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Effects of Almitrine Bismesylate on the Ionic Currents of Chemoreceptor Cells from the Carotid Body

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ABSTRACT

Almitrine is a drug used in the treatment of hypoxemic chronic lung diseases such as bronchitis and emphysema because it is a potent stimulant of the carotid bodies in human and different animal species that produces a long-lasting enhancement of alveolar ventilation, ameliorating arterial blood gases. However, the mechanism of action of almitrine remains unknown. We investigated the effect of almitrine on ionic currents of chemoreceptor cells isolated from the carotid body of rat and rabbits by using the whole-cell and inside-out configurations of the patch-clamp technique. Almitrine at concentrations up to 10 μM did not affect whole-cell voltage-dependent K^+, Ca^{2+}, or Na^+ currents in rat or rabbit cells. However, this concentration of almitrine significantly inhibited the Ca^{2+}-dependent component of K^+ currents in rat chemoreceptor cells. This effect of almitrine on the Ca^{2+}-dependent component of K^+ currents was investigated further at the single-channel level in excised patches in the inside-out configuration. In this preparation, almitrine inhibited the activity of a high-conductance (152 ± 13 pS), Ca^{2+}-dependent K^+ channel by decreasing its open probability. The IC_{50} value of the effect was 0.22 μM. The inhibitory effect of almitrine on Ca^{2+}-dependent K^+ channels also was observed in GH3 cells. We conclude that almitrine inhibits selectively the Ca^{2+}-dependent K^+ channel and that in rat chemoreceptor cells, this inhibition could represent an important mechanism of action underlying the therapeutic actions of the drug.

The CB is an arterial chemoreceptor origin of ventilatory reflexes directed to maintain blood levels of O_2, CO_2, and H^+ under physiological limits. Chemoreceptor cells are the CB elements that sense blood P_{O_2} and $P_{CO_2}/[H^+]$, being activated when P_{O_2} decreases and $P_{CO_2}/[H^+]$ increases. Activated chemoreceptor cells release neurotransmitters in amounts that are proportional to the decrease in P_{O_2} and to the increase in P_{CO_2}/H^+; parallel increases in the action potential frequency of the sensory nerve of the CB and in ventilation follow (Gonzalez *et al.*, 1994).

The coupling of the decrease in P_{O_2} to the exocytotic machinery responsible for the release of neurotransmitters in chemoreceptor cells (i.e., the chemotransduction process) is incompletely understood, but it is well documented that plasma membrane mechanisms are involved. The presence in rabbit chemoreceptor cells of O_2-sensitive K^+ channels (López-Barneo *et al.*, 1988; López-López *et al.*, 1989), whose open probability decreases as a function of P_{O_2} (Ganfornina and López-Barneo, 1991), led to the proposal that hypoxia could control the excitability of the cells, causing cell depolarization, activation of Na^+ and Ca^{2+} channels, an increase in $[Ca^{2+}]_i$, and release of neurotransmitters (Gonzalez *et al.*, 1992; Gonzalez *et al.*, 1994). O_2-sensitive K^+ channels also have been found in neonatal (Peers, 1990; Buckler, 1997) and adult (Hatton *et al.*, 1997; López-López *et al.*, 1997) rat chemoreceptor cells, and a similar transduction sequence has been proposed. The detailed characterization of the different CB chemoreceptor cell preparations showed some discrepancies that have been reported to be mainly species related [see López-López and Peers (1997) for a review]. In particular, O_2-sensitive K^+ channels seem to be different between rabbit and rat chemoreceptor cells. Although hypoxia inhibits a transient voltage-dependent I_K in rabbit cells (López-Barneo *et al.*, 1988), the O_2-sensitive currents in rats are both a ChTX-sensitive Ca^{2+}-dependent I_K (Peers, 1990; Wyatt and Peers, 1995; López-López *et al.*, 1997) and a leak I_K (Buckler, 1997).

In patients with chronic respiratory failure, acute exacerbations brought about by respiratory infections may further impair their blood gas levels. Treatment with central respi-

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ABBREVIATIONS: CB, carotid body; BK, large-conductance Ca^{2+}-dependent K^+ channel; ChTX, charybdotoxin; TTX, tetrodotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I-V, current-voltage; I_{Na}, Na^+ current(s); I_{Ca}, Ca^{2+} current(s); I_K, K^+ current(s); IK_{Ca}, Ca^{2+}-activated component of I_K; I_{peak}, maximal amplitude of K^+ current; $125K_i$, intracellular K^+ solution; IK_V, voltage-dependent component of I_K.

ratory stimulants provides limited clinical success and many side effects; long term oxygen therapy, being more successful, is both expensive and hard for patients. A third possibility to improve oxygenation to the blood is to stimulate the CBs with a drug such as almitrine bismesylate, which improves ventilation without central nervous system disturbances. The effect of almitrine enhancing alveolar ventilation through stimulation of the CB has been reported in several studies (Laubie and Schmitt, 1980; Bisgard, 1981; McQueen *et al.*, 1989; Lahiri *et al.*, 1989), but its mechanism of action remains unknown. It was reported recently that almitrine produces a long-lasting increase in the release of catecholamines from chemoreceptor cells in resting normoxic conditions and potentiates low P_{O_2}-induced catecholamine release (Almaraz *et al.*, 1992). It also has been shown that almitrine inhibits I_K in neonatal rat cells (Peers and O'Donnell, 1990), although this effect has not been characterized. These results led to the suggestion that the O_2-sensitive K^+-channels could be possible targets for almitrine in chemoreceptor cells (Almaraz *et al.*, 1992).

The aim of the current work was to characterize the effects of almitrine on the ionic currents of rabbit and rat chemoreceptor cells. After previous suggestions, our main thrust was the description of the effects of almitrine on O_2-sensitive K^+ channels, but possible effects on other ionic currents of chemoreceptor cells also were studied to uncover additional potential targets for the drug. Due to the well-documented species-related differences in the properties of ionic currents from CB chemoreceptor cells (López-López and Peers, 1997), the effects of almitrine on I_{Na}, I_K, and I_{Ca} were studied in freshly or acutely cultured cells isolated from adult rabbits or rats using the whole-cell configuration of the patch-clamp technique. Almitrine did not modify voltage-dependent I_{Na}, I_K, or I_{Ca} from rabbit or rat cells. However, almitrine inhibited the Ca^{2+}-dependent component of the I_K recorded from rat cells in the whole-cell configuration. This selective effect was characterized further at the single-channel level in membrane patches excised from rat chemoreceptor cells.

Materials and Methods

Cell isolation and culture. Experiments were performed on cultured rat and rabbit CB chemoreceptor cells. Adult Wistar rats (3–4 months old) or adult New Zealand White rabbits (1.5–2 kg) were anesthetized with pentobarbital sodium (100 mg/kg administered intraperitoneally to the rats or 40 mg/kg administered through the lateral vein of the ear to the rabbits). After tracheostomy, the carotid artery bifurcations were removed, and the animals were killed by an intracardiac bolus injection of pentobarbital sodium. The CBs were cleaned of surrounding connective tissue and enzymatically dispersed as described previously (Pérez-García *et al.*, 1992; López-López *et al.*, 1997). Dispersed cells were plated onto small poly-L-lysine-coated coverslips and maintained in culture for up to 36 hr.

