Augmentation of Cav3.2 T-Type Calcium Channel Activity by cAMP-Dependent Protein Kinase A

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ABSTRACT

Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels is crucial for important physiological activities such as hormone secretion and neuronal excitability. However, it is not clear whether these channels are regulated by cAMP-dependent protein kinase A (PKA). In the present study, we examined whether PKA modulates Cav3.2 T-type channels reconstituted in Xenopus oocytes. Application of 10 μM forskolin, an adenylyl cyclase stimulant, increased Cav3.2 channel activity by 40 ± 4% over 30 min and negatively shifted the steady-state inactivation curve (V_{50} = −61.4 ± 0.2 versus −65.5 ± 0.1 mV). Forskolin did not affect other biophysical properties of Cav3.2 channels, including activation curve, current kinetics, and recovery from inactivation. Similar stimulation was achieved by applying 200 μM 8-bromo-cAMP, a membrane-permeable cAMP analog. The augmentation of Cav3.2 channel activity by forskolin was strongly inhibited by preincubation with 20 μM N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89), and reversed by subsequent application of 500 nM protein kinase A inhibitor peptide. The stimulation of Cav3.2 channel activity by PKA was mimicked by serotonin when 5HT\(_7\) receptor was coexpressed with Cav3.2 in Xenopus oocytes. Finally, using chimeric channels constructed by replacing individual cytoplasmic loops of Cav3.2 with those of the Na\(_{1.4}\) channel, which is insensitive to PKA, we localized a region required for the PKA-mediated augmentation to the II-III loop of the Ca\(_{3.2}\).

Low-voltage-activated T-type Ca\(^{2+}\) channels play a key role in elevating intracellular calcium ion concentration around the resting membrane potential. Ca\(^{2+}\) influx via T-type Ca\(^{2+}\) channels regulates the pacemaker activities of sino-atrial myocytes and neuronal cells, the low-threshold calcium spikes crowned by bursting of Na\(^{+}\)-dependent action potentials in thalamic neurons, smooth muscle contraction, aldosterone and cortisol secretion in the adrenal cortex, the potentials in thalamic neurons, smooth muscle contraction, and Houser, 1993; Martinez et al., 1999), epilepsy (Tsakiridou et al., 1995), and neurogenic pain (Kim et al., 2003).

To date, molecular biological studies have identified 10 genes encoding the voltage-activated calcium channel \(\alpha_1\) subunits that determine the primary biophysical and pharmacological properties of the channels. Expression studies have revealed that the Ca\(_{3.1}\) (\(\alpha_{1C}\)), Ca\(_{3.2}\) (\(\alpha_{1H}\)), and Ca\(_{3.3}\) (\(\alpha_{1I}\)) genes encode low-voltage-activated T-type Ca\(^{2+}\) channel \(\alpha_1\) subunits, whereas the other genes encode high-voltage-activated Ca\(^{2+}\) channel \(\alpha_1\) subunits. The three T-type Ca\(^{2+}\) channels have been produced in expression systems and have been shown to possess the following biophysical and pharmacological properties:

1) activation thresholds around a resting membrane potential of −60 to −70 mV, 2) inactivation at low voltages, 3) slow deactivation, 4) tiny single channel conductance, and 5) high sensitivity to kurtoxin and mibefradil. The Ca\(_{3.1}\) and Ca\(_{3.2}\) channels generate typical T-type channel currents with transient kinetics because of fast activation and subsequent inactivation. In contrast, Ca\(_{3.3}\) channels produce atypical T-type channel currents with much slower kinetics.

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ABBREVIATIONS: PKA, cAMP-dependent protein kinase A; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; 5-HT, 5-hydroxytryptamine; 8-Br-cAMP, 8-bromo-cAMP; RpBrCAMPs, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; PKI, protein kinase A inhibitor peptide; PCR, polymerase chain reaction; I-V, current-voltage; AKAP, protein kinase A-anchoring protein.
T-type channels are primarily regulated by dynamic changes in membrane potential. Numerous studies have revealed that they are also affected by hormones and/or neurotransmitters via Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (Welsby et al., 2003) and protein kinase C (Park et al., 2003). Interestingly, G protein βγ subunits have been shown to make a negative regulation effect on Ca\textsubscript{3,2} T-type channel activity by interacting with the cytoplasmic loop connecting domain II and III (Wolfe et al., 2003). It has recently shown that treatment of cAMP or β-adrenergic stimulation could increase T-type channel activity in rat chromaffin cells through Epac-dependent recruitment of T-type channels (Novara et al., 2004; Giancippoli et al., 2006).

