

Capsaicin Binds to the Intracellular Domain of the Capsaicin-Activated Ion Channel

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Capsaicin (CAP) excites small sensory neurons, causing pain, neurogenic inflammation, and other visceral reflexes. These effects have been proposed to be the result of CAP activation of a nonselective cation current. It is generally assumed that CAP binds to an extracellular domain of the membrane receptor. However, the exact binding site is not known because of the lipophilic nature of CAP. To determine whether the binding domain is extracellular or intracellular, we tested the effect of a synthetic water-soluble CAP analog, DA-5018·HCl, on current activation. CAP activated the 45 pS (at –60 mV) nonselective cation channel from either side of the membrane. However, DA-5018·HCl, which had a greater potency and efficacy than CAP, activated the channels only from the cytosolic side of the patch membrane in a capsazepine, a CAP receptor antagonist, reversible manner. When ap-

plied extracellularly, DA-5018·HCl did not, but CAP did, activate whole-cell currents in sensory neurons, as well as in oocytes expressing vanilloid receptor 1, a recently cloned CAP receptor. Hydrogen ions, reported as a possible endogenous activator of cation current, failed to elicit any current when acidic medium (pH 5.0–6.0) was applied intracellularly, indicating that H⁺ does not mediate the CAP effect. These results indicate that CAP and its analog bind to the cytosolic domain of the CAP receptor and suggest that an endogenous CAP-like substance other than H⁺ may be present in the cell.

Key words: capsaicin receptor; VR1; binding domain; DA-5018; capsazepine; acid; pain

Capsaicin (CAP), a pungent chemical present in hot peppers, produces an immediate pain or hyperalgesia when applied cutaneously or intradermally (Simone et al., 1987, 1989; Geppetti et al., 1988; Park et al., 1995). CAP also causes neurogenic inflammation mediated by release of neuropeptides, such as substance-P or calcitonin gene-related peptide, from sensory nerve endings (for review, see Szolcsanyi, 1996). In addition, CAP paradoxically induces desensitization of sensory neurons to various types of noxious stimuli, thus producing a long-lasting and naloxone-resistant analgesia (for review, see Holzer, 1991). Because of this analgesic action, CAP is often used for alleviating pain caused by diabetic or herpetic neuropathy or arthritis, and CAP analogs are being explored as potential analgesics (Watson et al., 1988; Bernstein et al., 1989; Donofrio et al., 1991; Szallasi and Blumberg, 1996).

The excitation of sensory neurons induced by CAP is believed to result from large influxes of cations, such as Na⁺ or Ca²⁺ (Bevan and Szolcsanyi, 1990). CAP produces an influx of Ca²⁺ and other cations in a dose-dependent manner in cultured sensory neurons (Wood et al., 1988; Bevan et al., 1992). Studies on cultured sensory neurons further show the presence of specific ion currents acti-

vated by CAP application (Marsh et al., 1987; Liu and Simon, 1994). Previously, we identified and characterized a ligand-gated cation channel specifically activated by CAP and antagonized by capsazepine (CZP), a functionally defined CAP receptor antagonist in cultured dorsal root ganglion (DRG) neurons (Bevan et al., 1992; Oh et al., 1996). Recently, a gene encoding for CAP receptor, vanilloid receptor 1 (VR1), was cloned from rat DRG neurons (Caterina et al., 1997). The VR1, having six putative transmembrane domains and two cytosolic domains in both ends of the protein, exhibits inward currents sensitive to CAP, as well as noxious heat when expressed in oocytes. The channel property of the expressed CAP receptor resembles that observed in native channels in DRG neurons (Oh et al., 1996; Caterina et al., 1997). Thus, activation of this channel may primarily account for the ionic responses of sensory neurons to CAP.

In previous studies, current responses to CAP were tested by applying CAP only to the extracellular space, with the assumption that CAP binds to an extracellular site (Liu and Simon, 1994; Oh et al., 1996; Caterina et al., 1997; Koplak et al., 1997). Because CAP or its analogs, such as CZP or resiniferatoxin, are lipid-soluble, it is possible that they pass through the cell membrane and act on binding sites present in the intracellular surface of the receptor. Thus, the location of CAP binding has yet to be determined. In this study, to understand further the signaling pathways involved in CAP-induced cellular effects, we used a salt form of a synthetic analog of CAP, DA-5018·HCl (DA) (see Fig. 1) as an experimental tool to locate the binding domain of CAP. The results of our study indicate that the binding sites for CAP are present at the intracellular side of the cell membrane.

Received July 14, 1998; revised Oct. 15, 1998; accepted Oct. 23, 1998.

This work was supported by Creative Research Initiatives of the Korea Ministry of Science and Technology.

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MATERIALS AND METHODS

Cell preparation. Cultured DRG neurons were prepared as described previously (Oh et al., 1996). Briefly, DRGs were dissected from all levels of lower cervical, thoracic, and lumbar spinal cord of 1- or 2-d-old neonatal rats. DRGs were collected in cold culture medium (4°C) containing DMEM-F-12 mixture (Life Technologies, Grand Island, NY), fetal bovine serum (10%; Life Technologies), 1 mM sodium pyruvate, 25 ng/ml nerve growth factor (Sigma, St. Louis, MO), and 100 U/ml of penicillin–streptomycin (Sigma). Ganglia were washed three times with DMEM-F-12 medium and incubated for 30 min in the DMEM-F-12 medium containing 1 mg/ml collagenase (Type II; Worthington, Freehold, NJ). The ganglia were then washed three times with Mg^{2+} - and Ca^{2+} -free HBSS and incubated with gentle shaking in the warm (37°C) HBSS containing 2.5 mg/ml trypsin (Life Technologies). The solution was centrifuged at 1,000 rpm for 10 min, and the pellet was washed two or three times with the culture medium to inhibit the enzyme. The pellet was suspended in the culture medium and gently triturated with a Pasteur pipette. The suspension was plated on round glass coverslips (Fisher, Pittsburgh, PA) placed in small Petri dishes. The glass coverslips were treated overnight with poly-L-lysine (Sigma) and dried before use. Cells were incubated at 37°C in a 95% air–5% CO_2 gas mixture. Cells were used 2–4 d after plating.

Current recording. Borosilicate glass pipettes (Narishige, Tokyo, Japan) were pulled and coated with Sylgard (Dow Corning, Midland, MI). Tip resistances were ~2 and 5 M Ω for whole-cell and single-channel current recordings, respectively. After gigaseals were formed with the glass pipettes, cell-attached and inside-out patch configurations were used to study single-channel currents as described by Hamill et al. (1981). A salt bridge (1% agar in 300 mM KCl) immersed in bath and an Ag/AgCl reference electrode in pipette solution was used to minimize changes in junctional potentials. Junctional potentials were canceled before gigaseals were formed. For whole-cell recording, the cell membrane under a glass pipette was ruptured by a gentle suction. After forming a whole cell, capacitive transient was canceled. Single-channel currents were recorded using a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) and filtered at 5 kHz with an eight-pole low-pass Bessel filter. Data were digitized at 37 kHz with a digital data recorder (VR-10B; Instrutech, Great Neck, NY) and stored on videotapes for later analysis. For chart recording, output of amplifier was filtered at 500 Hz (Frequency Device, Havenhill, MA) and fed into a thermal array chart recorder (TA-240; Gould Instrument System, Valley View, OH). The digitized data stored on videotapes were imported to a personal computer (IBM pentium-compatible) for computer analysis of single-channel currents.

