Calcium and avian intrapulmonary chemoreceptor response to CO₂

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¹Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona; ²Division of Neurobiology, Physiology and Behavior, University of California at Davis, Davis, California; and ³Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York

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Hempleman, S. C., S. X. Egan, J. Q. Pilarski, T. P. Adamson, and I. C. Solomon. Calcium and avian intrapulmonary chemoreceptor response to CO₂. J Appl Physiol 101: 1565–1575, 2006. First published August 10, 2006; doi:10.1152/japplphysiol.00088.2006.—Intrapulmonary chemoreceptors (IPC) are highly responsive respiratory chemoreceptors that innervate the lungs of birds and diapsid reptiles. IPC are stimulated by low levels of lung PCO₂, inhibited by high levels of lung PCO₂, and their vagal afferents serve as a sensory limb for reflex adjustments of breathing depth and rate. Most IPC exhibit both phasic and tonic sensitivity to CO₂, and spike frequency adaptation (SFA) contributes to their phasic CO₂ responsiveness. To test whether CO₂ responsiveness and SFA in IPC is modulated by a Ca²⁺-linked mechanism, we quantified the role of transmembrane Ca²⁺ fluxes and Ca²⁺-related channels on single-unit IPC function in response to phasic changes in inspired PCO₂. We found that 1) broad-spectrum blockade of Ca²⁺ channels using cadmium or cobalt and blockade of L-type Ca²⁺ channels using nifedipine increased IPC discharge; 2) activation of L-type Ca²⁺ channels using BAY K 8644 reduced IPC discharge; 3) blockade of Ca²⁺-activated potassium channels using charybdotoxin (antagonist of large-conductance Ca²⁺-dependent K⁺ channel) increased IPC discharge, but neither charybdotoxin nor apamin affected SFA; and 4) blockade of chloride channels, including Ca²⁺-activated chloride channels, with niflumic acid decreased IPC discharge at low PCO₂ and increased IPC discharge at high PCO₂, resulting in a net attenuation of the IPC CO₂ response. We conclude that Ca²⁺ influx through L-type Ca²⁺ channels has an inhibitory effect on IPC afferent discharge and CO₂ sensitivity, that spike frequency adaptation is not due to apamin- or charybdotoxin-sensitive Ca²⁺-activated K⁺ channels in IPC, and that chloride channels blocked by niflumic acid help modulate IPC CO₂ responses.

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were reduced when cadmium and cobalt were present. However, because both cadmium and cobalt are nonspecific Ca\textsuperscript{2+} channel blockers, with many pathophysiological and toxicological side effects, these results were suggestive but not definitive evidence for Ca\textsuperscript{2+} involvement in IPC responses. Therefore in series II and III, we used much more specific antagonists and agonists of Ca\textsuperscript{2+}-related ion channels to further test the hypothesis.

In series II, we quantified the effects of the specific L-type Ca\textsuperscript{2+} channel blocker nifedipine and the specific L-type Ca\textsuperscript{2+} channel agonist BAY K 8644 on IPC discharge in vivo. L-type Ca\textsuperscript{2+} channels have been found to contribute to CO\textsubscript{2} sensitivity in other respiratory chemoreceptor cells (11, 26). Based on our series I results, we hypothesized that IPC might also possess L-type Ca\textsuperscript{2+} channels, and if so nifedipine should increase IPC excitability and BAY K 8644 should decrease IPC excitability.

In series III, to explore the inhibitory effect of Ca\textsuperscript{2+} influx on IPC CO\textsubscript{2} response, we quantified the effects of the KC\textsubscript{a} channel blockers charybdotoxin [CBTX, antagonist of large-conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (BK\textsubscript{Ca}) channels] and apamin [antagonist of small-conductance KC\textsubscript{a} (sKC\textsubscript{a}) channels] and the chloride channel blocker niflumic acid [which also antagonizes Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} (Cl\textsubscript{ca}) channels] on IPC discharge in vivo. We hypothesized that, if any of these channels contributed to IPC function, changes in IPC discharge and phasic responses to CO\textsubscript{2} step stimuli should occur with antagonist treatment. For example, if CBTX or apamin reduced SFA in IPC, it would indicate that this adaptation is due to KC\textsubscript{a} channel activation.

**METHODS**

**General.** Experiments were approved by the Institutional Animal Care and Use Committee at Northern Arizona University, in accordance with The American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings” (1). Thirty-nine mallard ducks, Anas platyrhynchos, body mass 1.0–1.4 kg, of either sex, were studied. Animals were anesthetized by intravenous infusion of 35–40 mg/kg pentobarbital sodium (dissolved in 10% ethanol), and a polyethylene cannula was implanted in the left brachial vein for administration of supplemental dosages of pentobarbital sodium (3–5 mg/kg iv, as needed to maintain a deep level of surgical anesthesia, i.e., absence of withdrawal response to a strong toe pinch) and experimental drugs. A thermistor probe was inserted into the colon, and body temperature was regulated at 40 ± 1°C using a circulating hot water pad and hot water bottles. Electrocardiogram was monitored using a Grass P511K AC preamplifier coupled to a Grass AM5 audio amplifier and Hitachi analog oscilloscope.

The interclavicular air sac was opened through a skin incision, and a pediatric cuffed endotracheal tube was inserted into the trachea. During subsequent surgery, animals were unidirectionally ventilated with a continuous 1 l/min stream of humidified air supplemented with 3% CO\textsubscript{2}, which entered through the endotracheal tube and exited via the interclavicular air sac opening. Gases from tanks were mixed and delivered by a Cameron Instruments GF-1 electronic mass flow controller. Unidirectional ventilation and deep surgical anesthesia precluded any breathing movements.