Electrophysiological recording. Ionic currents were recorded at room temperature (20–25°) using the whole-cell and inside-out modes of the patch-clamp techniques (Hamill *et al.*, 1981). Whole-cell current recordings and data acquisition were made as described previously (López-López *et al.*, 1997). Patch pipettes used for single-channel recordings were made from borosilicate glass (0.8 mm; World Precision Instruments, New Haven, CT) and double-pulled (Narishige PP-83) and heat-polished (Narishige MF-83) to resistances of 12–20 MΩ when filled with the internal solution.

Recordings were made with an Axopatch-200A patch-clamp amplifier and a Digidata 1200 A/D interface, driven by pCLAMP version 6.02 software (Axon Instruments, Burlingame, CA) with a Pentium computer. Single-channel records were filtered at 1 kHz and digitized at 10 kHz.

Analysis. Analysis of the data was performed with the CLAMPFIT and FETCHAN subroutines of the pCLAMP software. Single-channel amplitudes and open probabilities were measured from amplitude histograms generated with FETCHAN. The amplitude histograms consisted of 256 bins with each bin containing the number of sample points falling within the bin width. The amplitude of the single-channel currents was taken as the difference between the peaks for opened and closed currents levels. Because most of the patches had multiple channels, open probabilities were expressed as *N*Po, where *N* represents the number of single channels present in the patch, and Po represents the open probability of a single channel. *N*Po was calculated using the following expression (Kajioka *et al.*, 1991):

$$NPo = (A_1 + 2A_2 + 3A_3 + \ldots nA_n)/(A_0 + A_1 + A_2 + \ldots A_n)$$

where A_0 is the area under the curve of the amplitude histogram corresponding to current in the closed state, and $A_1 \ldots A_n$ represents the histogram areas reflecting the different open-state current levels for 1 to *n* channels present in the patch. Histogram parameters were obtained from multiple least-squares gaussian fits of the data using ORIGIN 4.0 software (MicroCal, Northampton, MA).

When pooled data are shown, they are expressed as mean ± standard error. Statistical comparisons were performed with the two-tailed *t* test for paired or unpaired data as appropriate, and values of $p < 0.05$ were considered statistically different.

Solutions. The compositions of the bathing and pipette solutions for all recording conditions are given in Table 1. Gigaseals were formed in the standard extracellular solution ($standard_e$). Whole-cell I_K were recorded in this same extracellular solution with $125K_i$ in the pipette. When we studied I_{Na} or I_{Ca}, the $0K_i$ solution was used in the pipette; for I_{Ca}, the external solution was

TABLE 1
Composition of recording solutions

	$Standard_e$	$10Ca_e$	$10Ba_e$	$125K_i$	$0K_i$	0Ca	1μMCa	10μMCa
NaCl	141	140	140					
CsCl					130			
KCl	4.7	2.7	2.7	125		125	125	125
$CaCl_2$	1.8	10					8.46	10.9
$BaCl_2$			10					
$MgCl_2$	1.2			4				
HEPES	10	10	10	10	10	10	10	10
Glucose	10	10	10					
EGTA				10	10	11.25	11.25	11.25
MgATP				5	4			
LiGTP					2			
pH	7.42 (NaOH)	7.42 (NaOH)	7.42 (NaOH)	7.2 (KOH)	7.2 (CsOH)	7.2 (KOH)	7.2 (KOH)	7.2 (KOH)

switched to 10 Ca_e (or 10 Ba_e), and in some experiments TTX was added to block I_{Na}.

For the solutions used in inside-out patch experiments, careful attention was paid to hold the Ca^{2+} concentration facing the internal side of the channel at fixed levels. Ca^{2+} chemical activity was fixed to the values indicated by buffering with EGTA according to the software CHELATOR (Schoenmakers *et al.*, 1992). Titrated stock solutions of $CaCl_2$ and EGTA were used to minimize errors due to impurities of EGTA and hydration of $CaCl_2$.

Chemicals and drugs. All chemicals used in pipette and bath solutions were obtained from Sigma Chemical (St. Louis, MO). ChTX (Alomone Labs, Jerusalem, Israel) was used as described previously (López-López *et al.*, 1997). Almitrine bismesylate [1-(4′,6′-diallylamino-2′-triazinyl)-4-(bis-4, 4′-fluorobenzydryl)piperazyne bismethane sulfonate; Vectarion, Servier International, Paris, France] was prepared in a 4.5 mM stock, with a solvent of a solution of 45 mM malic acid. At the highest concentration used (0.1 mM), malic acid alone had no effect on whole-cell ionic currents from chemoreceptor cells (data not shown)

Results

Effects of almitrine on ionic currents from rat chemoreceptor cells. The effect of almitrine (10 μM) on whole-cell I_K from rat CB chemoreceptor cells is shown in Fig. 1. After establishment of the whole-cell configuration, I-V relationships for IK were obtained every minute, with the application of groups of 200-msec depolarizing steps from −60 to +100 mV in 10-mV steps. The holding potential was −60 mV. After several minutes under control conditions, almitrine (10 μM) was applied for 5 min, and the recording continued for 6 min after removal of the drug. Peak current values obtained at +20 and +90 mV in one representative cell are plotted against time in Fig. 1a. The slope of the usual decay of I_K amplitude at +20 mV (△), due to washout of I_{Ca} (see López-López *et al.*, 1997), sharply increased after the application of almitrine 10 μM (Fig. 1, *hatched rectangle*). The effect of almitrine on the current elicited at +90 mV was much less pronounced. In the 6-min period after the removal of the drug from the bathing solution, there was no recovery from inhibition. Fig. 1b shows sample records and the whole I-V relationships obtained before (time 2 min) and after (time 12 min) application of the drug. It is evident that the prominent hump in the I-V curve, which is due to the activation of Ca^{2+}-dependent K^+ channels (Peers, 1990; López-López *et al.* 1997), disappeared in the presence of almitrine. Results obtained in 11 cells with the same protocol were averaged and presented in Fig. 1c as percentage of inhibition produced by the application of 10 μM almitrine. In seven cells (●), the inhibition was clearly voltage dependent, being maximal between the range of potentials of activation of Ca^{2+} channels (0–20 mV; $p < 0.001$ at 0 mV). In four cells (○), there was almost no effect of almitrine in the entire range of tested voltages; two of these four cells did not exhibit a clear hump in their I-V relationships, but we do not have any explanation for the lack of effect of almitrine in the other two cells with a clear Ca^{2+}-activated component. The voltage dependence of the inhibition strongly suggested that almitrine effectively inhibited IK_{Ca} in rat cells; indeed, the effect of the abolishment by almitrine of the hump of the I-V relationship is comparable to that observed with the IK_{Ca} blocker ChTX (Peers, 1990; López-López *et al.*, 1997). To confirm the specificity of almitrine on IK_{Ca}, we studied the effect of the drug on I_K recorded in the presence of 20 nM ChTX. Fig. 2 shows the I_{peak} at +20 mV elicited by voltage ramps (0.023 mV/ms) from −60 to +100 mV applied every 30 sec. This ramp protocol was used instead of step depolarizations to enhance the Ca^{2+}-dependent component of I_K (López-López *et al.*, 1997). The application of ChTX produces a marked decrease of the current amplitude that is maximal at voltages between +10 and +20 mV (Fig. 2, *inset*). In the presence of ChTX, almitrine did not modify I_K, suggesting a common target (IK_{Ca}) for the inhibitory effect of the two drugs. The same lack of effect of almitrine on ChTX treatment was observed in an additional three cells. However, because IK_{Ca} decayed slowly along the experiments (Fig. 1a, initial 4 min and △; see also López-López *et al.*, 1997) and because the washout of almitrine seemed to be very slow, it was very difficult to quantify the inhibition due to the drug; the two effects (current decay and current inhibition) combine in an apparently irreversible fashion. Moreover, the effect of almitrine can be due to a direct inhibition of Ca^{2+}-activated K^+ channels, an