Channel open probability (P_o), amplitude, and mean open time of single-channel currents were obtained using the pCLAMP software (version 6.02; Axon Instruments). P_o of single channels was obtained from the ratio of the areas under the curves representing open events divided by the sum of the areas under the curves representing open and closed events. The half-amplitude algorithm in the FETCHAN program (Axon Instruments) was used for the threshold amplitude for detecting open events. Channel activity (NP_o) was calculated as a product of the number of channel (N) in the patch and P_o . NP_o or P_o was collected only from patches that contained less than five functional CAP-activated channels.

VR1 expression in oocytes and two-electrode recording. Rat brain mRNAs were isolated using the FastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego, CA). VR1 cDNA was cloned into a pSDF vector by reverse transcriptase-PCR from rat brain mRNA using the SuperScript kit (Life Technologies). PCR primers, designed based on the sequence of VR1 (Caterina et al., 1997), are as follows: VR1-HindIII (sense), 5'-CCC AAG CTT GCC ACC ATG GAA CAA CCG GCT AGC-3'; and VR1-KpnI (antisense), 5'-CCG GTA CCT TAT TTC TCC CCT GGG AC-3'. cRNA transcripts were synthesized from *Xba*I-linearized VR1 cDNA templates using SP6 RNA polymerase of the MEGAscript kit from Ambion (Austin, TX) as suggested by the manufacturer. Defolliculated *Xenopus laevis* oocytes were injected with 1 ng of VR1 cRNA in 50 nl of water. Three to 5 d after injection, two-electrode voltage-clamp recording was performed ($E_{hold} = -60$ mV) using an oocyte-clamp amplifier (OC-725C; Warner Instrument, Hamden, CT). The recording chamber was perfused at a rate of 2 ml/min at room temperature with a solution containing (in mM): 96 NaCl, 5 HEPES, 2 KCl, 1.8 $CaCl_2$, and 1 $MgCl_2$, pH 7.5 (ND96).

Solutions. Solutions in bath and pipette for single-channel recordings contained (in mM): 140 Na^+ , 2 Mg^{2+} , 144 Cl^- , 5 EGTA, and 10 HEPES,

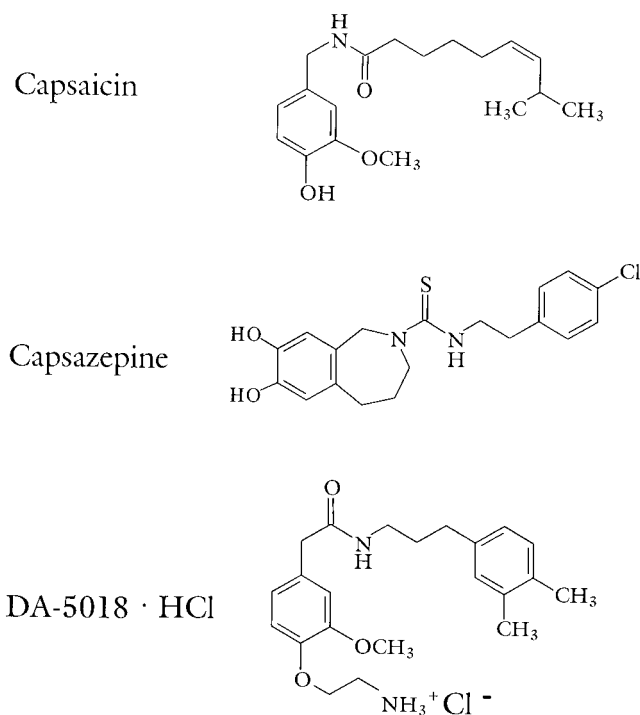


Figure 1. Chemical structure of capsaicin analogs.

pH 7.2. For whole cell, pipette solution contained (in mM): 140 K^+ , 2 Mg^{2+} , 144 Cl^- , 5 EGTA, 10 HEPES, and 4 ATP, pH 7.2. The control perfusion solution for whole-cell recording contained (in mM): 140 Na^+ , 5 K^+ , 2 Mg^{2+} , 1 Ca^{2+} , 151 Cl^- , and 10 HEPES. CAP and CZP (Research Biochemicals, Natick, MA) were dissolved and stored in 100% ethanol to make 10 mM stock solutions. A synthetic analog of CAP, DA (406.9 MW) (Fig. 1), was obtained from Dong-A Pharmaceutical (US patent 5242944; Seoul, Korea), dissolved in distilled water, and stored as a stock solution (10 mM). All other reagents used in cell culture or electrophysiological experiments were purchased from Sigma. All values were expressed as mean \pm SE.

RESULTS

Activation of single-channel currents by intracellular CAP

Previously, we showed that CAP activates a nonselective cation channel when applied to the outer surface of the patch membrane of cultured DRG neurons (Oh et al., 1996). Because CAP is highly lipid-soluble and thus can cross the membrane easily by diffusion, we predicted that CAP applied to the inner surface of inside-out patches would also lead to the channel activation. As shown in Figure 2A, channel activity was not observed when control solution was applied to the bath (intracellular side) in an inside-out patch. When 1 μ M CAP was introduced to the bath solution, a rapid activation of single-channel currents was observed within a few seconds ($n = 21$). This activation by intracellular CAP was completely antagonized by 10 μ M CZP, a competitive CAP receptor antagonist (Fig. 2A). As summarized in Figure 2B, P_o of single-channels induced by intracellular CAP increased from 0.00 to 0.42 ± 0.09 ($n = 5$). This P_o of single-channels, however, decreased to 0.03 ± 0.01 ($n = 5$) when 1 μ M CAP was applied to the bath together with 10 μ M CZP. Intracellular application of resiniferatoxin, a potent agonist of CAP receptors (Szallasi and Blumberg, 1989), was also tested for

