L- and N-type Ca^{2+} channels in adult rat carotid body chemoreceptor type I cells

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1. Whole-cell voltage-dependent Ca^{2+} currents recorded from chemoreceptor type I cells of the adult rat carotid body had maximum amplitudes of −94 pA in 10 mM Ca^{2+} and were half-inactivated at a holding potential of −38 mV. Somatostatin and dopamine inhibited whole-cell Ca^{2+} current in type I cells.

2. The dihydropyridine agonist (+)202-791 increased the Ca^{2+} current amplitude by 106% at a step potential of −18 mV. The dihydropyridine antagonist nimodipine decreased the Ca^{2+} current amplitude by 40% from a holding potential of −80 mV, and by 74% from a holding potential of −60 mV. The nimodipine-sensitive current had a maximum amplitude at a membrane potential of −12 mV. ω-Conotoxin GVIA (ω-CgTX GVIA) blocked the whole-cell Ca^{2+} current by 40%. The ω-CgTX GVIA-sensitive current had a maximum amplitude at a membrane potential of +2 mV.

3. In summary, type I cells of the adult rat carotid body have dihydropyridine-sensitive L-type and ω-conotoxin GVIA-sensitive N-type voltage-dependent Ca^{2+} channels. These channels may play a role in the voltage-gated entry of Ca^{2+} necessary for stimulus–secretion coupling in response to changes in arterial P_{O_2}, P_{CO_2} and pH. Inhibition of the Ca^{2+} currents by somatostatin and dopamine may alter the chemotransduction signal in type I cells.

The mammalian carotid body is the principal arterial chemosensor for O_2, CO_2 and pH and is involved in the regulation of ventilation (Biscoe, 1971). An anastomosing network of blood vessels surrounds two kinds of cells in the carotid body, type I (glomus cells, 8–15 μm in diameter) and type II cells (sheath cells, 6 μm in diameter). Type I cells, the primary sensory cells in the carotid body, are excitable cells capable of generating Na^+- and Ca^{2+}-dependent action potentials (Ureña, López-López, González & López-Barneo, 1989). Changes in P_{O_2} and P_{CO_2} stimulate dopamine release from type I cells, and this release is highly dependent on external Ca^{2+} (Gronblad, Akerman & Eranko, 1979; Obeso, Rocher, Fidone & González, 1992; Pérez-García, Obeso, López-López, Herreros & González, 1992). Low P_{O_2} has been shown to depolarize type I cells, probably through inhibition of an O_2-sensitive K^+ current (López-Barneo, López-López, Ureña & González, 1988; Delpiano & Hescheler, 1989; López-López, González, Ureña & López-Barneo, 1989; Ganfornina & López-Barneo, 1991). The resulting depolarization then stimulates Ca^{2+} entry through activation of voltage-dependent Ca^{2+} channels. Increases in P_{CO_2} have also been shown to depolarize type I chemoreceptor cells and increase intracellular Ca^{2+} through activation of voltage-dependent Ca^{2+} channels (Buckler & Vaughan-Jones, 1994). The Ca^{2+} channel types responsible for this rise in intracellular Ca^{2+} are only partially sensitive to dihydropyridines. Dihydropyridines, L-type Ca^{2+} channel antagonists, do not fully block the rise in intracellular Ca^{2+}, indicating that other types of Ca^{2+} channels probably contribute to the rise in intracellular Ca^{2+}. Dihydropyridine-sensitive, L-type Ca^{2+} channels are present in type I cells (Hescheler, Delpiano, Acker & Pietruschka, 1989; Fieber & McCleskey, 1993), but the existence of other types of Ca^{2+} channels has not been investigated in detail. Therefore we have investigated the biophysical and pharmacological properties of voltage-dependent Ca^{2+} currents in type I cells from the adult rat carotid body in order to determine the types of voltage-dependent Ca^{2+} channels present in these cells.