There is controversy about whether they are regulated by cAMP-dependent protein kinase A (PKA). The majority of investigators have reported that T-type channels are little affected by PKA (Bean, 1985; Benham and Tsien, 1988; Hagiwara et al., 1988; Tytgat et al., 1988; Hirano et al., 1989; Tseng and Boyden, 1989; Fisher and Johnston, 1990). In contrast, T-type Ca\textsuperscript{2+} currents in frog atrial myocytes were reported to be increased by isoproterenol in two ways: a cAMP-dependent way and a cAMP-independent way (Alvarez and Vassort, 1992). In addition, Lenglet et al. (2002) also reported that T-type channel activity recorded in rat glomerulosa cells was augmented by PKA after stimulation of 5HT\textsubscript{7} receptors.

In the present investigation, we sought to resolve the question whether T-type channel activity is regulated by PKA, using T-type Ca\textsubscript{3,2} Ca\textsuperscript{2+} channels reconstituted in the Xenopus oocyte system. We found that Ca\textsubscript{3,2} channel activity was significantly increased by forskolin-activated PKA. This PKA effect could be mimicked by serotonin when 5HT\textsubscript{2} receptors were coexpressed with Ca\textsubscript{3,2} in Xenopus oocytes. In addition, we localized the region(s) of the Ca\textsubscript{3,2} channel responsible for PKA stimulation to the cytoplasmic loop connecting domains II and III.

**Materials and Methods**

**Materials**

Forskolin, H89, 5-hydroxytryptamine (5-HT; serotonin), 8-bromo (Br)-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate (RpBcAMP\textsubscript{s}), and protein kinase A inhibitor peptide (PKI) were purchased from Sigma-Aldrich (St. Louis, MO). Forskolin and H89 were diluted in dimethyl sulfoxide to generate 10 mM stock solutions. The concentration of dimethyl sulfoxide in the bath solution is expected to be less than 0.1%, which had no effect on T-type currents. PKI, 8-Br-cAMP, and RpBcAMP\textsubscript{s} were prepared at stock concentrations of 1, 100, and 1000 mM in double distilled water, respectively.

**Construction of Chimeric Channels and the Deletion Mutant**

The chimeric channels Ca\textsubscript{3,2}/Na\textsubscript{1,4}N-term, Ca\textsubscript{3,2}/Na\textsubscript{1,4}I-II, Ca\textsubscript{3,2}/Na\textsubscript{1,4}I-III, and Ca\textsubscript{3,2}/Na\textsubscript{1,4}III-IV were created by replacing individual cytoplasmic loops encoded by human Ca\textsubscript{3,2} cDNA (α\textsubscript{1G}; GenBank accession number AF051946) with the corresponding loops of rat Na\textsubscript{1,4} cDNA (μ-1; GenBank accession number NM_013178) by overlap extension PCR (Horton et al., 1989). A rat 5HT\textsubscript{7} receptor cDNA was obtained by reverse transcription-PCR from adrenal gland total RNA from Sprague-Dawley rats. All PCRs were performed using f/4 DNA polymerase (Genaxxon BioScience, Biberach, Germany). PCR products were inserted into TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced. Error-free PCR products were subcloned into the original Ca\textsubscript{3,2} pGEM-HEA using restriction enzyme sites (Chuang et al., 1998). Detailed information about the construction of the individual chimeric channels is given below.