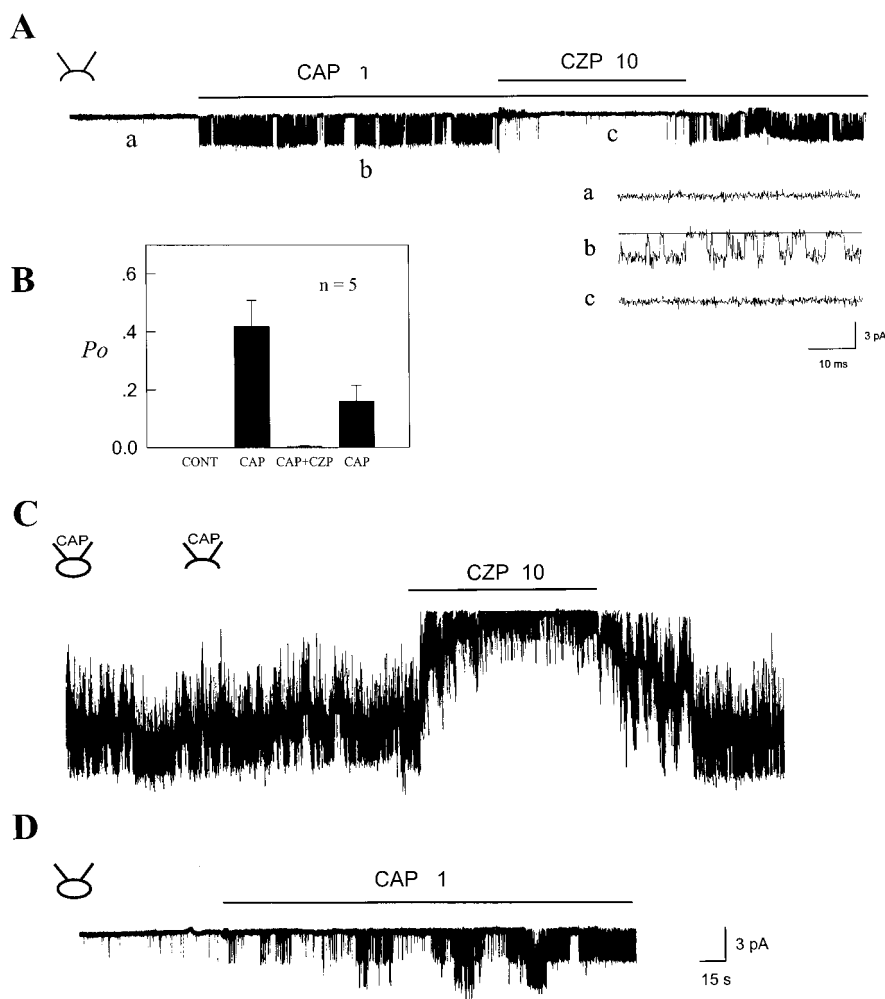


Figure 2. Activation of single-channel currents by intracellular CAP application and block by CZP. *A*, An inside-out membrane patch was held at -60 mV in a symmetrical 140 mM NaCl solution. After an inside-out patch was formed, CAP (1 μ M) or CAP plus CZP (1 and 10 μ M, respectively) was applied to bath. *Inset* shows single-channel current traces in an expanded time scale. *Straight line* in the *inset* represents the closed state of the channel. *B*, P_o of the channel obtained when CAP was applied to the intracellular surface of five different patch membranes. *C*, Blockade by intracellular CZP application of channel currents activated by extracellular application of CAP. The pipette contained CAP (1 μ M) in the 140 mM NaCl solution in a cell-attached or inside-out patch. CZP (10 μ M) was applied to bath (intracellular side) after an inside-out patch was formed. *D*, Activation of single-channel currents after bath perfusion of 1 μ M CAP in a cell-attached patch.

activation of the CAP-activated channel. In inside-out patches, perfusion of resiniferatoxin (1 – 2 nM) to the inner side of the membrane also caused rapid activation of single-channel currents ($n = 6$; data not shown). The rapid onset of activation after intracellular application of CAP suggests that the receptor for CAP may be at the inner membrane surface. Thus, channel activation by the extracellular application of CAP may be activating the channel by diffusing into the cell membrane and acting on binding domains present in the intracellular side of the patch membrane.

To examine whether the channel activated by extracellular CAP can be blocked by intracellular CZP, we applied CZP to the inner surface of the patch membrane while CAP was kept in the extracellular surface of the patch. As shown in Figure 2*C*, when a cell-attached patch was formed with a pipette containing 1 μ M CAP, activation of single-channel currents was observed ($P_o = 0.51 \pm 0.13$; $n = 7$). After forming an inside-out patch, the channel activity persisted, although it depressed slightly ($P_o = 0.38 \pm 0.13$; $n = 7$). In the same patch, 10 μ M CZP applied to the bath (intracellular surface) quickly blocked the CAP-induced single-channel currents ($P_o = 0.01 \pm 0.0$; $n = 7$) (Fig. 2*C*). The rapid activation of channel by CAP and rapid inhibition by intracellular CZP also suggest that the binding site may be at the intracellular side of the membrane. In other experiments, CAP was applied to the bath after forming cell-attached patches with

pipettes containing only the control 140 mM NaCl solution. When perfused to the bath, 1 μ M CAP activated single-channel currents 8.4 ± 2.6 sec ($n = 14$) after application (Fig. 2*D*). It, however, generally required several minutes for CAP to activate the channels maximally. Activation of the channel in this patch configuration further suggests that extracellular CAP can diffuse easily through the membrane to reach the target site.

Activation of single-channel currents by intracellular DA

To determine whether CAP activates the channel by acting at an intracellular or extracellular binding domain of the membrane, we used a CAP analog that is charged at physiological pH and thus cannot cross the cell membrane easily. A salt form of an analog of CAP, DA (Fig. 1), was synthesized (Park et al., 1993) and used as the water-soluble ligand for activating the channel. DA at physiological pH was applied to isolated patches under different configurations. As shown in Figure 3*A*, 0.5 μ M DA also caused activation of single-channel currents ($P_o = 0.73 \pm 0.11$; $n = 5$) when applied to the bath (intracellular side) in an inside-out patch. This activation by intracellular DA was blocked by 10 μ M CZP ($P_o = 0.0 \pm 0.0$; $n = 5$). To determine whether extracellular application of DA can activate the channel, we applied DA to the outer surface of the cell membrane. As shown in Figure 3*B*, 1 μ M DA in the pipette solution (extracellular side)