The left vagus nerve was exposed in the neck, freed from surrounding tissue, and draped over a small plastic platform submerged in light mineral oil. Single-unit recordings were made from fine filaments dissected from the exposed vagus nerve and placed on a 35-gauge monopolar platinum-iridium electrode (“fiber-picking” technique). Electrical activity in the filaments was referenced to an Ag-AgCl electrode on the nerve sheath through a differential high-impedance probe (Grass HIP). Signals were amplified ~10,000-fold with a Grass P511K AC preamplifier. IPCs were identified by their nearly immediate response to step changes in ventilatory gas PCO\textsubscript{2} and their inverse sensitivity to PCO\textsubscript{2}. Single-unit IPCs were identified by the constant amplitude and shape of their action potential waveforms using a Haer slope/height window discriminator. The window discriminator also generated a digital pulse (5 V, 10 μs) with each occurrence of the discriminated action potential. Interspike intervals and discharge rates were measured by timing the digital pulses from the window discriminator using a microcomputer sampling at 14,500 Hz (timing accuracy ±69 μs). Analog action potential signals were band-pass filtered between 100 and 3,000 Hz, notch-filtered at 60 Hz, visualized on an analog Hitachi oscilloscope, auditioned with a Grass AM5 audio amplifier, and recorded on a Vetter four-channel VHS tape system using pulse-code modulation.

**Measuring IPC response to CO\textsubscript{2}.** Experiments were conducted using a repeated-measures design. We first monitored the normal IPC responses to CO\textsubscript{2} during a 15- to 20-min control period to ensure that the IPC discharge was stable (e.g., Fig. 1A). We then administered test drug(s) listed in series I–III below and measured the IPC response again. Vagal filaments were tested for CO\textsubscript{2}-linked action potential activity by stepping inspired CO\textsubscript{2} between 0 and 6% with an 11-s period cycle, in a continuous unidirectional air flow of 2 l/min. When an IPC was identified, single-unit spike discharge responses were recorded on tape and computer during 10 complete CO\textsubscript{2}-step stimulus cycles (300–2,000 action potentials). Digital timing pulses triggering the analog CO\textsubscript{2} step stimulus were logged simultaneously with times of action potential occurrence by computer (20). These recordings were analyzed to produce cycle-triggered stimulus histograms of IPC discharge responses to the stimulus cycle. Consistency and signal strength of the IPC response to the repetitive stimulus were quantified using a two-way ANOVA (20). IPC with significant (P < 0.05) predrug discharge entrainment to the CO\textsubscript{2} step stimuli were accepted for further analysis (10, 20).

**Series I: effects of inorganic calcium channel blockers on IPC discharge.** Stock solutions of CdCl\textsubscript{2} and CoCl\textsubscript{2} (Sigma Aldrich, St. Louis, MO) were prepared in normal saline. Either 0.1 mmol/kg CdCl\textsubscript{2} (n = 5) or 1 mmol/kg CoCl\textsubscript{2} (n = 7) was slowly infused over a period of 1–2 min, via the brachial vein cannula, while IPC discharge and CO\textsubscript{2} stimulus steps were recorded. IPC recording continued for 5 min after CdCl\textsubscript{2} or CoCl\textsubscript{2} infusion. The IPC discharge responses reported for CdCl\textsubscript{2} and CoCl\textsubscript{2} occurred immediately following blocker injection. Cardiac function was compromised with CdCl\textsubscript{2} administration, and cardiac failure often occurred within 10 min. Animals were less impaired by CoCl\textsubscript{2}.

**Series II: effects of L-type calcium channel antagonist and agonist on IPC discharge.** Nifedipine (N-7634, Sigma RBI, St. Louis, MO) was dissolved in 95% ethanol and diluted 1:100 with normal saline immediately before use. S-(−)-BAY K 8644 (B-133, Sigma RBI) was dissolved in 10% ethanol and then diluted 1:10 with normal saline immediately before use. After quantifying an IPC’s normal response to CO\textsubscript{2} steps (see above), either 1 mg/kg nifedipine (n = 7) or 2 mg/kg BAY K 8644 (n = 6) were slowly infused over a period of 1–2 min, via the brachial vein cannula, while IPC discharge and CO\textsubscript{2} stimulus steps were recorded. IPC discharge was allowed to stabilize for 5 min, reaching a new steady state, and then IPC discharge response to CO\textsubscript{2} steps was again measured.

**Series III: effects of KC\textsubscript{a} and Cl\textsubscript{ca} channel blockers.** CBTX (C-7802, Sigma RBI) and apamin (A1289, Sigma RBI) were dissolved in water, aliquoted, and frozen before use. Nifumic acid (N-0630, Sigma-RBI) was dissolved in saline immediately before use. After an IPC’s normal response to CO\textsubscript{2} steps was quantified (see above), either 1–9 nmol/kg CBTX (n = 5), 100 nmol/kg apamin (n = 5), or 100 μmol/kg nifumic acid (n = 4) were slowly infused over a period of 1–2 min, via the brachial vein cannula, while IPC discharge and CO\textsubscript{2} stimulus steps were recorded. IPC discharge was allowed to...
of stimulus cycles given. These discharge rates produced separate cycle-triggered histograms of discharge frequency for each IPC under control and drug-treated conditions.