It is possible that voltage-dependent Ca^{2+} channels in type I cells could be modulated by neurotransmitters released from nerve terminals within the carotid body, by substances released from type I cells themselves, by blood-borne substances or by substances released from endothelial cells. If these substances modulate the Ca^{2+} currents in

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type I cells, then alterations in the ventilatory response to changing arterial P_{O_2}, P_{CO_2} and pH may occur. Consequently, we have tested a variety of substances for their ability to modulate Ca^{2+} currents in type I cells.

METHODS

Cell isolation

Experiments were performed on enzymatically isolated cells from the rat carotid body. Adult Wistar rats (150–350 g) were decapitated without prior anaesthesia using a laboratory guillotine and the whole region of the carotid artery bifurcation was removed and placed in cold Hanks' balanced salt solution (HBSS). The carotid body was cleaned with forceps and cut using fine scissors. The isolated carotid bodies were then incubated in an enzymatic solution containing 0·4 mg ml^{-1} collagenase type D, 0·1 mg ml^{-1} trypsin (both from Boehringer-Mannheim Biochemicals) and 0·1 mg ml^{-1} DNAase type I (Sigma) in Earle's balanced salt solution (EBSS) at 35 °C for 1 h in a shaking water bath. After incubation, the cells were dissociated by vigorous shaking of the flask for 10 s. The enzymes were inactivated by adding an equal volume (6 ml) of culture medium composed of minimum essential medium (MEM) containing 10% fetal bovine serum, 1% penicillin–streptomycin (50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin), 1% glutamine (all from Gibco) and 10 μg ml^{-1} insulin (Boehringer-Mannheim Biochemicals). The cells were centrifuged twice at 100 g for 6 min and resuspended in culture medium. The cells were then plated onto 35 mm poly-L-lysine-coated polystyrene culture dishes (Falcon), incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and used within 2–8 h. Only type I cells (glomus cells) of approximately 10 μm diameter, or clusters of such cells, were used in the experiments.

Electrophysiological techniques

Ionic currents from type I cells were recorded at room temperature (22–26 °C) using conventional whole-cell patch clamp recording techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with an Axopatch-1D patch clamp amplifier (Axon Instruments). Patch pipettes were made from borosilicate glass capillaries (1·65 mm outer diameter, 1·2 mm inner diameter, Corning 7052, Garner Glass Co., Claremont, CA, USA) using a model P-87 Flaming-Brown micropipette puller (Sutter Instrument Co., San Rafael, CA, USA). The patch electrodes were coated with Sylgard 184 (Dow Corning) and fire-polished on a microforge (Narishige, Tokyo, Japan). Pipette resistances ranged from 3 to 5 MΩ when filled with the internal solution described below. Cell culture dishes were placed on the stage of an inverted microscope (Nikon Diaphot) and were superfused with external solution by gravity at a rate of approximately 1 ml min^{-1}. After a gigaohm seal was formed between the pipette and the membrane, the patch was ruptured by steady suction. Mean access resistance and membrane capacitance as determined from the time constant and area of the uncompensated capacitive transient were $12 \pm 0{\cdot}3$ MΩ and $5 \pm 0{\cdot}1$ pF, respectively ($n = 110$). The cell membrane capacitance and series resistance were electronically compensated to > 80%. Whole-cell currents were low-pass filtered at 2–5 kHz (−3 dB) using the 4-pole Bessel filter of the clamp amplifier. A holding potential of −80 mV was employed for the voltage clamp experiments unless otherwise stated.

Voltage protocols were generated by a Macintosh IIci computer (Apple) equipped with a MacAdios II data acquisition board (GW Instruments Inc., Cambridge, MA, USA). Data acquisition and protocol generation software were written by Dr Stephen R. Ikeda (Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, USA).

Sustained Ca^{2+} current amplitudes were measured as the average current between 50 and 55 ms during the voltage step. These amplitudes were used to generate current–voltage (I–V) curves using Igor software (WaveMetrics, Lake Oswego, OR, USA). I–V curves were corrected for linear leakage as determined from hyperpolarized test potentials. Changes in current amplitudes between control and drug treatment conditions were calculated as per cent inhibition = (1 − drug/control) × 100, using leak-subtracted values. Data are presented as means ± S.E.M. where appropriate. Statistical significance was determined by Student's t test. The differences were considered significant when $P < 0{\cdot}05$.