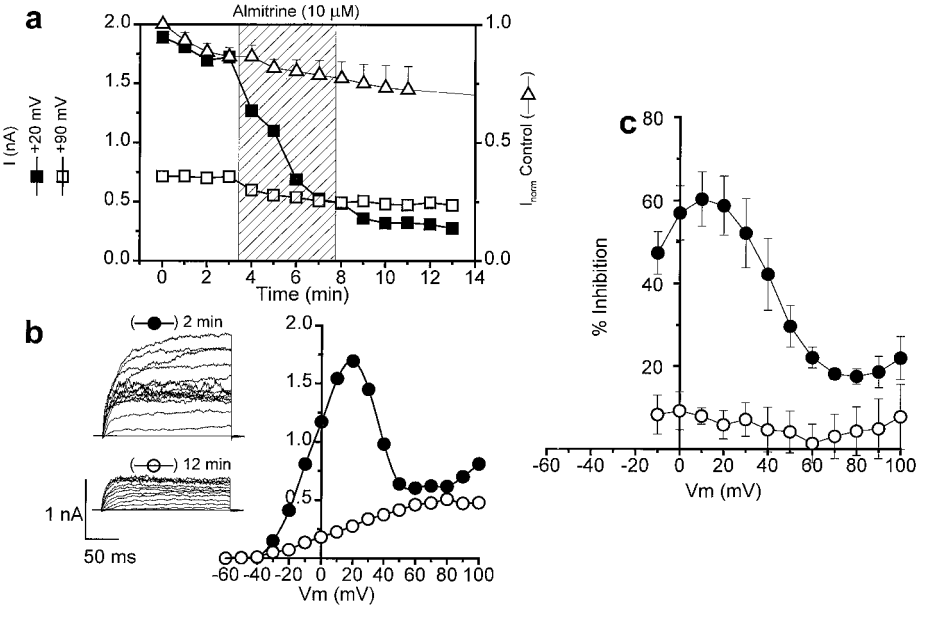

Fig. 1. Effect of almitrine (10 μM) on whole-cell I_K from rat chemoreceptor cells. Families of I_K at different potentials were obtained every minute, and (a) peak currents measured at +20 and +90 mV are plotted against the time when the first pulse of the corresponding family was applied. *Hatched area*, application of almitrine. The averaged normalized decay of the current amplitude at +20 mV due to the washing-out of I_{Ca} for four control cells also is plotted in the figure. b, Current families and I-V relationships corresponding to the families recorded at times 2 and 12 min. c, Percentage of inhibition at potentials over −10 mV was computed according to the expression: Inhibition (%) = $100 \cdot (I_{control} - I_{Almitrine})/I_{control}$. A total of 11 cells were studied and grouped according the shape of the voltage dependence of the inhibition (●, seven cells; ○, four cells). The effect of washing-out was not corrected. Solutions: $standard_e$, $125K_i$.

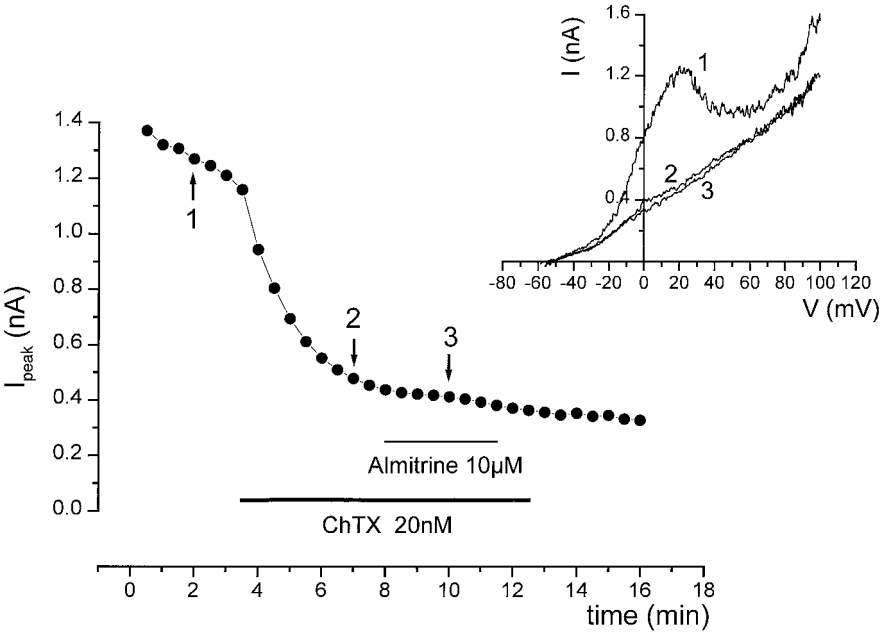

Fig. 2. Effect of almitrine (10 μM) on whole-cell I_K from rat chemoreceptor cells after ChTX application. Peak current amplitude at +20 mV obtained from 7-sec depolarizing ramps from −60 to +100 mV is plotted against time. *Solid bars*, periods of time in which the indicated drugs (ChTX or almitrine) were present in the bath solution. *Inset*, I-V relationships for the ramps taken at the times indicated (*arrows 1*, control; *2*, 20 nm ChTX; *3*, 20 nM ChTX plus 10 μM almitrine). Currents were not leak-subtracted. Solutions: standard$_e$, 125K$_i$.

inhibition of the entry of Ca^{2+} through the Ca^{2+} channels, or both.

This latter possibility was tested by studying the effect of almitrine on whole-cell I_{Ca}. The effect of almitrine on I_{Ca} in rat chemoreceptor cells is shown in Fig. 3. Families of I_{Ca} were obtained through the application every 2 min of a group of 7-msec depolarizing pulses from a holding potential of −80 mV to +60 mV in 10-mV steps. I_K were blocked with Cs^+ in the pipette (solution 0K$_i$), and I_{Ca} were maximized with 10 mM Ba^{2+} in the bath (solution 10 Ba$_e$; see Table 1). Fig. 3a shows a typical experiment in which current amplitudes were measured immediately before the end of pulses to three different potentials and plotted against time. Almitrine (10 μM) was applied for 5 min (*hatched bar*). Actual records at the indicated times illustrating the progressive run-down of the currents also are shown; again, almitrine did not modify the time course of this rundown. Mean I-V relationships obtained in five cells during the application of almitrine were normalized to the averaged control and recovery I-V curves and are shown in Fig. 3b. TTX was not present in the bath solution in these recordings, but the observation that the currents were completely blocked by 100 μM Cd^{2+} excludes the possibility of any contaminating I_{Na} in our records, confirming previous reports that indicate that Na^+ channels are either absent in rat CB chemoreceptor cells or present in only a low percentage (López-López *et al.*, 1997).