**Ca\textsubscript{3,2}/Na\textsubscript{1,4}N-term**

The N terminus (‘5-polylinker-849) of Na\textsubscript{1,4} was amplified from rat Na\textsubscript{1,4} cDNA using forward primer 5′-TTAATCAGCTCTATATGGG-3′ (T7 primer) and reverse primer 5′-CAGGTGCTGCAAACATGGGAACGCGCGTGAATGAG-3′. By overlap extension PCR, the amplified N-terminal cDNA was connected to the domain I portion (377–1227) of Ca\textsubscript{3,2} amplified with forward primer 5′-CTCTATCCGGCGTGTTCCATGTCGTCGAGCAGCTG-3′ and reverse primer 5′-GATGTCGACCCAGCT-TCCAG-3′, respectively. The extended cDNA was digested with Clai (‘5-polylinker) and BamHI (729, Ca\textsubscript{3,2}) and ligated into Ca\textsubscript{3,2} pGEM-HEA opened with Clai (‘5-polylinker) and BamHI (729, Ca\textsubscript{3,2}).

**Ca\textsubscript{3,2}/Na\textsubscript{1,4}I-II**

The cytoplasmic I-II loop (1360–2434) of Ca\textsubscript{3,2} was replaced with the corresponding loop (1782–2162) of Na\textsubscript{1,4}. The I-II loop (1782–2162) of Na\textsubscript{1,4} was amplified with forward primer 5′-TTCCTGGAGCACAGGCTGAGGAAATGCCC-3′ and reverse primer 5′-CTCGACGATTCGCGGCGAAGTGTC-3′. The upstream portion (nucleotides 305-1360) preceding the Ca\textsubscript{3,2} I-II loop was amplified with forward primer 5′-GCCGCGCACTGCTTCTCTTG-3′ and reverse primer 5′-AGCTTCTATCTTGCTGACCTGTTCTCTTG-3′. The downstream portion (2435–3116) of the Ca\textsubscript{3,2} I-II loop was amplified using forward primer 5′-GCCGCGCACTGCTTCTCTTG-3′ and reverse primer 5′-GCCGCGCACTGCTTCTCTTG-3′. The three PCR products were joined together by overlap extension PCR, and the extended cDNA was digested with NotI and BspEI and ligated into Ca\textsubscript{3,2} pGEM-HEA, which was opened with NotI (341) and BspEI (2637).

**Ca\textsubscript{3,2}/Na\textsubscript{1,4}I-III**

The II-III loop (3134–3958) of Ca\textsubscript{3,2} was replaced with the corresponding loop (2847–3518) of Na\textsubscript{1,4}. The II-III loop (2847–3518) of Na\textsubscript{1,4} was amplified from Na\textsubscript{1,4} cDNA using forward primer 5′-ATCTCTGTTGGAGGCTTCTAGCTGTCGACGGCG-3′ and reverse primer 5′-AGCTTCTATCTTGCTGACCTGTTCTCTTG-3′. The upstream portion (2435–3133) preceding the Ca\textsubscript{3,2} II-III loop was amplified using forward primer 5′-CTCCGGAGACCTGTCGTTGGACGCGA-3′ and reverse primer 5′-GCCAGCAGCTGACCTGACCTGACGCGA-3′. The downstream portion (3957–4401) of the Ca\textsubscript{3,2} II-III loop was amplified using forward primer 5′-TTCAGAGTGTGGCAGCAGAGAGAGGTGTCG-3′ and reverse primer 5′-AGAAGAGCAGAGAGGAATGGGCAGGAC-3′. The II-III loop cDNA and its upstream and downstream cDNAs were joined by further PCR. The extended cDNA was digested with BspEI and EcoRV and ligated into Ca\textsubscript{3,2} pGEM-HEA that was digested with BspEI (2637, Ca\textsubscript{3,2}) and EcoRV (4350, Ca\textsubscript{3,2}).