Treatment effects of drugs and CO₂ on IPC discharge frequency were quantified using a two-way repeated-measures ANOVA. Main effects were time (“α”), mapping to the CO₂ stimulus as indicated above), drug (“β”, control or treatment), and IPC identifier (“γ”, to quantify inter-IPC variation). Interaction terms time * drug (αβ), time * IPC (αγ), and drug * IPC (βγ) completed the models. Tests were run in JMP-IN version 4.0 (SAS, Cary, SC) on a PC. The statistical model was:

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\beta\gamma)_{jk} + (\alpha\gamma)_{ik} + \epsilon_{ijk} \]  

where \( Y_{ijk} \) is the observed discharge frequency for IPC \( k \), with drug \( j \), at time \( i \); \( \mu \ldots \) is the grand mean IPC discharge over all IPC, drugs, and times; and \( \epsilon_{ijk} \) is the residual error associated with \( Y_{ijk} \), which was the three-way interaction term \((\alpha\beta\gamma)_{ijk}\) in this repeated-measures model. Type I error rate of \( \alpha = 0.05 \) was used for all tests. Post hoc multiple comparisons were made with Tukey’s honestly significant difference test in JMP-IN version 4 that controlled for the overall type I error rate (\( \alpha = 0.05 \)).

Statistical interpretation: modulation of IPC discharge and/or modulation of IPC CO₂ sensitivity. Two statistical effects on IPC discharge were important in the present analysis: the “main effect of the drug treatment relative to control”; and the “interaction effect” of the drug treatment with CO₂-time relative to control. The “main effect” of the drug measured whether the drug significantly modulates mean IPC discharge rate compared with control (Δdischarge frequency), but it does not specifically quantify whether the drug affects the CO₂ sensitivity of IPC discharge. The “interaction effect” of the drug with CO₂-time specifically measures whether the drug significantly modifies the IPC CO₂ sensitivity (Δdischarge frequency/ΔPCO₂). For example, cadmium treatment was found to be significant as a main effect (see RESULTS), meaning that the mean IPC discharge rate (averaged over all CO₂ stimulus times) was significantly different during cadmium treatment compared with control. (Interpretation: cadmium significantly modifies IPC discharge.) There was also a significant interaction between cadmium and CO₂-time (see RESULTS), which indicates that the magnitude of the cadmium effect on IPC discharge rate was significantly different during different parts of the CO₂-time stimulus cycle (cadmium had a greater stimulatory effect at high CO₂ than at low CO₂, Fig. 2). [Interpretation: cadmium significantly changes IPC CO₂ sensitivity (Δdischarge frequency/ΔPCO₂).]

RESULTS

Control measurements: stability of discharge. In the repeated-measures design, each IPC served as its own control: the IPC discharge response to CO₂ after drug injection was compared with response of the same IPC before drug injection. To ensure that IPC CO₂-step responses were stable during the predrug control period, we monitored IPC discharge for 15–20 min on the computer or oscilloscope before administering test drugs. If the IPC’s discharge was not stable during this 15- to 20-min control period, we stopped recording from that IPC and searched for another IPC; thus only IPC with stable discharge rates were used in the present investigation. During the 15- to 20-min control period, anesthesia level was also checked and adjusted as necessary with 3–5 mg/kg pentobarbital (dissolved in 10% ethanol) flushed with 1–3 ml saline. In 15 IPC, we recorded cycle-triggered stimulus histograms of IPC discharge at both the beginning (control) and end (repeat control) of this 15- to 20-min period. The stability of remaining IPC was verified online during the control period and recorded at the
end of the stabilization period. Figure 1A shows the average stability of the 15 IPC recorded at both the beginning and end of the control period by computer, which included an intervening injection of intravenous pentobarbital to adjust anesthesia. No difference in CO2 effect on IPC discharge was seen between the beginning and end of the control period ($P > 0.7$), showing the stationarity of the IPC response and the lack of effect of pentobarbital/ethanol injections.

**Vehicle control: ethanol.** Figure 1B shows that intravenous injection of 1 ml of 10% ethanol in saline (vehicle control) in two different IPC had no effect on discharge response to CO2 ($P > 0.6$). This dosage of ethanol was 10-fold greater than the 1 ml of 1% ethanol used as a vehicle for nifedipine and BAY K 8644 in series II. Ten percent ethanol (volume 0.2–0.4 ml) was also used as a vehicle for pentobarbital anesthesia given in Fig. 1A between the control and repeat control measurements, also with no effect on IPC CO2 response ($P > 0.7$).

**Series I: effects of CdCl₂ and CoCl₂ on IPC discharge.** The overall statistical fits of the repeated-measures ANOVA model (Eq. 1) to CdCl₂ and CoCl₂ data were significant (CdCl₂: $P < 0.0001$, $R^2 = 0.877$; CoCl₂: $P < 0.0001$, $R^2 = 0.849$). Figures 2, 7, and 8 show that both cadmium and cobalt increased the mean IPC discharge averaged over the entire stimulus cycle. The ANOVA model revealed significant main effects of CdCl₂ and CoCl₂ compared with controls ($P < 0.0001$ for each), indicating that both CdCl₂ and CoCl₂ modulated (increased) mean IPC discharge rate, by ~50 and 30%, respectively (see Fig. 7). ANOVA showed significant interaction between CdCl₂ * CO₂-time ($P < 0.05$), indicating CdCl₂ reduced IPC CO₂ sensitivity by ~30% (see Fig. 8). No significant CoCl₂ * CO₂-time interaction was seen ($P > 0.9$), indicating that CoCl₂ did not significantly alter IPC CO₂ sensitivity.