Effects of almitrine on rat Ca^{2+}-activated K^+ channels. The inhibition of the Ca^{2+}-dependent component of IK and the lack of effect of almitrine on I_{Ca} strongly suggested that Ca^{2+}-activated K^+ channels were in fact the targets for the action of the drug. This possibility was tested by studying the effect of almitrine on the activity of single Ca^{2+}-activated K^+ channels present in excised chemoreceptor cell membrane patches and recorded in the inside-out configuration of the patch-clamp technique. It is well known that Ca^{2+}-activated K^+ channels present in rat cells are mainly ChTX-sensitive BKs (Peers and Buckler, 1995; Wyatt and Peers, 1995; López-López *et al.*, 1997). BK channels in the isolated patches were identified in this study on the basis of their voltage dependence, large conductance, and Ca^{2+} sensitivity (Fig. 4). In asymmetrical solutions (4.7 mM K^+ at internal membrane face, 125 mM K^+ at external membrane face), the I-V relationship of the isolated channels showed some inward rectification (Fig. 4a, □), which is well described by the Goldman-Hodgkin-Katz current equation. The extrapolated reversal potential was close to the K^+ equilibrium potential, which was +84 mV in these experiments. When the bath solution was changed and single-channel currents were recorded under symmetrical conditions (125 mM K^+ at both sides of the membrane), the rectification disappeared and the reversal potential shifted to 0 mV, which is as expected for a K^+-selective channel (Fig. 4a, △). Fig. 4a also shows the averaged I-V relationships obtained with symmetrical high K^+ conditions from different patches (●). The average slope conductance under these conditions was 152 ± 13 pS (five cells).

The other requirement used to classify these high-conductance K^+ channels as BK channels was their dependence on bath ("intracellular") Ca^{2+}, as shown in Fig. 4b. Channel activity was recorded under symmetrical K^+ conditions (125 mM) at +60 mV with 0, 1, or 10 μM Ca^{2+} in the bathing solution. *N*Po in each situation (see Materials and Methods) was calculated from all-points histograms generated in all cases from ≥4-min recording of channel activity. Mean *N*Po values of 4–12 patches at the three Ca^{2+} concentrations are represented in the figure. Although the Ca^{2+} dependence of the channels has not been characterized thoroughly, it is evident that channels were almost silent in 0 Ca^{2+} and that on increasing Ca^{2+} concentration, channel activity increased markedly.

When 10 μM almitrine was added to the bathing solution, BK activity recorded under symmetrical K^+ conditions at +60 mV clearly was inhibited, in both 1 μM and 10 μM Ca^{2+} (Fig. 5). Lower doses of the drug (0.01 μM) or malic acid (0.1 mM) did not affect the activity of the channels (Fig. 5). This malic acid concentration corresponds to that in the bathing solution with 10 μM almitrine.

The dose dependence of the effect of almitrine on BK

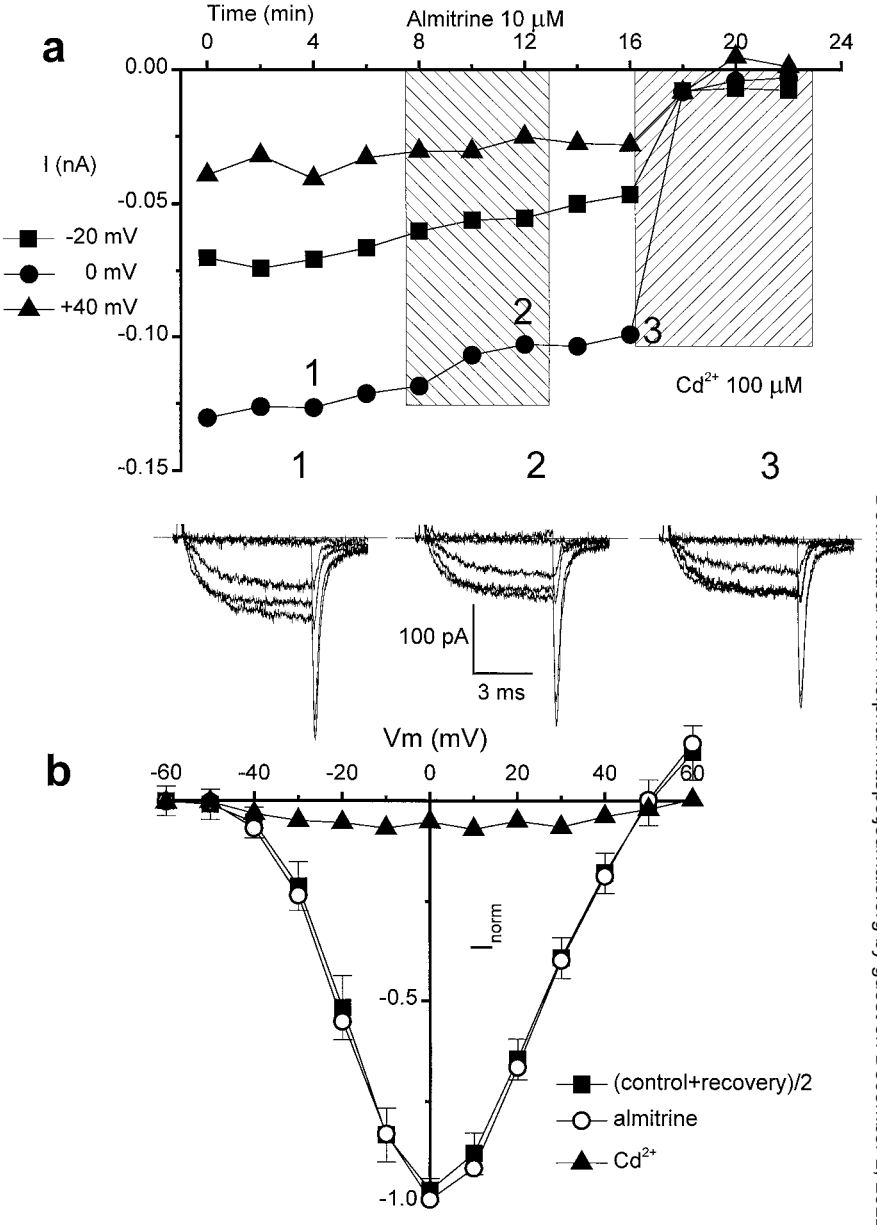

Fig. 3. Effect of 10 μM almitrine on whole-cell I_{Ca} from rat chemoreceptor cells. a, Families of I_{Ca} at different potentials were obtained every 2 min, and peak currents measured at −20, 0, and +40 mV were plotted against the time when the first pulse of the corresponding family was applied. *Hatched area*, application of almitrine or Cd^{2+} 100 μM. Whole-cell currents were obtained with pulses to −40, −20, 0, +20, and +40 mV during the families *1–3*. b, I-V relationships obtained during the application of almitrine or Cd^{2+} and normalized against the average of those obtained in control and recovery conditions. Values are mean ± standard error from five cells. Solutions: $10Ba_e$, $0K_i$.