Solutions

HBSS solution (Gibco) was made from the powder to which 4·2 mM $NaHCO_3$ was added and the pH adjusted to 7·4. EBSS solution was made from ×10 concentrated liquid (Sigma) with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (1 M Hepes buffer, Sigma), 20 mM glucose and 2·6 mM $NaHCO_3$. The pH was adjusted to 7·4 with 1 N NaOH.

To isolate Ca^{2+} currents for whole-cell recording, cells were bathed in an external solution that contained (mM): 140 methanesulphonic acid ($MeSO_3H$) (Aldrich Chemical Co.), 140 tetraethylammonium hydroxide (TEA-OH) (Aldrich Chemical Co.), 10 Hepes, 15 glucose, 10 $CaCl_2$, 0·0001 tetrodotoxin (Calbiochem Corporation, La Jolla, CA, USA); pH 7·4, 330 mosmol kg^{-1}. The intracellular solution consisted of (mM): 120 *N*-methyl-D-glucamine (NMG), 20 tetraethylammonium chloride, 11 ethylene-glycol-bis-(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 Hepes, 14 phosphocreatine (Tris salt), 1 $CaCl_2$, 4 magnesium adenosine 5′-triphosphate (MgATP) and 0·1 sodium guanosine 5′-triphosphate (Na_2GTP); pH 7·2, 305 mosmol kg^{-1}.

Drugs were locally applied by gravity to isolated cells or clusters of type I cells from a micropipette (10–15 μm diameter, type N51A glass, Garner Glass Co.) lowered into the bath to within 10–20 μm of the cell. Removal of the micropipette from the bathing medium terminated drug application. All compounds were diluted into the external solution from concentrated aqueous or absolute ethanol stock solutions and diluted to their final concentrations just before use. These stock solutions were all stored at −50 or −80 °C. Nimodipine (Research Biochemicals International, Natick, MA, USA), (+)202-791 (2,6-dimethyl-3-carbomethoxy-5-nitro-4-(2-trifluoromethyl-phenyl)-1,4-dihydropyridine), which was a generous gift from Kathleen D. Roskaz (Sandoz Research Institute, East Hanover, NJ, USA), and L-(+)-muscarine chloride (Research Biochemicals International) were diluted into external solution daily from stock solutions of 10 mM in absolute ethanol. The final concentration of ethanol was 0·01%, which had no effect on the Ca^{2+} currents from type I cells. The following stock solutions were prepared in distilled water: 1 mM ω-conotoxin GVIA (ω-CgTX GVIA, Bachem Inc., Torrance, CA, USA), 100 μM ω-agatoxin IVA (ω-Aga IVA, Peptides International, Louisville, KY, USA), 100 μM ω-conotoxin MVIIC (Peptides International), 1 mM [D-Trp^8]-somatostatin (Bachem Inc.), 0·5 mM vasoactive intestinal peptide (VIP, Peninsula Laboratories, Belmont, CA, USA), 10 mM Met-enkephalin (Sigma), 1 mM substance P (Sigma), 100 mM (−)-noradrenaline hydrochloride (NA, Sigma), 10 mM 9-β-D-ribofuranosyladenine (adenosine, Sigma), 10 mM 5-hydroxytryptamine hydrochloride (5-HT, Sigma), 10 mM

3-hydroxytyramine hydrochloride (dopamine, Sigma), 1 mM human angiotensin II (Ang. II, Sigma), 1 mM endothelin-3 (Peptides International), 10 mM 8-bromo-guanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP, Sigma), 1 M cadmium chloride ($CdCl_2$, Fluka, Ronkonkoma, NY, USA), 1 M barium chloride ($BaCl_2$, Sigma) and 1 M nickel chloride ($NiCl_2$, Sigma). Phorbol 12-myristate,13-acetate (PMA, Sigma) was diluted into the external solution from a 1·62 mM stock solution in dimethyl sulphoxide (DMSO). Final concentrations of DMSO were 0·03%. Stock solutions of PMA were kept at −80 °C. Stock solutions of NA, adenosine, 5-HT, 8-Br-cGMP, $CdCl_2$ and $NiCl_2$ were prepared just before use. Solutions of NA and (+)202-791 were protected from light whenever possible.