The average cycle-triggered stimulus histograms of IPC discharge before and after 100 μmol/kg CdCl₂ infusion and before and after 1.0 mmol/kg CoCl₂ infusion are shown in Fig. 2, A and B. The effects of CdCl₂ on IPC discharge were larger (increasing mean discharge by 50%) and most noticeable at high CO₂ during the CO₂ stimulus cycle (Fig. 2A), while the effects of CoCl₂ were smaller (increasing average IPC discharge by ~30%) and were seen at both high and low CO₂ during the CO₂ stimulus cycle (Fig. 2B). Before infusion of either Ca²⁺ channel blocker, IPC discharge showed typical phasic response to the CO₂ step: discharge was highest when intrapulmonary CO₂ was low, and discharge was nearly silenced by high CO₂. Following infusion of CdCl₂, IPC discharge was raised significantly during the high-CO₂ portion of the stimulus step, and the overall magnitude of the phasic IPC response to CO₂ was reduced. The paired difference in IPC

![Figure 2](https://example.com/figure2.png)

**Fig. 2. Series I: cycle-triggered averages of IPC discharge rate (means ± SE) during repetitive CO₂ step stimuli.** A: before and after infusion of 100 μmol/kg CdCl₂ ($n = 5$). B: before and after infusion of 1 mmol/kg CoCl₂ ($n = 7$). Paired differences (means ± SE) comparing effects of drug infusion with control are shown for cadmium (C) and for cobalt (D).
discharge frequency between control and CdCl2 treatment is summarized in Fig. 2C. In one IPC, CdCl2 completely eliminated the response to CO2, and the IPC discharged at monotonically high rates (Fig. 3). In two additional IPC (not shown), CdCl2 was infused while animals were ventilated with steady inspired CO2, and a marked increase in IPC discharge rate was observed; however, responses to cyclic CO2 steps could not be measured due to rapid deterioration of IPC recording quality. In most animals, infusion of CdCl2 resulted in cardiac arrhythmia and death after ~10 min.

Although both CdCl2 and CoCl2 elevated IPC discharge, the effects of these blockers were not identical. With infusion of CoCl2, the increase in IPC discharge was seen during both the high- and low-CO2 portions of the CO2 stimulus cycle (Fig. 2B), in contrast to the effects of CdCl2, which were only apparent at high CO2. The difference in IPC discharge frequency between control and CoCl2 treatment is summarized in Fig. 2D and was smaller than the effect of CdCl2.

Series II: effects of nifedipine and BAY K 8644 on IPC discharge. The overall statistical fits of the repeated-measures ANOVA model (Eq. 1) to nifedipine and BAY K 8644 data were significant (nifedipine: $P < 0.0001$, $R^2 = 0.952$; BAY K 8644: $P < 0.0001$, $R^2 = 0.915$). Figures 4, 7, and 8 show that blockade of the L-type Ca2+ channels with nifedipine and activation of the L-type Ca2+ channels with BAY K 8644 exerted symmetrically opposing effects on IPC discharge. ANOVA indicated significant ($P < 0.0001$) main effects of nifedipine and BAY K 8644, indicating that both drugs modulated mean IPC discharge rate (nifedipine increased it by ~50%, BAY K 8644 decreased it by ~30%, see Fig. 7). ANOVA also indicated significant ($P < 0.0001$) interaction between nifedipine * CO2-time and BAY K 8644 * CO2-time, indicating that both drugs changed IPC CO2 sensitivity (nifedipine increased it by ~40%, BAY K 8644 decreased it by ~50%, see Fig. 8).

IPC discharge was increased at low CO2 following infusion of nifedipine and decreased at low CO2 following infusion of BAY K 8644. The average cycle-triggered stimulus histograms of IPC discharge before and after 1 mg/kg nifedipine infusion and before and after 2 mg/kg BAY K 8644 infusion are shown in Fig. 4, A and B. Following infusion of nifedipine, IPC discharge was increased at low CO2, but remained unchanged at high CO2 during the CO2 stimulus cycle. In addition, the relative amount of SFA was unaffected by nifedipine. The paired difference in IPC discharge frequency between control and nifedipine treatment is summarized in Fig. 4, C and E. In contrast to these effects, following infusion of BAY K 8644, IPC discharge was decreased at low CO2 and slightly elevated at high CO2 (i.e., BAY K 8644 exerted a slight excitatory effect at high CO2). BAY K 8644 also decreased SFA relative to control. The difference in IPC discharge frequency between control and BAY K 8644 treatment is summarized in Fig. 4, D and E. For direct comparison, the effects of nifedipine infusion and BAY K 8644 infusion on the paired changes in IPC discharge (drug-control) during the CO2 stimulus cycle are presented in Fig. 4E, emphasizing the nearly symmetrical opposing effects on IPC discharge. Infusions of nifedipine and BAY K 8644 were well tolerated by the animals, and the drugs produced stable changes in IPC response.