channel activity was explored further in several patches. The holding potential was +60 mV, and the Ca^{2+} concentration in the bath was kept at 1 μM. All-point histograms obtained during 4-min periods at different concentrations of almitrine in a single patch are shown in Fig. 6a. The concentration of almitrine was increased progressively from 0.1 to 10 μM in this particular case. At 8–12 min after removal of the drug, the channel activity recovered almost completely. Due to this slow recovery, washout of the drug could not be detected in the whole-cell experiments; run-down of IK_{Ca} usually is faster. Also evident in Fig. 6a is the fact that almitrine inhibited channel activity decreasing the open probability without affecting the channel conductance. The relationship between the normalized channel activity (*N*Po in the presence of almitrine divided by *N*Po under control conditions) and the concentration of almitrine obtained in several different patches are shown in Fig. 6b. The continuous curve was drawn by fitting all data to the following equation:

$$\frac{NPo}{NPo_{control}} = \frac{1}{1 + \left(\frac{IC_{50}}{[Almitrine]}\right)^{n}}$$

where IC_{50} and n were 0.22 μM and 0.68, respectively. Importantly, the mean blood levels attained with therapeutic doses of almitrine in patients have been estimated to be ~0.3 μM, which is very close to the IC_{50} value obtained in the current study (Campbell *et al.*, 1983).

We further characterize the inhibition of BK channels by almitrine by considering its dependence of the intracellular Ca^{2+} concentrations. Fig. 7 shows the percentage of decrease of *N*Po in the presence of 10 μM almitrine at two intracellular Ca^{2+} concentrations: 1 and 10 μM. Although this concentration of almitrine almost completely blocks BK channels recorded in 1 μM Ca^{2+}, there is only a 39% inhibition when the

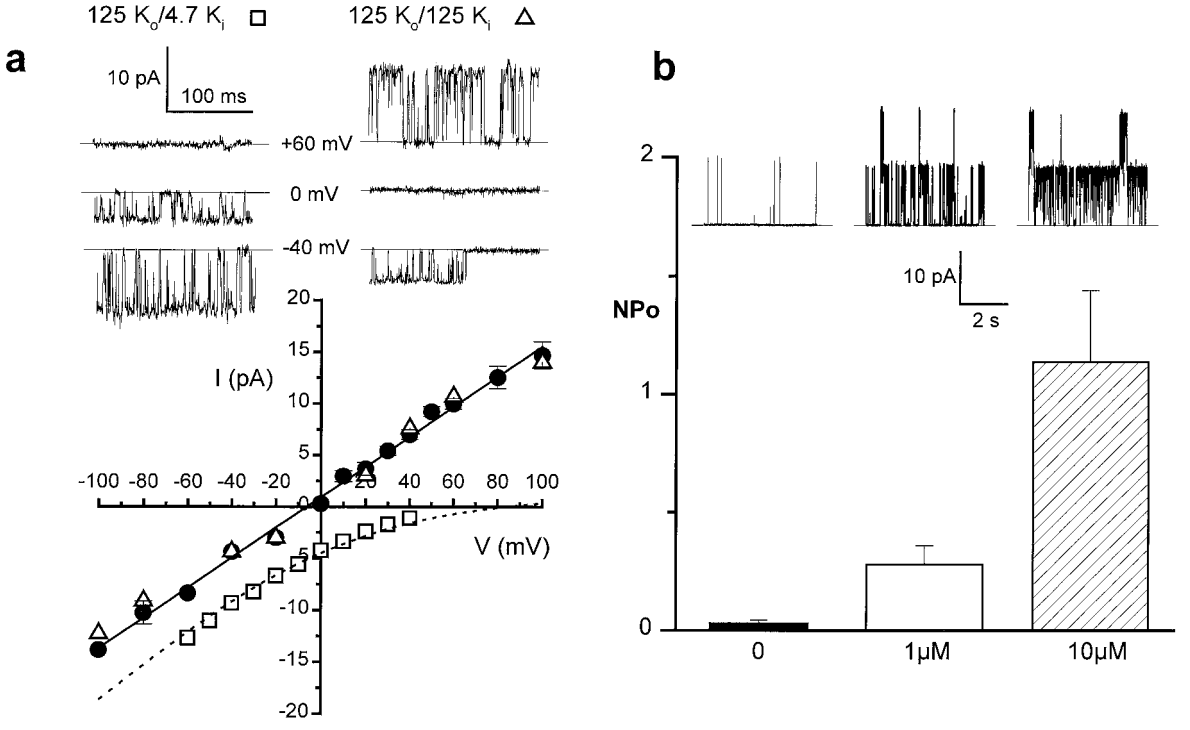

Fig. 4. High-conductance K^+ channels in inside-out excised patches. Ca^{2+} dependence. a, Current amplitudes were measured in a single patch at different potentials in 125K_o/4.7K_i [solutions: standard$_e$ (bath)/0Ca (pipette)] and in symmetrical high K^+ [solutions: 1 μM Ca (bath)/0Ca (pipette)]. *Top*, sample recordings at three different potentials in the two conditions. *Solid line*, closed level. *Downward deflections*, inward current. *Upward deflections*, outward current. *Bottom*, full I-V relationships under the two conditions. *Dotted line*, I-V relationship predicted by the Goldman-Hodgkin-Katz equation. Mean ± standard error values obtained in four different patches in symmetrical K^+ also are shown (●). *Solid line*, linear fit to average data. b, Ca^{2+} dependence of high-conductance K^+ channels. *Top*, sample currents corresponding to 5-sec recordings obtained from a single excised patch (inside-out) with 0, 1, or 10 μM Ca^{2+} in the bath solution; *solid line*, closed level. The patch holding potential was +60 mV. The patch was held in each condition for >4 min, and that time of recording was used to calculate *N*Po for each condition according to the equation in Materials and Methods. *N*Po in this patch amounted to 0.03, 0.31, and 0.95 when recording in solutions 0Ca, 1μMCa, and 10μMCa, respectively. Mean *N*Po values obtained in the three conditions also are plotted. Each value represents the mean ± standard error of 4–12 patches.

Ca^{2+} concentration is raised to 10 μM. Also illustrated in the figure is our observation that the effect of almitrine on BK channels in rat chemoreceptor cells is not tissue specific; BK channels recorded from GH3 cells also are inhibited by almitrine to a very similar extent. Furthermore, this effect of almitrine on BK channel activity in GH3 cells shows the same Ca^{2+} dependence.