**Ca\textsubscript{3,2}/Na\textsubscript{1,4}III-IV**

The III-IV loop (4759–4915) of Ca\textsubscript{3,2} was replaced with the corresponding loop (4329–4485) of Na\textsubscript{1,4}. The III-IV loop (4329–4485) of Na\textsubscript{1,4} was amplified from rat Na\textsubscript{1,4} cDNA using forward primer 5′-GTCGAGACTCCAAAGAGAACTCAGTGGTGTGACTG-3′ and reverse primer 5′-GTCGAGACTCCAAAGAGAACTCAGTGGTGTGACTG-3′. The preceding portion (3957–4759) of the Ca\textsubscript{3,2} III-IV loop was amplified using forward primer 5′-AACAAGTTTGGTGGGAGGAGAGCTGACGACCTGACGACCTGACGAC-3′ and reverse primer 5′-CCGCGCGAGAGCTGACGACCTGACGACCTGACGAC-3′. The three amplified fragments were extended by additional PCR. The extended cDNA was digested with EcoRV and AvrII, and ligated into Ca\textsubscript{3,2} pGEM-HEA, which was opened with EcoRV (4350, Ca\textsubscript{3,2}) and AvrII (6170, Ca\textsubscript{3,2}).

**Ca\textsubscript{3,2}AcR**

Ca\textsubscript{3,2}AcR was constructed by deleting the carboxy-terminal portion (5774–6906) of Ca\textsubscript{3,2}. The portion (3957–5773) preceding the Ca\textsubscript{3,2} carboxy terminus was amplified by PCR using forward primer 5′-CCTCCATTGGTGGACAGGACGACGACGAC-3′ and reverse primer 5′-GGGATCTGCCGCTCCCGAGCGC-3′. The PCR product
Cloning of Rat 5HT<sub>7</sub> Receptor cDNA

Rat adrenal gland RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method. The first strand cDNA was synthesized from 0.5 μg of rat adrenal gland RNA with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) by incubation at 22°C for 10 min and then at 42°C for 50 min. The reaction was terminated by heating at 95°C for 5 min. PCR was performed using a pair of PCR primers designed based on the rat 5HT<sub>7</sub> receptor sequence (GenBank accession number NM_022938). The primer sequences were as follows: forward primer 5′-GCGGCTGGCCAGGATGATGGA-3′ and reverse primer 5′-AGCCAA TGATTCTTGGTTGTTG-3′. The PCR reaction consisted of initial denaturation at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min. The resulting PCR products were separated on a 1% agarose gel and purified using a gel extraction kit. The purified products were ligated into TOPO TA cloning vector and transformed into competent cells. One of three PCR products sequences was identical to the open reading frame of the rat 5HT<sub>7</sub> receptor cDNA. The cloned 5HT<sub>7</sub> receptor cDNA was subcloned into pGEM-HEA.

Expression of the Ca<sub>3.2</sub> Channel, the Rat Na<sub>1.4</sub> Channel, Their Chimeric Channels, and the 5HT<sub>7</sub> Receptor in Xenopus Oocytes

Several ovary lobes were surgically isolated from mature female Xenopus laevis (Xenopus Express, Haute-Loire, France) anesthetized with 0.1% of 3-aminobenzoic acid ethyl ester (Sigma-Aldrich). The isolated lobes were manually torn into small clusters of five to six oocytes in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 2.5 mM pyruvic acid, and 50 μg/ml gentamicin, pH 7.6). Collagenase (type IA, 2 mg/ml; Sigma-Aldrich) dissolved in Ca<sup>2+</sup>-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.6) was treated for approximately 30 min to remove the follicle membranes of the isolated oocytes. Stage V to VI oocytes were manually selected under a stereomicroscope and recovered in SOS solution at 18°C for several hours or overnight. Each oocyte was injected with 3 to 10 ng of cRNA in a volume of 40 nl using a Nanoinjector (Drummond Scientific, Broomfield, CO) attached to a micromanipulator (Narishige, Tokyo, Japan). All cDNAs of the T-type channel, the sodium channel, their mutant channels, and 5HT<sub>7</sub> receptor were linearized with AflII or XbaI and transcribed by T7 polymerase using mMESSAGE mMACHINE T7 kits purchased from Ambion (Austin, TX). For the wild-type and chimeric channels (Ca<sub>3.2</sub>/Na<sub>1.4</sub> N-term, Ca<sub>3.2</sub>/Na<sub>1.4</sub> I-II, Ca<sub>3.2</sub>/Na<sub>1.4</sub> II-III, and Ca<sub>3.2</sub>/Na<sub>1.4</sub> III-IV), 3 to 10 ng of cRNA was injected into oocytes to compare their relative expression levels. There was no significant difference between current amplitudes of the wild-type and chimeric channels. In contrast, the expression level of Ca<sub>3.2</sub> was significantly smaller than that of the Ca<sub>3.2</sub> (P < 0.01, Student’s t test). The synthesized cRNA was resuspended in diethyl pyrocarbonate-treated H<sub>2</sub>O and stored at -70°C. The 5HT<sub>7</sub> receptor and Ca<sub>3.2</sub> channel cRNAs were injected in a molar ratio of 1:1.