Tyrosine hydroxylase immunocytochemistry

To confirm the identity of type I cells from the adult rat carotid body, we assayed for the presence of tyrosine hydroxylase, an enzyme used to synthesize catecholamines. Immunocytochemical studies were performed using the Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA) on cells obtained from the adult rat carotid body as described above. The cells were plated on gridded coverslips (Bellco Glass, Inc., Vineland, NJ, USA) coated with poly-L-lysine and placed on the bottom of 35 mm polystyrene culture dishes containing culture medium. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 4 h. The cultures were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature (22–26 °C). The cells were then washed extensively (three times for 5 min) in PBS and incubated for 15 min in 1·5% horse serum to block non-specific binding sites. The blocking serum was replaced with 2 ml per dish of a monoclonal anti-tyrosine hydroxylase antibody (Boehringer-Mannheim Biochemicals) diluted 1:1000 in PBS containing 0·1% Tween and incubated overnight at 40 °C. The cells were washed three times for 5 min each with PBS and then incubated with biotinylated secondary antibody for 15 min. Cells were washed extensively and incubated in an avidin and biotinylated horseradish peroxidase macromolecular complex solution (as per manufacturer's instructions) for 30 min. The cells were washed three times with PBS and then reacted with 2 ml per dish of a DAB (3,3′-diaminobenzidine) solution with nickel intensification (as per manufacturer's instructions). The development of the chromagen was watched under a microscope for 2 min. Cells were then washed extensively with PBS. Background staining was assessed by following the same protocol with the omission of the primary antibody.

RESULTS

Morphological and immunohistochemical identification of type I cells from the adult rat carotid body

The data presented in this paper were obtained from type I chemoreceptor cells. These cells were distinguished from non-type I cells by morphological and immunocytochemical criteria. Enzymatically dissociated cells of the carotid body consisted of small numbers of both isolated cells and of cell clusters of different sizes. Immediately after plating, the cells were round and phase bright. After several hours in culture, the cells appeared as a quite homogeneous population, with the majority having soma sizes of about 10 μm in diameter and short processes. These cells were identified as type I cells by morphological criteria.

Type I cells are adrenergic cells which exhibit tyrosine hydroxylase activity (reviewed by González, Almaraz, Obeso & Rigual, 1994). Immunoreactivity to tyrosine hydroxylase (TH) was used to confirm that the cells from the adult rat carotid body used in our experiments to record Ca^{2+} currents were type I cells. Anti-TH-positive cells were approximately 10 μm in diameter and could be easily distinguished from the smaller TH-negative cells (data not shown). These results were expected since similar results were obtained by Nurse (1990) in rat pups. Omission of the primary antibody resulted in the absence of immunoreactivity in the cultures.

Characteristics of Ca^{2+} currents in type I cells from the adult rat carotid body

Whole-cell Ca^{2+} currents

The characteristics of the voltage-dependent Ca^{2+} currents were studied in 143 acutely isolated type I cells from the adult rat carotid body. Whole-cell patch clamp recordings of type I cells were performed with external and internal solutions that effectively isolated Ca^{2+} currents (see Methods). Under these conditions, prominent Ca^{2+} currents were recorded in an external solution containing 10 mM Ca^{2+}. Three lines of evidence show that the currents studied were indeed Ca^{2+} currents: (1) the currents were increased and/or blocked by organic Ca^{2+} channel agonists and antagonists; (2) the currents were blocked by inorganic Ca^{2+} channel blockers; and (3) Ba^{2+} supported current through these channels.