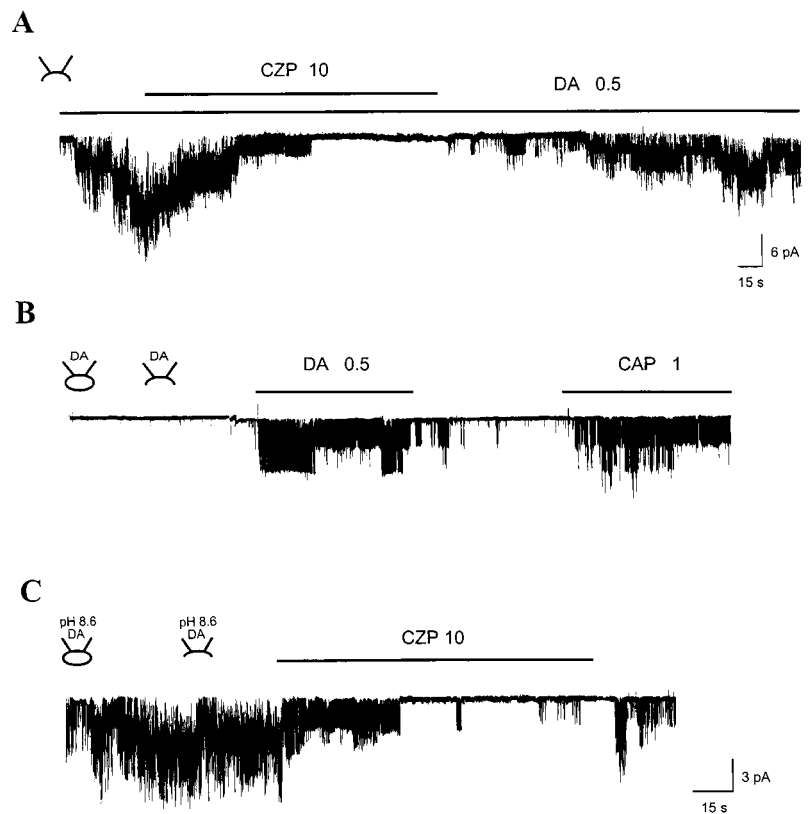


Figure 3. Differential responses of the CAP-activated channel to an ionized form of the CAP analog, DA, applied to the intracellular or extracellular surface of patch membranes. *A*, Activation and block by CZP of single-channel currents by 0.5 μM DA applied to bath (intracellular side) in an inside-out patch. *B*, Failure of DA in normal pH (pH 7.2) to activate the CAP-activated channel in a cell-attached or inside-out patch, with the pipette (extracellular side) containing 1 μM DA. Only when DA or CAP was perfused to the bath was the rapid activation of the channel currents observed. Time and current scales are same as shown in *C*. *C*, DA at pH 8.6 in the pipette-activated channel currents in a cell-attached or inside-out patch. Application of 10 μM CZP to the bath after forming an inside-out patch blocked the channel currents.

failed to activate the CAP-activated ion channel in either the cell-attached or inside-out patch state ($n = 5$). In the same patch, however, application of 0.5 μM DA to the bath (intracellular side) activated the channel currents (Fig. 3*B*). The response to the intracellular application of DA was reversible. After washout, application of 1 μM CAP to the bath caused openings of the same channels ($n = 5$). Because the ionic state of DA depends on the pH of the solution, we changed the pH of the extracellular DA. At pH 6.0, the proportion of un-ionized species of DA is dramatically reduced (0.16% of total DA, see Discussion) compared with that at physiological pH (2.5% of total DA). At pH 6.0, 1 μM DA in the pipette (extracellular side) did not activate the channel currents in cell-attached or inside-out patches ($n = 6$). However, intracellular application of DA at pH 6.0 readily activated the currents. In contrast, as shown in Figure 3*C*, at pH 8.6, at which the proportion of uncharged species is high (37% of total DA), extracellular application of 1 μM DA greatly activated channel currents in all patches tested ($n = 8$). The channel currents activated by extracellular DA at pH 8.6 were abolished by intracellular application of 10 μM CZP (Fig. 3*C*). These results clearly suggest that DA in the predominantly charged state can activate the channel only from the intracellular side and that binding of CAP and its analogs takes place at the intracellular side of the membrane.

Single-channel openings by CAP and DA

The channels activated by intracellular application of CAP exhibited single-channel properties similar to those obtained from the extracellular application of CAP (Oh et al., 1996). Channel openings with membrane potentials held at -80 to $+80$ mV in 40 mV increments in symmetrical Na⁺ solution are shown in Figure 4*A*. Amplitude histograms were obtained from channel openings

at each membrane potential held at -80 to $+80$ mV in 20 mV increments, and the mean amplitude was plotted against membrane potential to obtain a current–voltage (I – V) relationship. As shown in Figure 4*B*, single-channel currents activated by CAP exhibited outward rectification. Slope conductances at -60 and $+60$ mV were 44.7 ± 0.9 and 77.5 ± 2.3 pS, respectively ($n = 11$). Channel openings produced by intracellular application of CAP occurred in short and long bursts (Fig. 4*A*). Single-channel currents activated by DA at each membrane potential were also obtained, and an I – V relationship was compared with that obtained from CAP. As shown in Figure 4*B*, single-channel currents activated by DA were outwardly rectifying, similar to those activated by CAP. Slope conductances at -60 and $+60$ mV were 45.3 ± 0.7 and 75.4 ± 1.1 pS ($n = 7$), respectively (Fig. 4*B*). The similarity of single-channel properties also suggests that DA activates the same channel activated by CAP.

Ion selectivity of single-channel currents activated by DA

To characterize further the channel activated by DA, ion selectivity of the channel current was determined. Because the CAP-activated ion channel discriminates poorly among cations, permeability to K⁺ or Cs⁺ of the channel current activated by DA was determined. In inside-out patches containing multiple CAP-activated channels, a voltage ramp from -80 to $+80$ mV in 300 msec duration was applied to get the reversal potentials in various ionic conditions. Macroscopic current responses to the voltage ramp were recorded from inside-out patches with the pipette containing the 140 mM NaCl solution and the bath containing the symmetrical NaCl or equimolar KCl or CsCl solution. In each patch, 0.5 μM DA was added to the bath solution. As shown in Figure 5, replacing bath NaCl with KCl and CsCl did not shift the

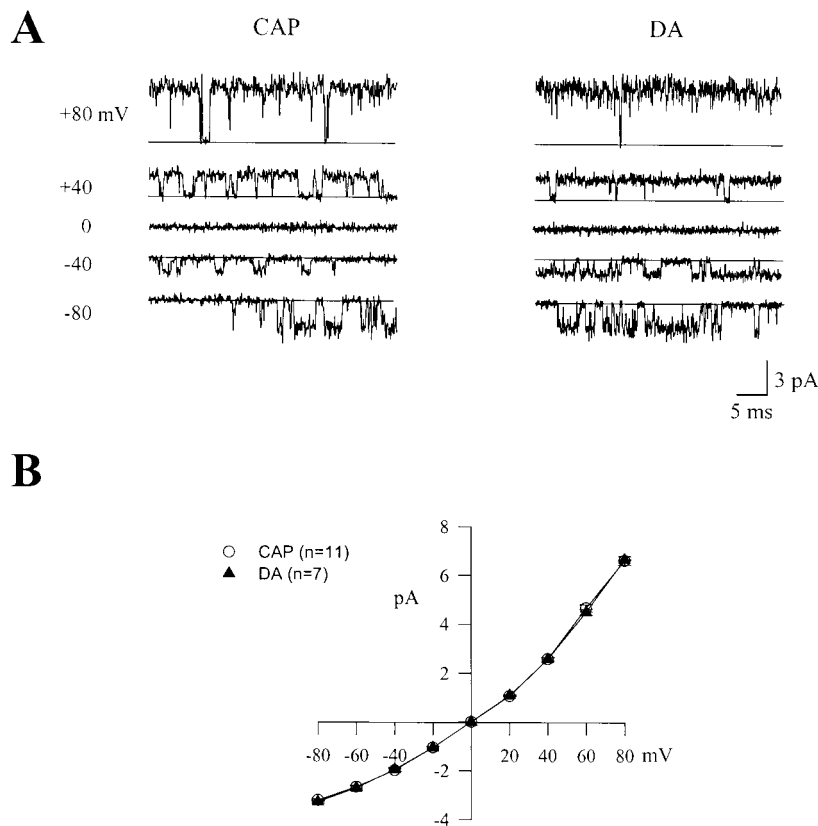


Figure 4. Single-channel openings obtained when CAP or DA was applied to the bath (intracellular surface) in inside-out patches. *A*, Traces of single-channel currents activated by $1 \mu\text{M}$ CAP (*right*) and $0.3 \mu\text{M}$ DA (*left*) held at different membrane potentials ranging from -80 to $+80$ mV. *B*, I - V relationships of single-channel currents. Each point was averaged from 11 (CAP) or seven (DA) inside-out patches. Bars representing SE are so small that they are inside the triangles or circles.