Effects of almitrine on ionic currents from rabbit chemoreceptor cells. The effect of almitrine (10 μM) on whole-cell voltage-dependent currents of rabbit CB chemoreceptor cells is shown in Fig. 8. Voltage-dependent I_K were studied in seven cells. A protocol similar to the one described above for I_K in rat cells was used, but current families were obtained every 2 min. For each cell, the I-V relationship was obtained by determining I_{peak} and the amplitude just before the end of the 200-msec pulses (I_{ss}). I-V relationships obtained before, during, and after the application of 10 μM almitrine were normalized to the peak current at +80 mV in control conditions (i.e., before the application of the drug), and the results obtained with the seven cells (mean ± standard error) are represented in Fig. 8a. In the presence of almitrine, there is a small reduction in the current amplitude at very depolarized values (>+40 mV). However, this reduction is due to the rundown of the currents along the experiment, and its time course is the same in almitrine-treated and untreated cells. Traces in Fig. 8a show the I_K elicited by depolarizing pulses to +40 mV before, during, and after the application of almitrine in one of the studied cells.

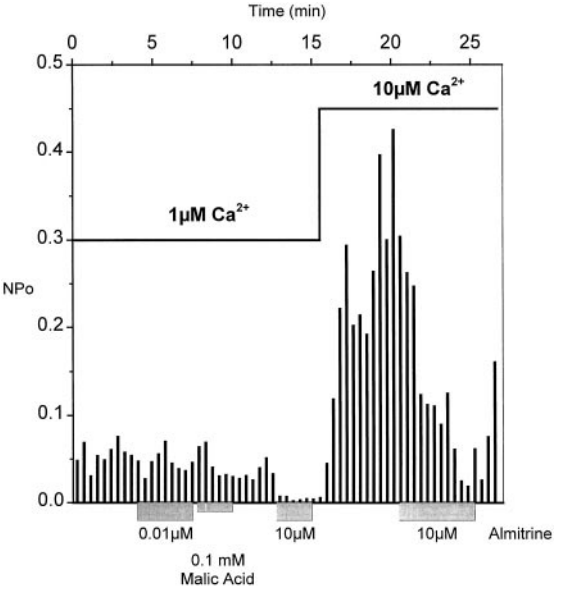

Fig. 5. Effect of almitrine on Ca^{2+}-dependent K^+ channel activity. *N*Po was measured every 20 sec and plotted against time in an excised patch with at least two channels present. Channel activity was recorded in symmetrical K^+, with 1 or 10 μM Ca^{2+} in the bath solution as indicated. Almitrine (0.01 or 10 μM) was applied when marked. The effect of 0.1 mM malic acid also was tested.

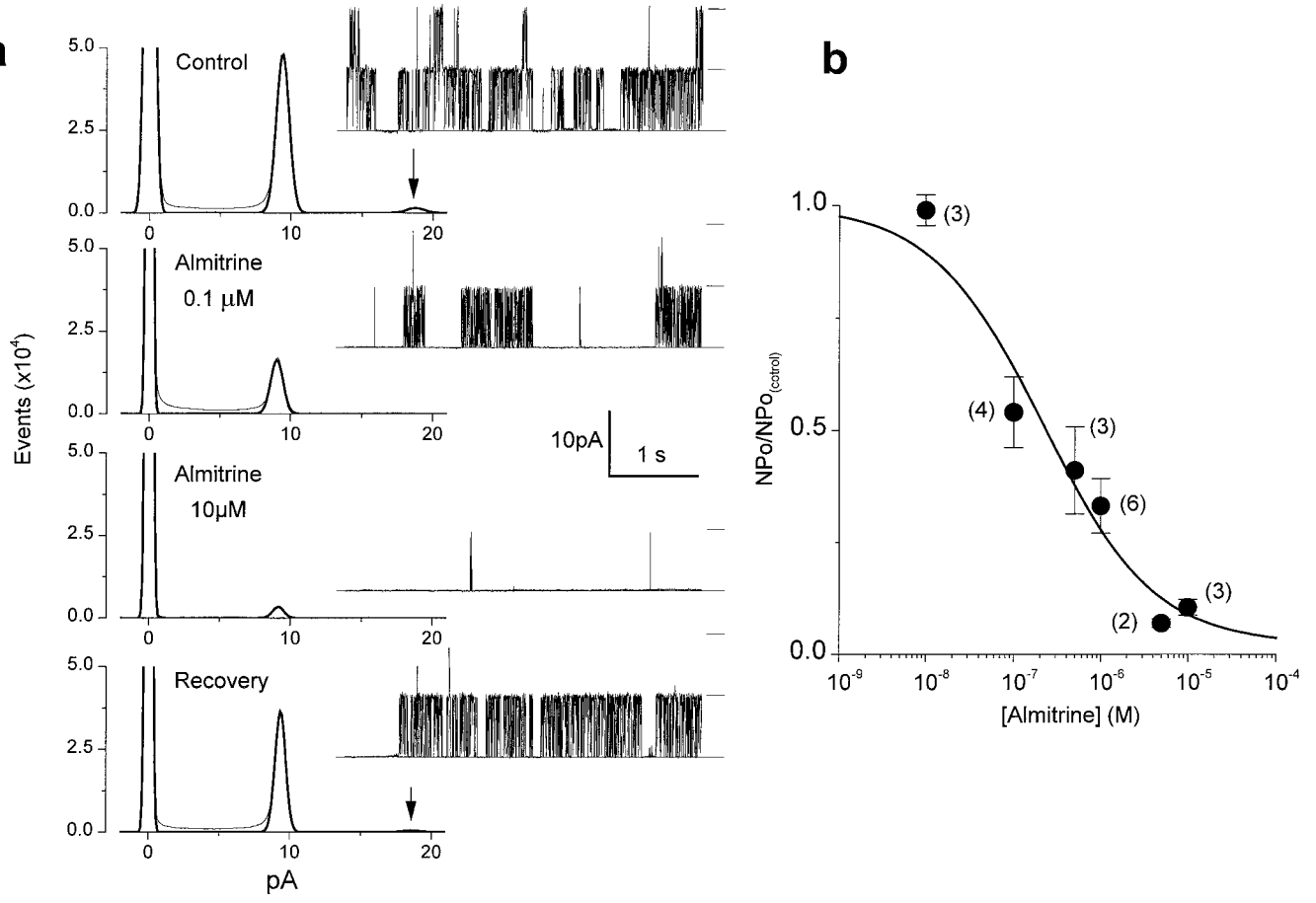

Fig. 6. Dose-response curve of Ca^{2+}-dependent K^+ channel activity in response to increasing doses of almitrine. a, Sample recordings of channel activity during 4 sec in the same excised patch in control conditions (symmetrical K^+ and 1 μM Ca^{2+} in the bath), with 0.1 and 10 μM almitrine, and 10 min after removal of the drug from the bath solution. *Solid line*, closed state. *Short marks on the left*, two opening levels in this patch. All-points histograms obtained in each condition from ≥4 min of recording also are shown. Pooled data from several patches were expressed as $NPo/NPo_{control}$ and represented as a function of almitrine concentration. *Numbers in parentheses*, number of averaged data. *Solid line*, nonlinear fit to the sigmoidal equation described in Results. Solutions: 0 Ca (pipette)/1 μM Ca (bath).