Series III: effects of CBTX, apamin, and niflumic acid on IPC discharge. The overall statistical fits of the repeated-measures ANOVA model (Eq. 1) to CBTX, apamin, and niflumic acid data were each significant (CBTX: $P < 0.0001$, $R^2 = 0.983$; apamin: $P < 0.0001$, $R^2 = 0.978$; niflumic acid: $P < 0.0001$, $R^2 = 0.951$). Blockade of BKCa, sKCa, and ClCa channels with CBTX, apamin, and niflumic acid, respectively, differentially affected IPC discharge (Fig. 5, KCa, Fig. 6, ClCa, also see Figs. 7 and 8). ANOVA showed significant main effects of CBTX ($P < 0.0001$) and niflumic acid ($P = 0.0003$), but no main effect of apamin ($P = 0.17$). The main effect results indicate that both CBTX and niflumic acid modulated mean IPC discharge rate (an 18% increase with CBTX, and a 10% decrease with niflumate), but apamin did not modulate mean IPC discharge rate. Interaction was seen for apamin * CO2-time ($P = 0.03$) and niflumate * CO2-time ($P = 0.0008$), indicating that apamin and niflumate changed IPC CO2 sensitivity (apamin slightly increased it, niflumate decreased it 30%). There was no interaction between CBTX * CO2-time, indicating that CBTX did not alter IPC CO2 sensitivity ($P = 0.88$).

The effect of CBTX was generally excitatory, while that of apamin was nearly imperceptible. Neither CBTX nor apamin significantly reduced SFA in IPC. The average cycle-triggered stimulus histograms of IPC discharge before and after 1–9 nmol/kg CBTX infusion and before and after 100 nmol/kg apamin infusion are shown in Fig. 5, A and B. The difference in IPC discharge frequency between control and CBTX treatment is summarized in Fig. 5, C and E. Also illustrated in Fig. 5E is the direct comparison of the changes in IPC discharge during the CO2 stimulus cycle observed for CBTX and apamin, emphasizing that CBTX had generally excitatory effects and that apamin’s effect was usually indistinguishable from control. Infusions of CBTX and apamin were both well tolerated by the animals.

In contrast to the effects of blockade of BKCa and sKCa channels, infusion of niflumic acid increased IPC discharge at high CO2 and decreased it at low CO2, thereby reducing the overall phasic response of IPC to CO2. The average cycle-triggered stimulus histograms of IPC discharge before and after 100 μmol/kg niflumic acid infusion is shown in Fig. 6A. The
difference in IPC discharge frequency between control and niflumic acid treatment is summarized in Fig. 6B.

Summary data. The magnitudes of the drug-induced changes in overall mean (±SE) IPC discharge for the different experimental series are summarized in Fig. 7. Figure 8 shows the average (±SE) maximum and minimum IPC discharge rate responses to step CO2 stimuli to illustrate any interaction of drug treatment with the CO2 response. Cadmium, cobalt, nifedipine, and BAY K 8644 treatments modulated mean IPC discharge by greater than ±20% (shown by the magnitude of the “main effect” of the drug, Fig. 7). Cadmium, nifedipine, BAY K 8644, and niflumic acid treatments produced greater than ±20% change in IPC CO2 sensitivity (shown by the magnitude of the “interaction effect” between drug and CO2 level, Fig. 8).

DISCUSSION
This study shows that influx of calcium through membrane ion channels modulates IPC response to phasic CO2 stimul-
In series I, IPC excitability increased in the presence of nonspecific Ca\(^{2+}\) channel blockers, but series I was complicated by the toxicity and nonspecificity of the heavy metal blockers CdCl\(_2\) and CoCl\(_2\) (see below). Therefore series II experiments further tested this observation using specific, relatively nontoxic dihydropyridine agents targeting L-type Ca\(^{2+}\) channels. Series II revealed that Ca\(^{2+}\) influx through L-type channels inhibits IPC excitation; mean IPC discharge rate and IPC CO\(_2\) sensitivity were augmented by L-type antagonist nifedipine and diminished by the L-type agonist BAY K 8644.

Series III experiments explored the role of Ca\(^{2+}\)-modulated ion channels in IPC excitability. Neither apamin (an sK\(_{Ca}\) channel blocker) nor CBTX (a BK\(_{Ca}\) channel blocker) influenced SFA in IPC, but CBTX produced a modest excitation of IPC without significantly affecting IPC CO\(_2\) sensitivity. Niflumic acid, a blocker of Cl\(^{-}\) channels, including calcium-modulated Cl\(^{-}\) channels, significantly reduced IPC CO\(_2\) sensitivity.

Taken together, these results reveal the physiological importance of Ca\(^{2+}\) ions for modulating IPC discharge and contributing to overall CO\(_2\) sensitivity. Further studies are needed to determine whether Ca\(^{2+}\) ions modulate IPC sensory end organs directly or modulate some unidentified synaptic input to IPC.

**Critique of systemic drug administration.** Single-unit IPC were studied in intact, anesthetized animals, and IPC discharge responses were measured before and after intravenous injection of various drugs that affect Ca\(^{2+}\)-related ion channels. Because...
the drugs were administered systemically, we could not limit their effect to the IPC in the lung. This was especially evident in series I experiments, because cadmium produced cardiovascular collapse ~10 min after infusion. We tried to avoid the complicating cardiovascular influences by recording from IPC within a few minutes of cadmium infusion. However, since IPC are sensitive to both ventilation and perfusion of the lung (11), a reduction in cardiac output (with the same level of unidirectional lung ventilation) would be expected to increase the relative importance of ventilation in determining the lung CO2 tension and the IPC discharge response. For example, when the pulmonary artery is ligated to completely occlude blood flow to a lung, IPC discharge typically becomes higher during low-inspired CO2 stimuli, and discharge becomes decreased more than usual during high-inspired PCO2 stimuli (11). This pattern was not seen with cadmium: it increased IPC discharge frequency during low-inspired CO2 but not high-inspired CO2, so the effect of cadmium on IPC is not attributable simply to changes in lung perfusion.