reversal potential (-3.0 ± 0.7 and -5.2 ± 0.6 mV, respectively; $n = 6$) from 0 mV, suggesting that the channel activated by DA is permeable to monovalent cations. The permeability ratios, $P_{\text{K}}/P_{\text{Na}}$ and $P_{\text{Cs}}/P_{\text{Na}}$, under these bi-ionic conditions calculated from the constant-field equation (Fatt and Ginsborg, 1958; Hille, 1992) were 1.1 and 1.2, respectively. To determine whether the channel activated by DA was permeable to Ca^{2+} , the pipette with 140 mM Na^+ was replaced with 100 mM Ca^{2+} , and the bath contained $0.5 \mu\text{M}$ DA in the 140 mM Na^+ solution (Fig. 5). The average reversal potential with 100 mM Ca^{2+} in eight experiments was 26.6 ± 2.2 mV, indicating the high Ca^{2+} permeability over Na^+ ($P_{\text{Ca}}/P_{\text{Na}} = 2.9$) as reported previously (Wood et al., 1988; Caterina et al., 1997; Zeilhofer et al., 1997). These results further suggest that the channel activated by DA is nonselectively permeable to cations, as observed in the CAP-activated channel.

Concentration dependency

We determined the concentration-effect curves for CAP and DA to compare their efficiencies on channel activation from the intracellular side. A steady-state level of channel activity was achieved during intracellular perfusion of agonists at different concentrations. As shown in Figure 6*A*, CAP concentration $< 0.1 \mu\text{M}$ rarely activated the channel, but CAP at $\geq 0.3 \mu\text{M}$ progressively increased the channel activity, showing a maximal activation at $10 \mu\text{M}$. The open probability (P_o) was plotted as a function of agonist concentration (Fig. 6*C*). Each data point was fitted by a nonlinear regression to the Hill equation:

$$P_o = P_{\text{omax}} \frac{1}{1 + (K_D/[\text{Agonist}])^n}$$

In this equation, K_D is the half-maximal concentration of agonists in activating the channel, $[\text{Agonist}]$ is the concentration of CAP

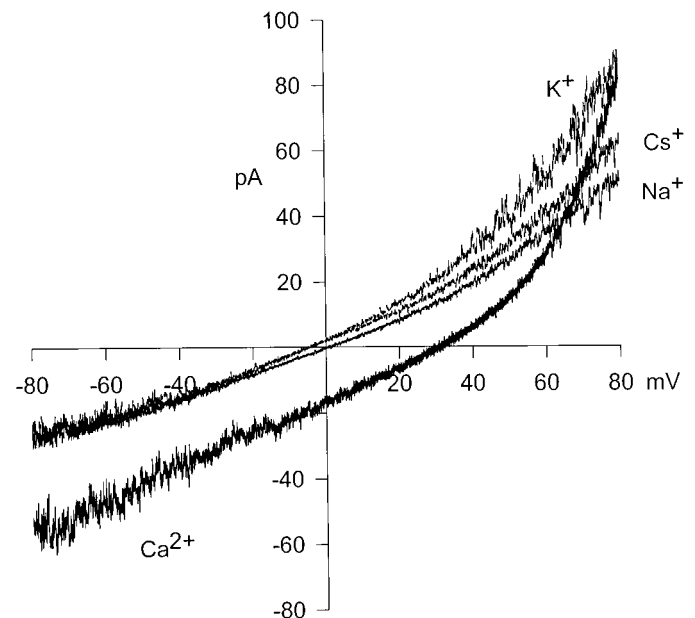


Figure 5. Ion selectivity of the channel current activated by $0.5 \mu\text{M}$ DA applied intracellularly. Macroscopic current responses to voltage ramps (300 msec) from -80 to $+80$ mV in a patch containing multiple CAP-activated channels after the bath solution of 140 mM NaCl was changed to equimolar KCl and CsCl. The pipette contained 140 mM NaCl, and $0.5 \mu\text{M}$ DA was added to each bath solution. To get a macroscopic current in $\text{Ca}^{2+}/\text{Na}^+$ bi-ionic condition, the voltage ramp was applied to a patch containing 100 mM CaCl_2 in the pipette and 140 mM NaCl and $0.5 \mu\text{M}$ DA in the bath.

