well as representative current traces, were recorded before (●, ▲) and 5 min after (○, △) application of 1 μM angiotensin. *Arrows*, times at which angiotensin (AT_{II}) was applied. A and B, Examination of the role of intracellular Ca^{2+} release. Oocytes were incubated overnight with 10 nM thapsigargin (A, ●, ○, n = 3; B, □) or injected 30–60 min before measurements with 50 nl of 94 mM Cs_4BAPTA (A, ▲, △, n = 6; B, ■). C and D, Examination of the role of G proteins. Oocytes were injected 30–60 min before measurements with 50 nl of 10 mM GDPβS (C, ●, ○, n = 4; D, □) or 5 μg/ml PTX (C, ▲, △, n = 5; D, ■). E and F, Examination of the involvement of IP_3 receptors. Oocytes were injected 30–60 min before measurements with 50 nl of 10 μM heparin (molecular weight, 3000) (E, ●, ○, n = 4; F, □). We also show the response of a control oocyte, coexpressing $\alpha_{1C,77}$ channels and AT_{1A} receptors (E, ▲, △, n = 6; F, ■), to angiotensin before the interventions described in A–F.

compared with the $\alpha_{1C,77}$ channel, we conclude that this locus is largely responsible for the angiotensin-induced modulation of the $\alpha_{1C,77}$ channel coexpressed with the AT_{1A} receptor. This modulation takes place when Ba^{2+} is the charge carrier through the channel and is apparently independent of permeation of Ca^{2+} through the pore.

Our data on the differential modulation of Ca^{2+} channels by pore-permeating Ca^{2+} and Ca^{2+} released in the cytosol might indicate critical steps in cross-signaling between the angiotensin receptor and IP_3-gated Ca^{2+} stores. Such dual control adds to the complexity of the mechanisms of cross-talk between Ca^{2+} channels and G protein-coupled receptors and may be of fundamental physiological significance, considering that signaling may take place in confined cellular microdomains.

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