The effect of almitrine on I_{Ca} in rabbit chemoreceptor cells was investigated as well (Fig. 8b). The recording protocol is the same used for I_{Ca} in rat chemoreceptor cells. Internal solution was solution $0K_i$ and, because of the higher density of Ca^{2+} channels in rabbit compared with rat chemoreceptor cells (López-López and Peers, 1997), the bath solution contained Ca^{2+} as the charge carrier instead of Ba^{2+} (solution 10 Ca_e, see Table 1). The bath solution also contained 300 nM TTX, a concentration known to completely block I_{Na} in rabbit chemoreceptor cells (López-López and Gonzalez, 1992). Almitrine (10 μM) was applied for ≥4 min. Traces obtained in a typical experiment with the pulses to +10 mV are shown. Current amplitudes decayed along the experiment, due to the well-documented progressive washing-out of the Ca^{2+} channels in chemoreceptor cells (Duchen *et al.* 1988; Ureña *et al.* 1989), and almitrine did not modify the time course of the washing-out. I-V relationships obtained in several cells (six) during the application of almitrine were normalized to the average between the maximal currents elicited in control and recovery conditions to correct for washing-out.

Finally, the effect of almitrine on I_{Na} was tested in rabbit chemoreceptor cells (Fig. 8c). A protocol similar to that described for I_{Ca} was used, but the cells were bathed with the solution $standard_e$ containing 100 μM Cd^{2+}, and I-V relationships were obtained every 30 sec. Almitrine (10 μM) was added to the bathing solution for >5 min. The I-V relationships obtained in four cells before, during, and after the application of the drug were normalized to the maximal current obtained in control conditions (i.e., before drug application); averaged; and represented in Fig. 8c. The normalized I-V relationships obtained in the absence and presence of the drug are not statistically different. The current traces obtained in one cell with the depolarizing pulses to +10 mV also are shown in Fig. 8c.

Discussion

We examined the effect of almitrine on ionic currents from chemoreceptor cells from rat and rabbit carotid bodies. In whole-cell recordings, the only significant effect observed has been an inhibition of IK_{Ca} in rat cells. Almitrine does not affect IK_v in rabbit cells (Fig. 8), or in rat cells, as suggested for the voltage dependence of the drug effect (Fig. 1b) and for the lack of effect in the presence of ChTX (Fig. 2). Furthermore, the effect on IK_{Ca} is not due to an inhibition of I_{Ca} (Figs. 3 and 8) but to a direct action of the drug on high-conductance Ca^{2+}-activated K^+ channels (Figs. 5–7). The effect of almitrine on BK channels is fully reversible, but the

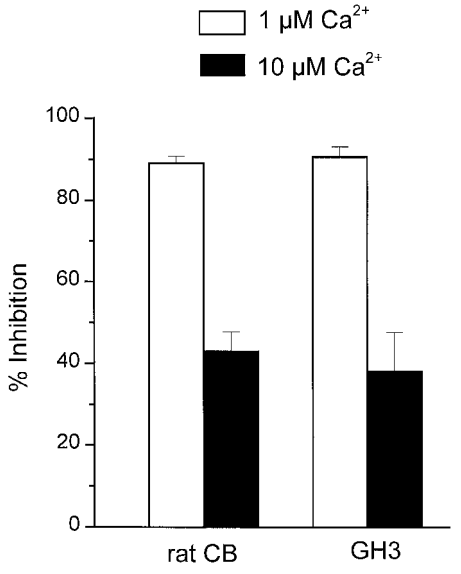

Fig. 7. Ca^{2+} dependence of the effect of 10 μM almitrine on BK channel activity recorded in excised membrane patches from rat CB chemoreceptor cells or GH3 cells at two different Ca^{2+} concentrations: 1 and 10 μM. The percent inhibition was calculated as $100 \cdot (NPo_{control} - NPo_{Almitrine})/NPo_{control}$, with $NPo_{control}$ being the average open probability before and after almitrine application. In all cases, almitrine was present in the bath for 4–6 min. Each *bar*, mean ± standard error of three to six patches. Solutions: 0 Ca (pipette)/1 μM Ca or 10 μM Ca (bath).

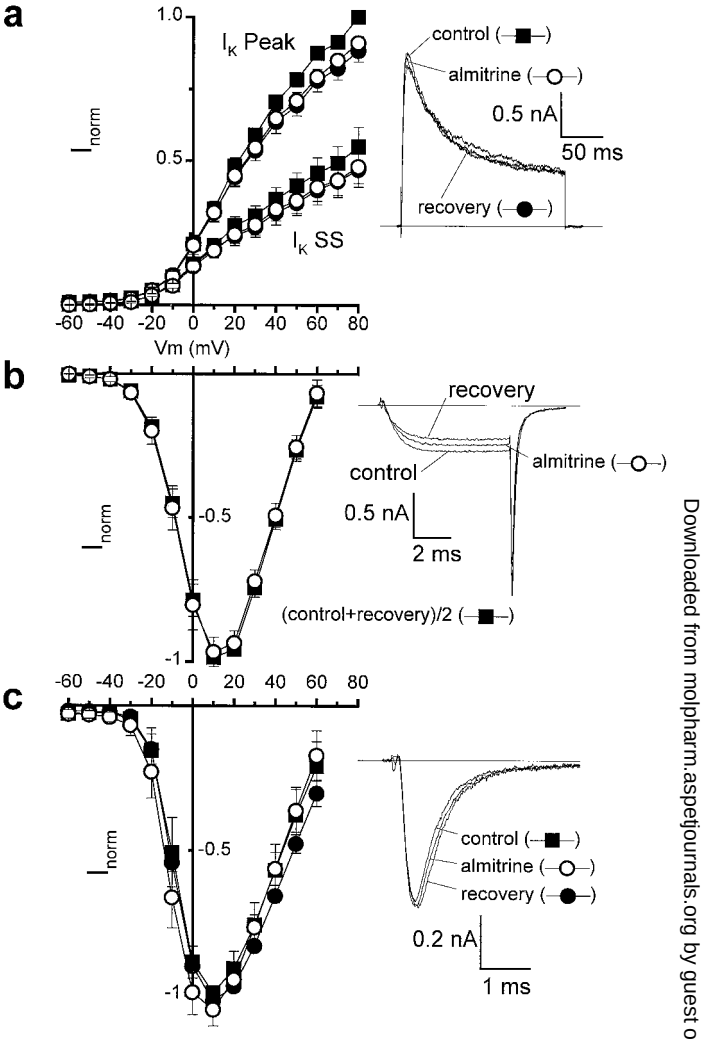

Fig. 8. Effect of 10 μM almitrine on whole-cell currents from rabbit chemoreceptor cells. a, I-V relationships of I_K measured at the peak (I_{peak}) or at 200 msec (I_{SS}) and obtained before, during, and after the application of 10 μM almitrine. Current amplitudes were normalized in respect to I_{peak} at +80 mV under control conditions. Values are mean ± standard error for seven cells. Solutions: standard$_e$/125K$_i$. *Traces* obtained in a single cell during a 200-msec step from −60 to +40 mV are shown. b, I-V relationships of I_{Ca} measured during the application of almitrine were normalized against the average of those obtained under control and recovery conditions to correct for rundown. Values are mean ± standard error for six cells. Solutions: 10Ca$_e$, 300 nM TTX/0K$_i$. *Traces* obtained in one cell in steps to +10 mV before, during, and after the application of 10 μM almitrine are shown. c, I-V relationships of I_{Na} measured under control conditions, during the application of almitrine, and after washing of the drug were normalized against the maximal current under control conditions. Values are mean ± standard error for four cells. Solutions: standard$_e$, 100 μM $CdCl_2$/0K$_i$. *Traces*, obtained in one cell with voltage steps to +10 mV are shown.