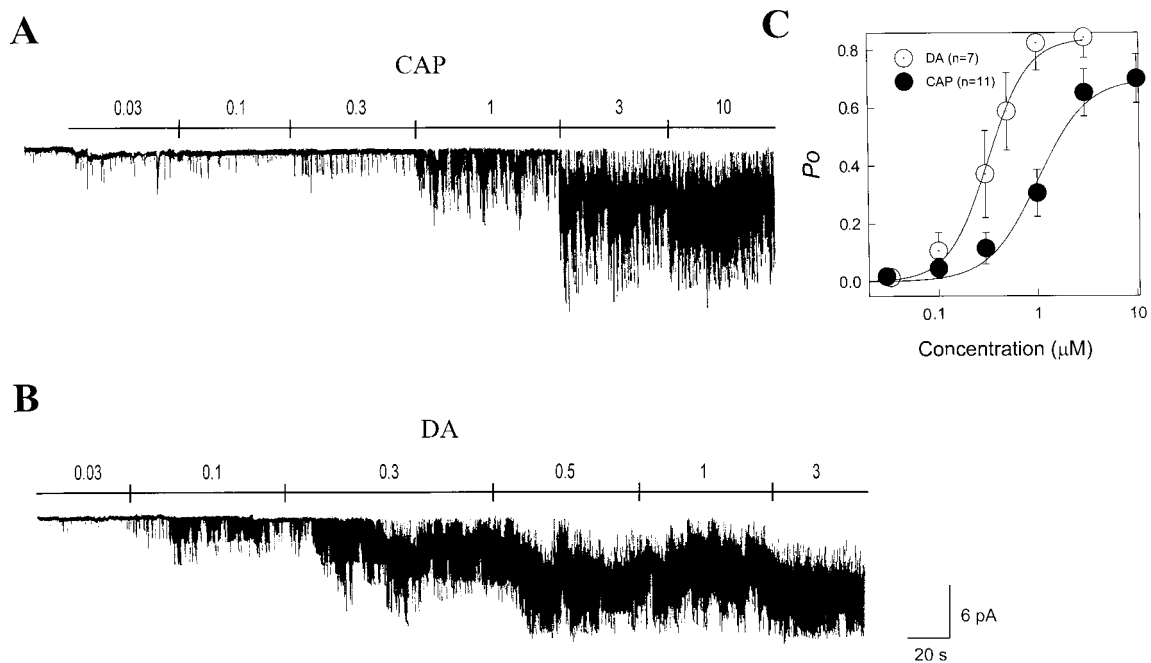


Figure 6. Concentration-dependent activation of the channel by intracellular CAP or DA. *A*, CAP, at different concentrations ranging from 0.03 to 10 μM , was perfused to the bath in an inside-out membrane patch in a symmetrical 140 mM NaCl condition. *B*, DA, at different concentrations ranging from 0.03 to 3 μM , was perfused to the bath in an inside-out patch in the symmetrical salt solution. *C*, A summary of the concentration–response relationship expressed in P_o of the channel versus concentration of intracellular agonists. Data points were fitted to the Hill equation, as described in Results.

or DA, $P_{o\text{max}}$ is the maximal P_o , and n is the Hill coefficient. K_D values for CAP and DA in activating the channel were 1.0 ($n = 11$) and 0.32 ($n = 7$) μM , respectively, indicating that DA has a greater potency in activating the channel. The intracellular application of DA also exhibited a greater efficacy as well, because the maximal current response ($P_o = 0.84 \pm 0.07$; $n = 7$) to DA (3 μM) was $\sim 24\%$ greater than that observed after 10 μM CAP application ($P_o = 0.70 \pm 0.09$; $n = 11$). The Hill coefficients for CAP and DA were 1.8 and 2.1, respectively. K_D and Hill coefficients obtained by intracellular CAP were not different from those obtained by extracellular CAP application ($K_D = 1.1$ μM ; $n = 1.8$) (Oh et al., 1996). Furthermore, the slopes of the dose–response curves suggest that two binding sites may be present for CAP and DA and that the binding occurs in a cooperative manner.

Whole-cell and oocyte currents activated by CAP and its analog

As shown in Figure 7, extracellular application of 1 μM CAP (a half-maximal dose) to a DRG cell under whole-cell configuration whose membrane potential was held at -60 mV produced a rapid inward current. After washout of CAP, 0.3 μM DA (a half-maximal dose) was perfused to the bath to test the effect of the CAP analog on the CAP-induced current. The perfusion of 0.3 μM DA did not activate the whole-cell current. After washout, reapplication of 1 μM CAP produced an inward current again but in a smaller magnitude, showing apparent desensitization, a property of the CAP-activated channel (Fig. 7*A*) (Docherty et al., 1996; Liu and Simon, 1996; Oh et al., 1996; Koplak et al., 1997). However, the application of DA in a supramaximal concentration (10 μM) exhibited a large, slowly activating inward current (Fig. 7*B*), probably because a substantial amount of an un-ionized form

of DA diffused into the intracellular side. To confirm whether activation of the channel currents by extracellular DA depends on the amount of un-ionized fraction of DA, we changed pH of the bath solution containing DA. Application of 0.3 μM DA at pH 6.0, at which the majority of DA were ionized (99.8%), did not activate any current at all, whereas 0.3 μM DA at pH 8.6, at which the proportion of uncharged DA was $\sim 37\%$, exhibited inward currents with the magnitudes of $63 \pm 10\%$ ($n = 5$) of those of CAP-induced currents. Inability of DA at a half-maximal concentration in activating the channel was also tested in oocytes expressing a newly cloned CAP channel, VR1 (Caterina et al., 1997). As shown in Figure 7*C*, the perfusion of the ND96 solution (pH 7.5) containing 1 μM CAP to an oocyte injected with cRNA (1 ng) of VR1 produced a rapid and large inward current. In the same patch, however, the application of 0.3 μM DA failed to exhibit the current response. However, as observed in cultured sensory neurons, a supramaximal dose (10 μM) of DA to the oocyte greatly activated the current at a much slower rate. Because CAP and its analog differ only in lipid solubility, the different current responses of native, as well as oocyte-expressed, channels to extracellular CAP and DA may imply that the ligand-binding domain of the CAP receptor is present in the intracellular side of the cell membrane.

Effect of intracellular acidification

H^+ has been suggested as an endogenous activator of CAP receptors (Bevan and Geppetti, 1994). If this were the case, an acidic solution (pH < 6.0) applied to the inner side of the patch membrane should activate the CAP-activated ion channel. To test the action of H^+ , CAP (1 μM) or acidic control solution (pH 5.0) was applied to the inner surface of patch membrane in inside-out patch configuration. When acidic control solution of pH 5.0 was perfused to the bath, the acidic solution failed to activate the