Inward Ca^{2+} currents elicited by 70 ms depolarizing voltage steps first appeared at about −40 mV, grew to maximal amplitude at −3 mV and became smaller with progressively more depolarized voltage steps (Fig. 1*A*). Currents were sustained throughout the 70 ms voltage step at all test potentials (Fig. 1*B*). The peak of the *I–V* curve was somewhat variable from cell to cell, but the majority of the cells had a maximal current amplitude between −3 and +2 mV. Peak currents in 10 mM external Ca^{2+} ranged from −30 to −270 pA with a mean of −94 ± 4·5 pA ($n = 101$).

Steady-state inactivation of the whole-cell Ca^{2+} current was determined by measuring the amplitude of the current at the end of a 70 ms voltage step to −3 mV preceded by 30 s prepulses to potentials between −100 and −20 mV (Fig. 2*A*). Current amplitudes were normalized to the amplitude evoked from a holding potential of −100 mV. The mean amplitudes were fitted to a modified Boltzmann equation:

$$i(V) = I_{max}\,[1 + \exp((V - V_{0.5})/k)]^{-1},$$

where i is the current amplitude at voltage V, I_{max} is the maximum current amplitude, $V_{0.5}$ is the half-inactivation voltage and k is a slope factor. $V_{0.5}$ and k for inactivation of the inward Ca^{2+} current in type I cells were −38 and 11 mV, respectively. The results give a measure of the

voltage dependence of the whole-cell Ca^{2+} currents in type I cells from the adult rat carotid body.

Ba^{2+} (10 mM) substituted for 10 mM Ca^{2+} as a charge carrier and increased the peak current amplitude by 80 ± 26% ($n = 5$). Figure 2*B* shows superimposed *I–V* curves for a representative cell in the presence of 10 mM Ca^{2+} and after superfusion with 10 mM Ba^{2+}. Next, the sensitivity of the whole-cell Ca^{2+} current to two different concentrations of cadmium (Cd^{2+}) was tested. The inward Ca^{2+} current elicited throughout the entire range of voltage steps was blocked in the presence of 500 μM Cd^{2+}. The amplitude of the current measured at −3 mV was reduced by 93 ± 1% ($n = 5$) by 500 μM Cd^{2+}. A lower concentration of Cd^{2+} (25 μM) was used to test for the presence of low voltage-activated Ca^{2+} currents. The *I–V* curve in the presence of 25 μM Cd^{2+} was differentially affected (Fig. 2*C*). Currents at more negative voltages were more resistant than currents at more positive voltages. The Ca^{2+} current resistant to 25 μM Cd^{2+} had a broad peak between −17 and −3 mV. The current elicited by a voltage step to −3 mV in the presence of 25 μM Cd^{2+} was sustained throughout the 70 ms step (Fig. 2*D*). When measured at a step potential of −3 mV the current decreased by 63 ± 4% ($n = 4$) in the presence of 25 μM Cd^{2+}. Low concentrations of Cd^{2+} have been previously reported to block high threshold Ca^{2+} currents to a greater extent than low voltage-activated, T-type Ca^{2+} channels (Fox, Nowycky & Tsien, 1987; Narahashi, Tsunoo & Yoshii, 1987). Thus the Ca^{2+} current remaining in the presence of 25 μM Cd^{2+} may represent a different type of Ca^{2+} current. Finally, the sensitivity of the whole-cell Ca^{2+} current to Ni^{2+} was tested. The whole-cell Ca^{2+} current was relatively insensitive to Ni^{2+} (28 μM). The peak of the *I–V* curve was reduced 7 ± 3% ($n = 4$) in the presence of 28 μM Ni^{2+}.

Dihydropyridine-sensitive L-type Ca^{2+} channels

Ca^{2+} channel agonists and antagonists were used to characterize the types of Ca^{2+} channels present in type I cells. Figure 3*A* illustrates *I–V* curves recorded in the presence and absence of 1 μM (+)202-791, a dihydropyridine agonist. The current amplitude increased in the presence of (+)202-791 and the peak of the *I–V* relationship shifted from −3 to −13 mV. In addition, the tail current resulting from a voltage step from −18 to −80 mV was prolonged in the presence of (+)202-791 (Fig. 3*B*). (+)202-791 increased the Ca^{2+} current amplitude by 106 ± 11, 79 ± 8 and 35 ± 5% at −18, −13 and −3 mV ($n = 6$), respectively. This effect was reversible after washing.