Future experiments that include ligation of the pulmonary artery after infusion of calcium channel blockers or agonists might help resolve some of the uncertainty about systemic influences on IPC response. Ligation would isolate the IPC from any circulatory changes.

Critique of drug dosages. None of these drugs or toxins had been used previously in avian IPC studies, and there were no literature references for IPC-relevant dosages. However, all had been used extensively for in vitro or in vivo studies in various other species (see discussion below), so we used those literature reports to estimate the body fluid concentrations of each drug that should produce blocking (or agonist) effects. Approximate drug dosages for ducks were then calculated on the basis of the animal’s body mass, making the simplifying assumption that body mass (in kg) was approximately equal to the volume of drug distribution (in liters). We reasoned this would place trial dosages in the approximate range. We then performed pilot experiments on anesthetized ducks (not shown), giving each drug over approximately a 10-fold concentration range that bracketed average literature reports of effective in vitro and in vivo dosages. In this way, we settled on the dosage that gave a measurable drug effect on IPC discharge and was within reported range of effective values from the literature. We have no specific information on the relative permeability of these drugs to IPC sensory endings, so uncertainty about pharmacologically appropriate dosages still exists.

Series I: effects of inorganic calcium channel blockers on IPC discharge. The divalent cations cadmium (Cd2+) and cobalt (Co2+) are typically used at micro- to millimolar concentrations to block Ca2+ channels in cell membranes, and usually in vitro rather than in vivo (6, 13, 15, 27, 30). In IPC, 100 μmol/kg CdCl2 and 1 mmol/kg CoCl2 increased mean action potential discharge rate by ~50 and 30%, respectively (Figs. 2 and 7), indicating that Ca2+ channel block increases IPC excitability. These data suggest that, under normal conditions, Ca2+ flows through cell membrane channels down their electrochemical gradient and decreases IPC afferent excitability. Since the influx of Ca2+, a cation with a positive Nernst equilibrium potential, would by itself cause membrane depolarization, the observed Ca2+-dependent inhibition must occur through other means. Two candidate mechanisms we considered were activation of KCa or ClCa channels. (series III).
Cadmium and cobalt are known neurotoxins, genotoxins, and carcinogens and, therefore, relatively nonselective Ca$^{2+}$ channel blockers. Although much of the acute neurotoxicity of cadmium and cobalt is related to their ability to interfere with Ca$^{2+}$ channel function, their other toxic effects are numerous. In our animals, we saw stimulation of IPC discharge immediately after injection of Cd$^{2+}$ and Co$^{2+}$, but Cd$^{2+}$ (not Co$^{2+}$) also caused cardiac arrhythmia and death after ~10 min. The immediate stimulation of IPC discharge by Cd$^{2+}$ and Co$^{2+}$ suggested that Ca$^{2+}$ channels may be involved in IPC signal transduction, but the general toxicity made the experimental results unclear. It was also unclear whether the different toxicological profiles of Cd$^{2+}$ and Co$^{2+}$ and the severe cardiac depression caused by Cd$^{2+}$ may have contributed to the different types of excitatory responses observed (Fig. 2). Because of these results and associated uncertainties about the specificity of Cd$^{2+}$ and Co$^{2+}$, we thought it was essential to follow up series I using much more specific and relatively nontoxic Ca$^{2+}$ channel modulators: nifedipine and BAY K 8644 (series II).

Comparison to other studies. There are no previous reports of the effects of Cd$^{2+}$ or Co$^{2+}$ or any other inorganic Ca$^{2+}$ channel blocker on IPC function, but heavy metal ions are common probes for investigating Ca$^{2+}$ channel function in many other chemoreceptors, especially in vitro. For example, treating neurons in rat locus ceruleus with 2 mM cobalt (or 50 μM nifedipine) blocks spiking from dendritic L-type Ca$^{2+}$ channels that may participate in primary CO$_2$ sensing (6). In carotid body glomus cells, 200 μM Cd$^{2+}$ (30) and >1 mM Co$^{2+}$ (15) essentially abolish Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels (predominantly L-type) during hypoxic or hypercapnic stimulation. Mouse sour-taste chemoreceptors, which depend on Ca$^{2+}$ influx through voltage-gated channels during signal transduction, are completely blocked by 500 μM Cd$^{2+}$ (27). Interestingly, although Cd$^{2+}$ blockade of calcium channels inhibits these other acid chemoreceptors, Cd$^{2+}$ blockade excited IPC. We think this difference may be related to the inverse acid/CO$_2$ sensitivity of IPC (hypocapnia excites, hypercapnia inhibits) relative to the other chemoreceptors.

Series II: effects of L-type calcium channel antagonist and agonist on IPC discharge. Series I results suggested that Ca$^{2+}$ channel blockers used were not very specific. Therefore, we investigated the effects of more specific organic probes of Ca$^{2+}$ channels: the dihydropyridine L-type channel antagonist nifedipine and the L-type agonist BAY K 8644 (series II).

Fig. 8. Bars show effect of indicated drug treatments on the magnitudes of CO$_2$-induced IPC discharge oscillations relative to control. Pretreatment controls are represented by solid bars, with normalized discharge rates ranging from 0% (minimum) to 100% (maximum). Drug treatments are represented by shaded bars, with minimum and maximum discharge rates expressed as a percentage of control. Dotted lines trace the changes in minimum, mean, and maximum discharge rates with drug treatment. Nonparallel dotted lines for some drug treatments (e.g., nifedipine) reflect the interaction between the drug and the CO$_2$ stimulus (i.e., a change in IPC CO$_2$ sensitivity with the drug). Error bars indicate SEs of minima and maxima.