Electrophysiological Recordings and Data Analysis

Whole-cell currents were measured with a two-microelectrode voltage-clamp amplifier (OC-725C; Warner Instruments, Hamden, CT) between the days 3 and 8 after cRNA injection. Microelectrodes were pulled from capillaries (Warner Instruments), and their electrode resistance was 0.2 to 1.0 MΩ. After the oocytes had been pricked with microelectrodes filled with 3 M KCl in SOS solution, the bath solution was exchanged with 10 mM Ba<sup>2+</sup> solution (10 mM Ba(OH)<sub>2</sub>, 90 mM NaOH, 1 mM KOH, and 5 mM HEPES, pH 7.4 with methanesulfonic acid). However, Na<sup>+</sup> currents were recorded using SOS containing 100 mM NaCl. Barium currents were acquired at 5 kHz and low pass filtered at 1 kHz, and Na<sup>+</sup> currents were acquired at 20 kHz and low pass filtered at 5 kHz using the pClamp system (Digidata 1320A and pClamp 8; Axon Instruments, Foster City, CA). Data were analyzed with Clampfit software (Axon Instruments) and presented graphically using Prism software (GraphPad Software Inc., San Diego, CA). They are presented as means ± S.E.M. and tested for significance using Student’s unpaired t test.

Results

Expression and Characterization of Human Ca<sub>3.2</sub> T-Type Ca<sup>2+</sup> Channels. Using the two-electrode voltage-clamp method, expression of Ca<sub>3.2</sub> (α<sub>1H</sub>) channels in Ca<sub>3.2</sub> cRNA-injected oocytes was detected as robust transient Ba<sup>2+</sup> currents (>500 nA) elicited by a test potential of ~20 mV from a holding potential of ~90 mV (Fig. 1A). In contrast, measurable transient Ba<sup>2+</sup> currents were not detected in H<sub>2</sub>O-injected or uninjected oocytes (data not shown). The Ca<sub>3.2</sub> currents elicited by a voltage protocol consisting of serial test potentials that were increased by 10 mV from a holding potential of ~90 mV displayed the typical biophysical properties of T-type channel currents, such as a low-voltage threshold of approximately ~60 mV for activation, fast acti-

Fig. 1. Effect of 10 μM forskolin on human Ca<sub>3.2</sub> T-type channel. A, representative current traces before (a) and 30 min after forskolin application (b) are overlapped. B, time course of forskolin-mediated stimulation of Ca<sub>3.2</sub> channel activity. A representative time course is plotted. The Ca<sub>3.2</sub> current was evoked by a test potential of ~20 mV from a holding potential of ~90 mV every 20 s, using 10 mM Ba<sup>2+</sup> as charge carrier. Current amplitude was normalized to the control current amplitude before forskolin application. Ca<sub>3.2</sub> activity was increased by 40 ± 4% in response to 10 μM forskolin within 30 min (n = 20). C, current-voltage relationship. Currents were evoked by depolarizing voltage steps in 10-mV increments from ~70 to ~40 mV from a holding potential of ~90 mV. Peak currents obtained during test potentials were normalized to the maximum observed. D, peak currents were normalized to the maximum observed before the application of forskolin (control, ○, forskolin application, □).
vation and inactivation, criss-crossing pattern between current traces, peak current at -20 mV, and reversal potential around +40 mV. These properties are identical to those described in previous reports (Lee et al., 1999; Park et al., 2003).