washout of the drug is very slow. The apparent lack of reversibility in whole-cell recordings certainly is due to the overlapping of the slow recovery and the washout of IK_{Ca}. The effect of almitrine on IK_{Ca} from rabbit cells has not been studied because the Ca^{2+}-dependent component of IK is present in a very variable amount and I_K is mainly a voltage-dependent current (Ureña *et al.*, 1989; Pérez-García *et al.*, 1992). In fact, the lack of a marked hump in the I-V relationships measured in the rabbit cells in which almitrine was tested (Fig. 8a) clearly suggests a minor presence of IK_{Ca} in the studied cells. Analogously, the effect of almitrine on I_{Na} was studied only in rabbit CB cells because only a small percentage of rat cells (<10%) exhibits I_{Na} (López-López *et al.*, 1997). Taken together, our data show that in chemoreceptor cells, almitrine, at concentrations up to 10 μM, inhibits selectively BK channels.

The single-channel properties of BK currents in our preparation show several differences with a previous work in neonatal rat CB cells (Wyatt and Peers, 1995), including a smaller unitary conductance (152 versus 190 pS) and a higher open probability for a given intracellular Ca^{2+} concentration (NPo = 0.3 at 1 μM Ca^{2+} versus 0.04). These discrepancies could reflect differences in the developmental stage between both preparations, because the experimental conditions and recording solutions are quite similar.

The selective effect of almitrine on BK channels in chemoreceptor cells provides a molecular target that can contribute to understanding of the reported chemostimulant effects of the drug in several preparations. We know that the main function of BK in excitable cells is to contribute to action potential repolarization (Garcia *et al.*, 1995; Sah, 1996), and thereby selective inhibition of this channel in spontaneously active cells increases action potential frequency. In addition, there is clear evidence for the contribution of BK to the maintenance of resting membrane potential in some preparations (Carl *et al.* 1996).

The results of electrophysiological studies have shown that most rat CB chemoreceptor cells possess whole-cell I_K with a ChTX-sensitive Ca^{2+}-dependent (BK) component that represents a major percentage of the entire I_K at membrane voltages between −10 and +40 mV (Peers and Buckler, 1995; López-López *et al.*, 1997; see Fig. 1). It is a well-established fact that BK currents in rat chemoreceptor cells are reversibly inhibited by low Po_2 and that this inhibition may play an important role in the modulation of the response of the cells

to hypoxia (Peers and Buckler, 1995; Wyatt and Peers, 1995; López-López *et al.*, 1997). However, because rat CB chemoreceptor cells in normoxic conditions do not present spontaneous activity (López-López and Peers, 1997), the functional significance of this inhibition is in dispute. Although some workers have shown that BK contributes significantly to the genesis and maintenance of resting membrane potential (Wyatt and Peers, 1995), others found that ChTX does not affect membrane potential (Buckler, 1997), implying that BK inhibition produced by low Po_2 cannot represent the trigger for the chemotransduction process. The chemostimulant action of almitrine in normoxic rats (Behm *et al.*, 1993; Lagneaux, 1994), in light of the findings reported in the current work, could be accounted for if BK contributes to the genesis of membrane potential, but the action of almitrine potentiating hypoxic chemoreception (Lagneaux, 1994) can be satisfactorily explained regardless of whether BK participates in the genesis of membrane potential because rat chemoreceptor cells can generate action potentials during hypoxic stimulation (Peers and Buckler, 1995).

The data presented here indicate that almitrine behaves as a selective blocker of BK in rat chemoreceptor cells and that the inhibitory effect of almitrine is dependent on the intracellular Ca^{2+} levels, being less prominent at higher Ca^{2+} concentrations (Fig. 7). Although the mechanism of this blockade has not been characterized, it probably involves a direct interaction of almitrine with the channel, because the role of intracellular mediators can be excluded in the inside-out configuration. Regarding the tissue-specificity of the effect, we found that almitrine at 10 μM also inhibits BK in GH3 cells (Fig. 7), although a detailed characterization of this inhibition is lacking. Due to the variability of expression of BK in rabbit chemoreceptor cells (see above), we have not studied the effect of almitrine on this channel in this species, but our preliminary results in GH3 cells make conceivable that almitrine would have comparable effects. In the rabbit CB chemoreceptor cells, BK currents are not O_2 sensitive (Ganfornina and López-Barneo, 1991). However, contrary to those in rat, rabbit chemoreceptor cells generate action potentials both at rest and after hypoxic stimulation (López-López *et al.*, 1989; Montoro *et al.*, 1996), so BK currents, when present, should contribute to action potential repolarization, and their inhibition by almitrine would lead to increased Ca^{2+} entry and consequent activation of the cell. Moreover, considering that chemoreceptor cells are electrically coupled (Abudara and Eyzaguirre, 1994), cell activation could spread to adjacent cells and ultimately to the entire CB. This hypothesis is consistent with previous data showing that almitrine both promotes the release of neurotransmitters from rabbit CB and potentiates the secretory response induced by hypoxia (Almaraz *et al.*, 1992).

In addition to its chemostimulant actions, almitrine improves ventilation/perfusion mismatching and increases the oxygenation of arterial blood through potentiation of the hypoxic pulmonary vasoconstriction (Chardon *et al.*, 1980; Saadjian *et al.*, 1994). This therapeutically important effect of almitrine also could be accounted for by the inhibition of BK because it is well documented that BK plays a very important role in regulation of pulmonary artery tone (Weir and Archer, 1995). Thus, the findings reported in the current work might represent the description of a common cellular mechanism generating the therapeutic effects of almitrine.

Based on the ubiquitous distribution of BK in the organism, it seems that almitrine would produce a wide spectrum of unwanted effects; however, this is not the case in laboratory animals or humans, indicating that at clinically useful doses, the function of most cells is not altered by almitrine. How this specificity is achieved remains unknown. It could be related to the tissue specificity of the properties of BK (Bolton and Beech, 1992; Tseng-Crank *et al.*, 1994) or, more likely, to the conjunction of additional cell mechanisms that, as targets for almitrine, amplify the effects of BK inhibition. In fact, almitrine produces other cellular effects (Leverve *et al.*, 1994) that might contribute to sharpen the specificity of almitrine actions at the level of CB chemoreceptors and pulmonary artery smooth muscle cells, minimizing simultaneously its actions in other tissues.

In conclusion, we demonstrate that almitrine at therapeutically useful doses inhibits the O_2-sensitive high-conductance Ca^{2+}-dependent K^+ channel in rat CB chemoreceptor cells without affecting other ionic currents. This effect of almitrine could represent an important mechanism underlying the chemostimulant action of almitrine.

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