Although both Cd$^{2+}$ and Co$^{2+}$ increased IPC discharge, Cd$^{2+}$ was the stronger stimulant, and it exerted its effects mainly during high CO$_2$. The stimulatory effect of Co$^{2+}$ was weaker, but persisted during both high CO$_2$ and low CO$_2$ (Fig. 1). The reason for the differences between the Cd$^{2+}$ and Co$^{2+}$ stimulation patterns was unclear but may be related to differences in Ca$^{2+}$ channel blocking efficacy, or to other toxic mechanisms unique to the different heavy metal ions. For example, the stimulation pattern caused by Cd$^{2+}$ was similar to the stimulation pattern caused by the carbonic anhydrase inhibitor acetazolamide (12), and we thought perhaps Cd$^{2+}$ might inhibit carbonic anhydrase enzyme in avian IPC, as has been reported in certain tissues of some other animals, like eels (16) and estuarian crabs (31). Since we could not directly assay carbonic anhydrase activity in IPC, we tested for carbonic anhydrase inhibition using an in vitro carbonic anhydrase activity assay on duck blood (17, 24). We found no significant inhibition of avian erythrocytic carbonic anhydrase by 100 or even 1,000 μmol/L Cd$^{2+}$ (data not shown), so it seemed unlikely (but not impossible) that CdCl$_2$ affected IPC discharge by impairing carbonic anhydrase.

Critique of series I. Cadmium and cobalt are known neurotoxins, genotoxins, and carcinogens and, therefore, relatively nonselective Ca$^{2+}$ channel blockers. Although much of the acute neurotoxicity of cadmium and cobalt is related to their ability to interfere with Ca$^{2+}$ channel function, their other toxic effects are numerous. In our animals, we saw stimulation of IPC discharge immediately after injection of Cd$^{2+}$ and Co$^{2+}$, but Cd$^{2+}$ (not Co$^{2+}$) also caused cardiac arrhythmia and death after ~10 min. The immediate stimulation of IPC discharge by Cd$^{2+}$ and Co$^{2+}$ suggested that Ca$^{2+}$ channels may be involved in IPC signal transduction, but the general toxicity made the experimental results unclear. It was also unclear whether the different toxicological profiles of Cd$^{2+}$ and Co$^{2+}$ and the severe cardiac depression caused by Cd$^{2+}$ may have contributed to the different types of excitatory responses observed (Fig. 2). Because of these results and associated uncertainties about the specificity of Cd$^{2+}$ and Co$^{2+}$, we thought it was essential to follow up series I using much more specific and relatively nontoxic Ca$^{2+}$ channel modulators: nifedipine and BAY K 8644 (series II).

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opposite chemosensitivity (where hypocapnia excites, hypercapnia inhibits) compared with locus ceruleus neurons (where hypocapnia inhibits, hypercapnia excites).

The excitation of IPC discharge produced by nifedipine was quantitatively similar to that elicited by Cd$^{2+}$ and Co$^{2+}$ (~30–50% increase in overall mean discharge, Fig. 7), suggesting that most of the Ca$^{2+}$ influx in IPC that can be blocked by Cd$^{2+}$ or Co$^{2+}$ may be carried through L-type Ca$^{2+}$ channels. Other voltage-gated Ca$^{2+}$ channel types in IPC, however, have yet to be investigated, including fast-inactivating Ca$^{2+}$ channels like P/Q-, N-, R-, and T-types. In carotid body glomus cells, dihydropyridine-sensitive L-type Ca$^{2+}$ channels carry most of the transmembrane Ca$^{2+}$ current, but other voltage-gated Ca$^{2+}$ channels are also expressed that help shape chemoreceptor response (15, 26).

**Critique of series II.** Nifedipine and BAY K 8644 were administered intravenously in 1% ethanol. Vehicle controls (Fig. 1B) showed that ethanol had no significant effect on IPC discharge. There is also considerable evidence in the literature that pentobarbital, which is the most commonly used anesthetic for IPC studies, and which is injected in a 10% ethanol vehicle, has no direct effects on IPC discharge (Fig. 1A and Ref. 11). In addition, the effects of nifedipine and BAY K 8644 on IPC discharge were opposite each other, even though both used the same dilute ethanol vehicle, supporting the interpretation that effects of these drugs were not due to the ethanol vehicle.

**Series III: effects of $K_{Ca}$ and $Cl_{Ca}$ channel blockers.** Since the results of series I and II supported a role for Ca$^{2+}$-dependent inhibition in IPC discharge, an effect that could be explained by calcium-linked activation of $K_{Ca}$ or $Cl_{Ca}$ channels, we investigated the function of BK$_{Ca}$, sK$_{Ca}$, and Cl$_{Ca}$ channels in IPC discharge.

Blockade of BK$_{Ca}$ channels by CBTX enhanced IPC response to the CO2 step stimulus. This is consistent with the idea that IPC excitation normally involves Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels, which activates BK$_{Ca}$ channels to provide a negative feedback limit on excitation (13). In some vertebrate CO2 chemoreceptors, including carotid bodies (22) and cultured medullary chemoreceptors (32), $K_{Ca}$ channels are inhibited by acid pH, and these channels have been implicated in CO2 chemosensitivity. However, our IPC data are more consistent with the BK$_{Ca}$ channels modulating mean IPC discharge and protecting IPC from overexcitation, rather than being the primary site of CO2 chemosensitivity. CBTX did not significantly affect CO2 sensitivity (i.e., the CBTX * CO2-time interaction was nonsignificant).