**Augmentation of Ca\(_{3.2}\) T-Type Ca\(^{2+}\) Channel Activity by Forskolin.** When currents were evoked by a test potential to -20 mV from a holding potential of -90 mV every 20 s, no significant run-up or run-down was observed over ≥30 min (data not shown). However, application of 10 μM forskolin, an adenylyl cyclase stimulant, increased the amplitude of the Ca\(_{3.2}\) currents with a delay of approximately 2 to 3 min. The Ca\(_{3.2}\) current amplitude increased continuously over 30 min (Fig. 1B). Forskolin-induced stimulation of current amplitude reached a maximum level (saturation) within ~70 min, where they were stable or ran down slowly (n = 3; data not shown). Representative traces before and 30 min after addition of 10 μM forskolin effect were overlapped for comparison (Fig. 1A). On average, 10 μM forskolin increased the amplitude of Ca\(_{3.2}\) currents by 40 ± 4% within 30 min (n = 20). Comparison of current-voltage (I-V) relationships before and 30 min after forskolin addition showed that -fold stimulations at different potentials were similar and that the I-V curve was not shifted (Fig. 1, C and D).

We also examined the effects of forskolin on other biophysical properties of the Ca\(_{3.2}\) channels, including steady-state inactivation, recovery from inactivation, and current kinetics. As expected from the I-V relationship, the activation curves obtained from fitting cord conductance were very similar before and after forskolin treatment (\(V_{50} = -34.3 ± 0.5\) versus -34.4 ± 0.5 mV; n = 9). In contrast, the \(V_{50}\) values of the steady-state inactivation curves before and after forskolin application were -61.4 ± 0.2 versus -65.5 ± 0.1 mV, indicating that steady-state inactivation was shifted toward the negative direction (Fig. 2A) (n = 5; \(P < 0.05\), Student’s t test). Apart from the steady-state inactivation, other biophysical properties, such as recovery from inactivation and the activation and inactivation kinetics of current traces, were slightly affected (Fig. 2, B–D).

**Forskolin Stimulates Ca\(_{3.2}\) Activity via PKA.** We tested whether the forskolin effect occurs via conversion of ATP to cAMP because of stimulation of adenylyl cyclase and subsequent activation of PKA. Indeed, application of 200 μM 8-Br-cAMP, a membrane-permeable cAMP, enhanced the peak amplitude of Ca\(_{3.2}\) currents by 39 ± 8% (n = 4) over 30 min. The stimulation profile of 8-Br-cAMP, including delay, extent of stimulation, and time course, was very similar to that of forskolin (Fig. 3). We next tested whether the forskolin effect was mediated by activation of PKA. When oocytes were preincubated in SOS containing 20 μM H89, a PKA-specific inhibitor, superfusion of 10 μM forskolin enhanced
the amplitude of Cav3.2 currents by only 5% (Fig. 4A). Likewise, the forskolin enhancement effect was almost abolished by preincubation with 200 μM RpBrcAMPs, a competitive antagonist of cAMP binding to PKA (Fig. 4B). Furthermore, the increase in the Cav3.2 current amplitude in response to forskolin could be almost completely reversed by subsequent application of 500 nM PKI, a membrane-permeable PKA inhibitor peptide (Fig. 4C). Together, these findings indicate that the stimulation by forskolin arises from activation of the PKA signaling pathway.

Reconstitution of the PKA Cascade in Xenopus Oocytes. We coexpressed Cav3.2 channels and 5HT7 receptors in oocytes to mimic the stimulation of PKA through a physiological second messenger system. Application of 100 nM 5-HT increased Cav3.2 channel activity over 30 min without any tendency to saturate (Fig. 5A). Unlike the forskolin effect, the augmentation of the Cav3.2 current by 5-HT was initiated with a rapidly increasing response, without any detectable delay. The rapid uprising response was then followed by a slow increase over more than 30 min. On average, the percentage stimulation over 30 min was 60 ± 7% (n = 8).
tectable lag, but the initial increase was not sustained; on average, the percentage augmentation of channel activity was only $16 \pm 3\%$ over 30 min (Fig. 5A), showing that 5-HT stimulation effect was strongly inhibited by H89 pretreatment. Consistently, application of 100 nM 5-HT increased Ca$_{3.2}$ peak amplitude by $57 \pm 6\%$ ($n = 8$) within 30 min, and most of the 5-HT augmentation effect could be reversed by subsequent application of 500 nM PKI. A representative time course of Ca$_{3.2}$ peak amplitude in response to 100 nM 5-HT, washing, and 500 nM PKI is shown in Fig. 5B. These findings demonstrate that the 5-HT stimulation effect on Ca$_{3.2}$ current amplitude is mainly due to the PKA pathway.