The dihydropyridine antagonist, nimodipine, partially blocked the whole-cell Ca^{2+} current as illustrated in Fig. 3*C*. The *I–V* curve elicited from a holding potential of −80 mV was asymmetrically decreased in the presence of nimodipine, leaving a notable shoulder on the *I–V* curve in the range of membrane potentials between −40 and −18 mV (Fig. 3*C*). The Ca^{2+} channel type contributing to the shoulder currents may represent another Ca^{2+} channel type and may correspond to the current remaining in the presence of 25 μM Cd^{2+} (cf. Fig. 2*C*). Current traces from the shoulder of the *I–V* curve elicited by a step to −30 mV are shown in Fig. 3*D*. To determine if the block by nimodipine was voltage dependent, currents elicited from a

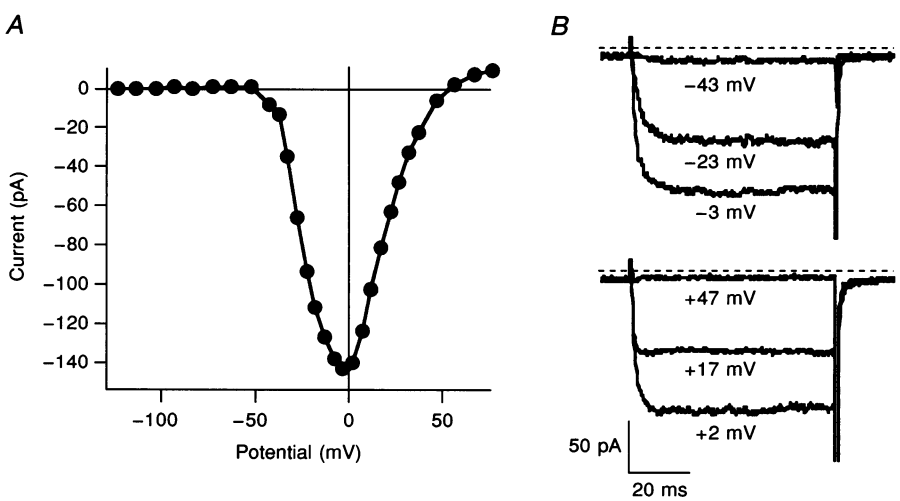

Figure 1. Ca^{2+} currents of isolated type I cells from the adult rat carotid body recorded with the whole-cell variant of the patch clamp technique

A, current–voltage (*I–V*) curve elicited by 70 ms depolarizing voltage steps to test potentials between −120 and +80 mV from a holding potential of −80 mV. Current amplitudes were measured as the average current between 50 and 55 ms during the voltage step and plotted as a function of the step potential. *B*, current traces elicited by depolarizing steps to the indicated voltages from the same cell shown in *A*. The zero current potential is indicated by the dashed line.

holding potential of −60 mV were compared with those elicited from a holding potential of −80 mV. The amplitude of the Ca^{2+} current decreased by 40 ± 5% ($n = 6$) and 74 ± 3% ($n = 11$) when measured during a voltage step to −13 mV from holding potentials of −80 and −60 mV, respectively. Nimodipine blocked significantly more current at −13 mV when tested from a holding potential of −60 mV compared with −80 mV ($P < 0.005$). In type I cells the effect of nimodipine was greater at less negative membrane potentials, consistent with the reported voltage dependence of inhibition by nimodipine (Cohen & McCarthy, 1987).