In contrast to the excitatory effect of blockade of BK$_{Ca}$ channels, blockade of sK$_{Ca}$ channels by apamin had no significant effect on mean IPC discharge rate, and a very small effect on IPC CO2 sensitivity, mostly at low CO2. In designing this experiment, we thought that SFA in IPC, which in many neurons is dependent on sK$_{Ca}$ channels (13), might be reduced or abolished by apamin. However, if anything, we saw slightly increased SFA with apamin, especially in those IPC that had little adaptation to begin with (Fig. 5, B, D, and E). Included within the mean responses of the five apamin-treated IPC shown in Fig. 5 were two IPC that showed 30–50% spike adaptation, and apamin also slightly increased adaptation of these IPC. We concluded that apamin-sensitive sK$_{Ca}$ channels do not contribute to IPC SFA. Other (apamin-insensitive) sK$_{Ca}$ channels (3), however, might contribute to IPC SFA; this requires further study.

Niflumic acid, a nonspecific blocker of chloride channels including Cl$_{Ca}$ channels, reduced the amplitude of IPC discharge during the CO2 step stimulus (Fig. 6). Niflumic acid inhibited IPC discharge at low CO2 when IPC were discharging most rapidly, and excited IPC at high CO2 when IPC were discharging more slowly. This brought IPC discharge closer to a median level during the CO2 stimulus cycle and reduced the extremes in IPC discharge rate (Figs. 6 and 8).

Significant interaction observed between niflumic acid and CO2 suggest that a Cl$^{-}$ current blocked by niflumic acid contributes to overall IPC CO2 sensitivity. The nfmolate-sensitive Cl$^{-}$ current appears to excite IPC during low CO2 and inhibit IPC during high CO2, but these results do not reveal the underlying mechanism. Two hypotheses seem testable for the future: 1) IPC may have H$^{+}$-modulated Cl$^{-}$ channels that are opened by acid (reducing excitability) and closed by alkalinity (increasing excitability), such that blockade of H$^{+}$-modulated Cl$^{-}$ channels by niflumic acid should reduce the amplitude of IPC response to CO2; and 2) blockade of Cl$^{-}$ channels by niflumic acid may reduce IPC response to CO2 by slowing the chloride shift through Cl$^{-}$ channels in the IPC cell membrane. IPC chemotransduction is known to be critically dependent on the carbonic anhydrase-catalyzed hydration-dehydration reaction: CO2 + H$_2$O $\leftrightarrow$ H$^+$ + HCO$_3^-$ Slowing the Cl$^{-}$/HCO$_3^-$ shift may slow this critical CO2 hydration-dehydration reaction. For example, during high CO2, rapid intracellular CO2 hydration would produce H$^+$ and HCO$_3^-$, and accumulating HCO$_3^-$ would likely move out of the cell while extracellular Cl$^{-}$ moves into the cell. During low CO2, intracellular HCO$_3^-$ would be rapidly converted to CO2, and extracellular HCO$_3^-$ would likely move into the cell in exchange for intracellular Cl$^{-}$ moving out of the cell. In either case, blocking Cl$^{-}$ channels and impairing the chloride shift might, by the law of mass action, slow the CO2 hydration-dehydration reaction by causing an intracellular deficit or excess of HCO$_3^-$.

**Critique of series III.** Repeated-measures ANOVA allows statistical detection of relatively small treatment-induced changes in IPC discharge, if the changes are consistent among IPC, just as a paired t-test offers greater detection power than an unpaired t-test. However, to determine biological significance, it is also important to consider the magnitude of the observed responses and how the responses may contribute to IPC function (Figs. 7 and 8). The IPC responses to apamin were small and relatively insignificant from a biological perspective; however, because apamin did not affect SFA, the hypothesis that sK$_{Ca}$ channels underlie IPC SFA was tested and seems unlikely. The effects of CBTX and niflumic acid on IPC discharge were much larger than the apamin effects, which indicated that their biological roles in IPC excitability are probably more important. The results suggest that BK$_{Ca}$ channels provide feedback inhibition of cellular excitability that may function to prevent IPC overexcitation at low Pco2, while niflumic acid-sensitive Cl$^{-}$ channels in IPC seem to contribute to CO2 sensitivity.

**Summary.** Our experiments show that transmembrane calcium influx through nifedipine and BAY K 8644-sensitive L-type Ca$^{2+}$ channels inhibits IPC discharge, but has no effect on IPC SFA. Our experiments suggest that Ca$^{2+}$-linked modulation of IPC discharge arises from several sources, including
CBTX-sensitive K\textsubscript{Ca} and niflumic acid-sensitive Cl\textsuperscript{-} channels, and most likely other Ca\textsuperscript{2+}-sensitive sources that remain to be investigated. Recent reviews of mammalian central and peripheral chemoreceptors (other than IPC) also suggest a modulatory role for calcium in CO\textsubscript{2} chemotransduction, which is consistent with the idea that multiple intracellular sites contribute to overall CO\textsubscript{2} sensitivity in respiratory chemoreceptors (14, 26). Although the modulatory effects of Ca\textsuperscript{2+} presented here for IPC are, in many cases, opposite those seen in carotid bodies and some central chemoreceptors, it is very likely that these differences reflect the evolutionary adaptation of IPC to their function in the avian lung, i.e., their marked inverse CO\textsubscript{2} sensitivity (hypocapnia excites, hypercapnia inhibits) and their extremely rapid phasic responsiveness (11, 18).

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