Localization of the Structural Region(s) Contributing to PKA-Mediated Stimulation. Previous studies have shown that rat Na$_{1.4}$ is not regulated by PKA (Smith and Goldin, 1996, 2000). Accordingly, we first confirmed that rat Na$_{1.4}$ channels expressed in oocytes were not affected by activation of PKA (Fig. 6). We then constructed chimeras of the Ca$_{3.2}$ and Na$_{1.4}$ channels to localize the structural regions required for PKA stimulation. We constructed Ca$_{3.2}$/Na$_{1.4}$N-term, Ca$_{3.2}$/Na$_{1.4}$I-II, Ca$_{3.2}$/Na$_{1.4}$I-III, and Ca$_{3.2}$/Na$_{1.4}$II-III by replacing individual cytoplasmic loops of Ca$_{3.2}$ with the corresponding loops of rat Na$_{1.4}$, and Ca$_{3.2}$C was generated by truncating its carboxyl tail (Fig. 6). The expression levels of the chimeric channels were not significantly different from that of the wild-type Ca$_{3.2}$. The activities of Ca$_{3.2}$/Na$_{1.4}$N-term, Ca$_{3.2}$/Na$_{1.4}$I-II, Ca$_{3.2}$/Na$_{1.4}$I-III, and Ca$_{3.2}$C were stimulated by forskolin by $36 \pm 5$, $34 \pm 2$, $35 \pm 6$, and $46 \pm 8\%$, respectively, within 30 min ($n = 4–5$). The stimulation profiles of the loop chimeras and the C-terminal truncation mutant were similar to that of wild-type Ca$_{3.2}$. In contrast, Ca$_{3.2}$/Na$_{1.4}$II-III activity was little changed by application of forskolin ($n = 6$; Fig. 6). On average, it was stimulated by only $2 \pm 4\%$ over 30 min, much less than that observed for the wild type ($P < 0.001$, Student’s $t$ test). Together, these results strongly suggest that the II-III loop contains structural element(s) critical for the PKA stimulation.

Discussion

Electrophysiological recordings have shown that low threshold T-type currents are mainly present in adrenal glomerulosa cells (Matsunaga et al., 1987; Cohen et al., 1988; Rossier et al., 1993). In situ hybridization analysis has shown that of the three T-type channel isoforms, Ca$_{3.2}$ is the major channel in these cells (Schrier et al., 2001). Lenglet et al. (2002) recently reported that T-type currents in rat glomerulosa...
loosa were stimulated by activation of 5-HT7 receptors via the
PKA signaling pathway. These findings prompted us to test
whether the channel activity of recombinant Ca3.2 reconsti-
tuted in the *Xenopus* oocyte system was regulated by PKA,
and we found that channel activity was indeed enhanced by
forskolin via activation of endogenous PKA.