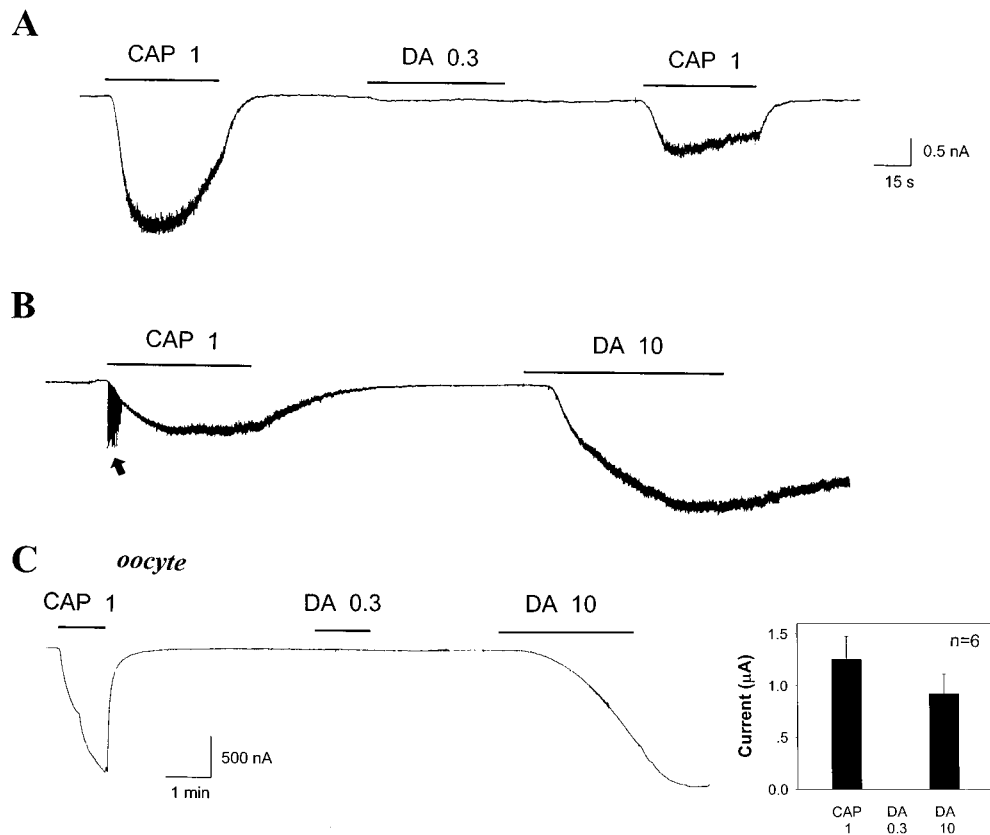


Figure 7. Current responses of cultured sensory neurons or oocytes expressing VR1 to the extracellular perfusion of CAP and its analog. *A*, Activation of whole-cell currents by bath application of 1 μM CAP but not by 0.3 μM DA. Note an apparent desensitization after CAP. *B*, A great activation of whole-cell current by bath application of 10 μM DA. *Arrow* indicates unclamped current spikes originating in narrow axon trunks. *C*, Activation by 1 μM CAP of a CAP receptor expressed in *Xenopus* oocytes. A half-maximal dose of DA (0.3 μM) failed to activate the current, but a supramaximal dose of DA (10 μM) activated the current. *Inset* shows a summary of current responses of oocytes expressing CAP receptors to CAP and DA ($n = 6$).

single-channel currents. However, application of CAP (1 μM) to the same patch elicited a rapid activation of the channel currents (Fig. 8*A*). In 30 additional experiments, none of the applications of acidic solution (pH 5.0–6.0) activated the single-channel currents when applied to the cytosolic surface of the patch membrane. Although acidic solution does not activate the channel directly, it is still possible that acid may activate the channel indirectly via cytosolic signal transduction pathways. To test this, we perfused acidic solution (pH 6.0) to the bath (extracellular side) in a cell-attached patch containing only control solution in the pipette. As shown in Figure 8*B*, application of acidic solution to the bath did not activate the channel ($n = 6$). However, 1 μM CAP perfused to the bath easily activated the channel. In this cell-attached patch, brief spontaneous openings of the channel were also observed before the application of acid or CAP (Fig. 8*B*). Because it is known that H^+ modulates activity of various ion channels (Hille, 1992), we tested whether acidic condition affected the CAP-evoked channel currents. As shown in Figure 8*C*, the CAP-activated channel activity was greatly augmented by acidic condition (pH 6.0) in five of eight patches.

Recently, an acid-sensitive channel was cloned in DRG neurons (Waldmann et al., 1997). Similar to the CAP-activated ion channel, the acid-sensitive ion channel is permeable to cations. The acid-sensitive channel has two putative transmembrane domains and sequence homology with amiloride-sensitive Na^+ -channels and is blocked by amiloride when expressed in *Xenopus* oocytes (Waldmann et al., 1997). In DRG neurons, intracellular

perfusion of 10 μM amiloride with 1 μM CAP failed to block the CAP-activated channel current in all six patches tested (Fig. 8*D*). This result again indicates that the CAP-activated channel is different from the acid-sensitive channel. These results suggest that H^+ does not activate the channel directly but possibly modulates the channel.

DISCUSSION

It is now clear that CAP activates a nonselective cation channel in cultured DRG neurons. Opening of the channel by CAP would cause influx of Na^+ and Ca^{2+} , resulting in depolarization of sensory neurons. It is generally assumed that CAP binds to a receptor site in the extracellular side of the membrane to produce its hyperalgesic effects. However, it is not clear whether the CAP binding site is present in the inner or outer surface of the cell membrane because of the high lipid solubility of CAP. In this study, we show that intracellular application of CAP can also activate the channel in a concentration-dependent manner, as expected of a lipophilic compound. A relatively membrane impermeable analog of CAP, DA, was able to activate the channel when the analog was applied to the inner, but not the outer, surface of the patch membrane, indicating that CAP and its analogs act on the channel from the intracellular side. The finding that agonists bind to the intracellular side of the membrane suggests that an endogenous activator of the channel may exist in the cytosol of cells.

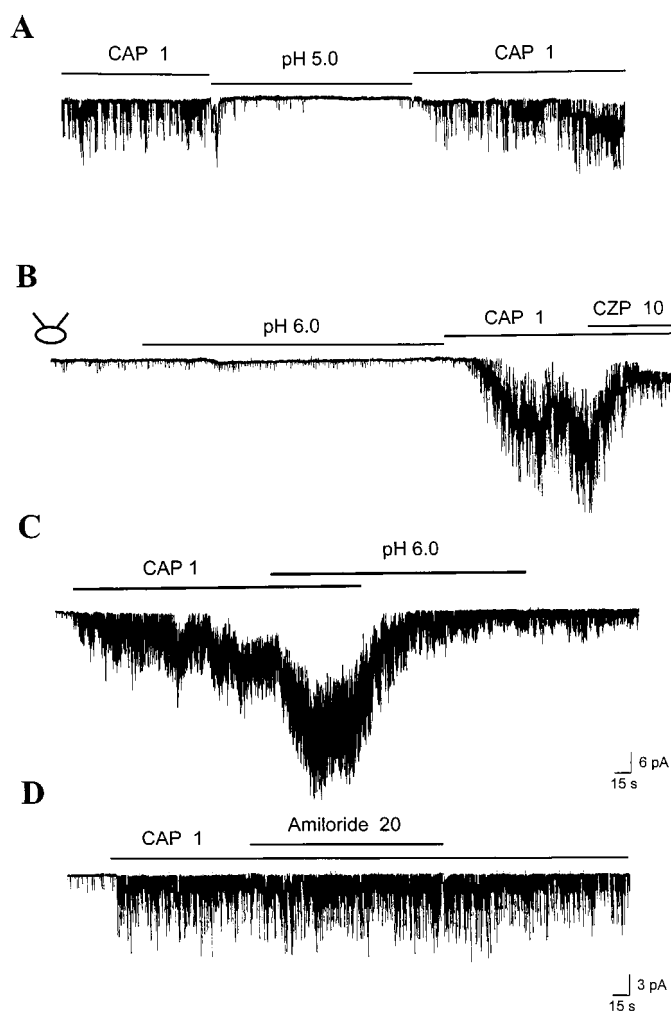


Figure 8. *A, B*, Failure of acid in activating the CAP-activated channel currents when perfused to the bath (intracellular surface) of an inside-out (*A*) or a cell-attached (*B*) patch. Time and current scales in *A* and *B* are same as shown in *D* and *C*, respectively. *C*, Modulation of the CAP-activated channel currents by acidic condition. *D*, Failure of 20 μM amiloride in inhibiting the CAP-activated channel currents.