Most previous workers have reported that T-type Ca2+
currents are little affected by PKA. For example, application
of isoproterenol, a β-adrenergic agonist, had no effect on
T-type currents recorded from either rabbit sinoatrial node
(Hagiwara et al., 1988), canine atrial myocytes (Bean, 1985),
guinea pig ventricular myocytes (Tytgat et al., 1988), rabbit
ear artery (Benham and Tsien, 1988), canine Purkinje neu-
ron (Hirano et al., 1989; Tseng and Boyden, 1989), or guinea
pig hippocampal CA3 neurons (Fisher and Johnston, 1990).
In contrast, T-type Ca2+ currents recorded from frog atrial
myocytes and rat glomerulosa cells were shown to be in-
creased by the cAMP-PKA pathway (Alvarez and Vassort,
1992; Lenglet et al., 2002). The glomerulosa T-type Ca2+
current was increased by 5-HT via activation of the PKA
signaling pathway. In contrast, Alvarez and Vassort (1992)
reported that T-type Ca2+ current in frog heart was in-
creased by cAMP treatment and the initial cAMP-mediated
increment could be further enhanced by subsequent applica-
tion of isoproterenol. The cAMP-mediated response was
shown to be relatively slow, whereas the subsequent iso-
proterenol response was fast. They also displayed that the
T-type Ca2+ current in response to isoproterenol was bi-
phasically enhanced with fast and slow time courses. The
cAMP-PKA pathway seemed to be involved in the slow
response, whereas the mechanism (possibly G protein-
mediated mechanism) responsible for the fast response
remained to be uncovered. The fast and slow increment
pattern of Ca3.2 channel activity by 5-HT shown in this
study was somewhat similar to the isoproterenol regulat-
ion pattern of frog T-type Ca2+ current. It is also similar
to the case of the isoproterenol regulation of frog T-type
channel current that the up-regulation of Ca3.2 current
activity in response to forskolin or 5-HT was obtained from
oocytes of 11 of 15 frogs. On the contrary, Ca3.2 T-type
channel currents of oocytes isolated from the other four
frogs did not show any enhancement to those drugs. To-
gether, these different regulation results suggest that the
PKA regulation effects on T-type channel currents can be
variable between tissues expressing T-type channels.

Native L-type Ca2+ currents recorded in cardiac myocytes
were strongly up-regulated by PKA, whereas recombinant
cardiac L-type Ca2+ channel currents recorded in HEK293
cells were essentially unaffected (Zong et al., 1995; Mikala et
al., 1998). This discrepancy was resolved by the finding that
stimulation of cardiac L-type channel currents depended on
the presence or absence of a PKA-anchoring protein (AKAP),
which played a crucial role in localizing PKA near to the
plasma membrane (Gao et al., 1997; Fraser et al., 1998).
However, AKAP is not likely to be a critical factor affecting
the regulation of T-type channel activity, because T-type
currents are insensitive to PKA in cardiac myocytes, in which
AKAP is expressed (Gray et al., 1998). The different effects
on T-type channels may depend on the presence or absence
of unidentified proteins that prevent PKA from interacting
with the channels. A related possibility is that T-type cur-
rents are up-regulated by activation of PKA via phosphoryl-
ation of an accessory subunit rather than the Ca3.2 α1
subunit itself, as shown for K,1.5 whose regulation by PKA
is reported to be via phosphorylation of K,β1.3 (Kwak et al.,
1999). Therefore, further efforts should be made to identify
regulatory proteins or auxiliary subunits of the T-type chan-
nel α1 subunit.

We localized a structural region contributing to the aug-
mentation of Ca3.2 activity to the II-III loop of the Ca3.2.
To identify a specific locus for phosphorylation by PKA in the
II-III loop, we tested the role of Thr1055, Ser1133, and
Ser1134 by making point mutation converting them individ-
ually into Ala because these sites are found in the motifs
[Arg-Arg-X-Ser/Thr] or [Arg-X-(X)-Ser/Thr] known to be phos-
phorylated by PKA and are conserved in the three T-type
channel isoforms. All of the mutant channels were regulated
by forskolin, and their regulatory profiles were similar to
that of wild-type Ca3.2, suggesting that these sites do not
contribute to the PKA-mediated stimulation. In addition to
the three sites examined, there are eight more putative sites
fitting the consensus motifs for PKA phosphorylation. Their
involvement remains to be investigated by making individual
point mutations.

In summary, we have shown that Ca3.2 channel activity
may be up-regulated by PKA, and we were able to reconstitute
the PKA signaling pathway augmenting T-type channel ac-
tivity in *Xenopus* oocytes. In addition, we localized the struc-
tural region involved in the PKA stimulation to the II-III
loop. The PKA stimulation of cloned T-type Ca2+ channels
in the *Xenopus* oocyte system demonstrated here may con-
tribute to understanding T-type channel regulation by neu-
rotransmitters and hormones.

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