Physicochemical property of DA

The pK_a of DA is 8.8. Therefore, the molar ratio of the ionized form ($-\text{NH}_3^+$) (Fig. 1) of DA to the un-ionized form ($-\text{NH}_2$) calculated using the Henderson–Hasselbalch equation ($pK_a = \text{pH} - \log[\text{Base}/\text{Acid}]$) is ~ 40 in the 140 mM NaCl solution at pH 7.2. This indicates that 97.5% of DA at this pH become ionized. Thus, at the applied concentration of 1 μM , 0.975 μM DA would be charged. Only a fraction (0.025 μM) of DA remains uncharged and thus can pass through the cell membrane under this pH condition. Once passing through the cell membrane, the uncharged DA will dissociate and equilibrate again with charged species. However, the concentration of DA, whether it is charged or not, would not be high enough to activate the channel, because $\text{DA} \leq 0.1 \mu\text{M}$ rarely activates the channel in excised membrane patches, as shown in the concentration–response relationship (Fig. 6). We further showed that application of DA at physiological pH to the extracellular side of the patch membrane failed to activate the channel, whereas intracellular application of the DA readily activated the channel (Fig. 3). In addition, when pH of the solution became basic (pH 8.6), thus increasing uncharged species

of DA (37% of total DA), the extracellular application of DA activated the channel currents in whole-cell and isolated membrane patches. These results strongly suggest that the ligand-binding domain of the receptor is present in the intracellular surface of the cell membrane and that activation of the channel by extracellular application of CAP or DA is caused by its diffusion through the cell membrane.

Binding property of the CAP-activated channel

High-affinity binding sites for CAP, as measured by specific binding of radio-labeled ^3H -resiniferatoxin, were distributed in the DRG, spinal cord, cerebellum, and retina (Szallasi et al., 1995). Furthermore, Szallasi et al. (1993) showed that ^3H -resiniferatoxin bound to the CAP receptor in a cooperative manner, with a Hill coefficient of 1.7. This is in good agreement with our results that CAP and DA activate the channel in a positive cooperative manner (Hill coefficients, 1.8 and 2.1, respectively). This positive cooperativity with the Hill coefficient of ~ 2 was also seen in VR1 when expressed in oocytes (Caterina et al., 1997). Judging from the data obtained from both binding assays and current recordings from native or heterologously expressed channels, the channel complex has at least two binding sites for CAP. Thus, the binding of a molecule of CAP to one subunit of the channel may accelerate binding of CAP to another subunit, exhibiting a positive cooperativity. Hydrophobicity analysis of VR1 predicts two relatively long cytosolic loops at both ends of the receptor protein and four short extracellular segments between putative transmembrane domains (Caterina et al., 1997). As suggested in part by the present study, these cytosolic domains may interact with CAP to cause an opening of the channel.

Endogenous activator of the CAP-activated channel

Analogous to what has been described for opiate receptors, distribution of high-affinity binding sites for CAP (Szallasi et al., 1995) and the presence of the CAP-activated ion channel in sensory neurons (Liu and Simon, 1994; Oh et al., 1996) suggest the existence of an endogenous ligand. Such an endogenous substance, however, has not yet been identified. Previous studies have suggested that proton (H^+) is a possible candidate for the endogenous substance, because acidic solution and CAP exhibit similar effects on various tissues, such as sensory neurons, muscle, and bladder (for review, see Bevan and Geppetti, 1994). Application of acidic solution has been reported to cause inward whole-cell currents that are similar to the CAP-induced currents (Bevan and Yeats, 1991; Geppetti et al., 1991). CAP and H^+ even show similar responses to NGF deprivation in primary cultures of DRG neurons (Bevan and Winter, 1995). Although these findings suggest that proton may be an endogenous ligand for the CAP receptor, there is no direct evidence indicating that proton is the activator of the CAP receptor. There are reports against the assumption that H^+ activates the CAP-activated channel (Bevan et al., 1992; Steen et al., 1992). For example, Rb^+ efflux induced by low pH from cultured DRG neurons was not blocked by CZP (Bevan et al., 1992). In the previous study, we failed to observe the activation of a CAP-activated channel by the extracellular application of acidic solution (pH 6.0) (Oh et al., 1996). Recently, both acid-sensitive and CAP-sensitive channels were cloned in DRG neurons (Caterina et al., 1997; Waldmann et al., 1997). In addition to the difference in their primary structures, there are clear lines of physiological evidence suggesting that the two

channels are not identical. First, the acid-sensitive domain of the channel is present at the extracellular side of the membrane. Second, the acid-sensitive channel shows high permeability of Na^+ over K^+ ($P_{\text{Na}}/P_{\text{K}} = 13$). Last, the acid-sensitive channel is blocked by amiloride, but the CAP-activated channel is not (Fig. 8). Thus, together with other reports, the results of the present study further indicate that proton may not be the endogenous ligand that binds to the CAP receptor. However, we could not exclude the possibility that protons would modulate the channel activity allosterically, partly as shown by Caterina et al. (1997). We also observed the modulatory effect of protons on the channel currents, because CAP applied in acidic solution (pH 6.0) exhibited a greater channel activity than CAP in normal pH (Fig. 8).

Clinical implication of DA

DA was developed as an analgesic, similar to other CAP analogs (Dray et al., 1990; Dray, 1992; Szallasi and Blumberg, 1996). These analogs have agonistic activities for CAP receptors and induce desensitization of sensory neurons to noxious stimuli. The desensitization of sensory neurons is believed to be responsible for the analgesic effect of the CAP analogs. Cellular mechanisms underlying the desensitization of sensory neurons by the CAP analogs are not known clearly. However, swelling of sensory neurons or cellular damage induced by Ca^{2+} -dependent cytosolic enzymes caused by a large influx of Na^+ and Ca^{2+} through CAP receptor is suggested (for review, see Bevan and Szolcsanyi, 1990; Szolcsanyi, 1993). Because DA is soluble in physiological solution, it can hardly pass through the cell membrane to activate the channel. Thus, our finding that the ligand binding site is present in the intracellular side of the CAP receptor suggests that DA is not suitable for clinical use. This appears true especially when the concentration of DA is low, but as the concentration of DA increases, the concentration of the un-ionized form of DA gets greater so that it is able to affect the CAP-activated channel from outside the cell membrane. For example, we observed in the present study that DA at half-maximal dose ($0.3 \mu\text{M}$) did not activate, but at $10 \mu\text{M}$, it greatly activated whole-cell current, even when applied to the bath (extracellular side) (Fig. 7). Under this experimental condition, the un-ionized form of DA reached $\sim 0.25 \mu\text{M}$, which is close to a half-maximal dose in activating the channel. Therefore, these results indicate that depending on its dose present in extracellular matrix systemic application of DA can have pharmacological actions on sensory neurons and, thus, can be applicable clinically.

In summary, we report that CAP activates a nonselective cation channel by acting on binding sites present in the intracellular side of the membrane receptor. We assume that the binding of CAP is directly on the channel, because a cloned gene, VR1, alone confers the sensitivity to CAP when expressed in oocytes. The present results suggest the possible presence of an endogenous CAP-like substance in the cytosol of sensory neurons. Because the CAP-activated current accounts for the cellular responses of sensory neurons to CAP, the present finding will help to further elucidate the mechanisms underlying sensory transduction induced by CAP.

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