ω-Conotoxin GVIA-sensitive N-type Ca^{2+} channels

To determine the contribution of N-type Ca^{2+} channels to the whole-cell current of type I cells, ω-conotoxin GVIA (ω-CgTX GVIA), a toxin isolated from the marine snail *Conus geographus* (Oliveira, McIntosh, Cruz, Luque & Gray, 1984), was tested for its ability to block the Ca^{2+} current. ω-CgTX GVIA has been shown to produce a potent and irreversible block of N-type channels (McCleskey *et al.* 1987; Aosaki & Kasai, 1989; Plummer, Logothetis & Hess, 1989). When type I cells were exposed to 10 μM ω-CgTX GVIA, the *I–V* curve showed an asymmetric inhibition such that currents to step potentials ≤ −18 mV were less affected (Fig. 4*A*). The current elicited by a step to −3 mV showed no kinetic changes in the presence of ω-CgTX GVIA (Fig. 4*B*). ω-CgTX GVIA produced an irreversible inhibition of the Ca^{2+} currrent in every cell tested with a mean inhibition of 40 ± 0·1% ($n = 10$).

Effects of ω-agatoxin IVA and ω-conotoxin MVIIC on the Ca^{2+} currents of type I cells

The following experiments were performed to determine the identity of other Ca^{2+} channel types that contribute to the whole-cell Ca^{2+} current in type I cells. The presence of

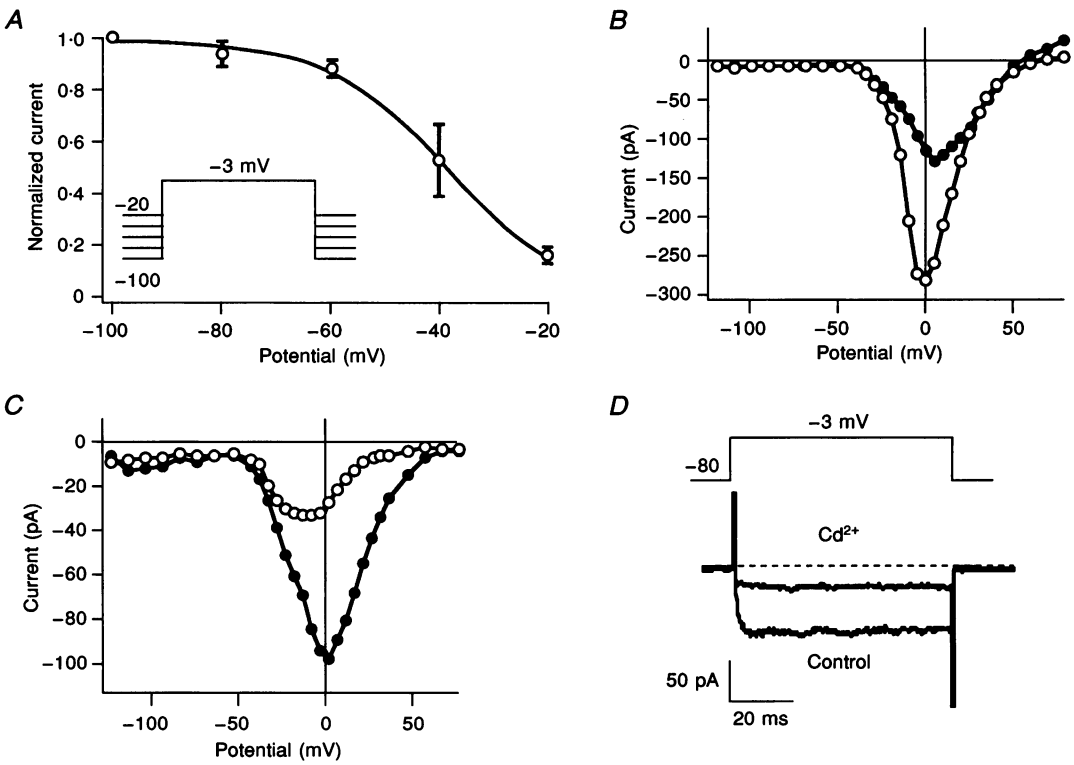

Figure 2. Inactivation, Ba^{2+} conductance and Cd^{2+} sensitivity of the Ca^{2+} currents in type I cells

A, steady-state inactivation of the Ca^{2+} current was determined by measuring the current amplitude at a test potential of −3 mV when preceded by 30 s prepulses to holding potentials between −100 and −20 mV (inset). Current amplitudes were normalized to the amplitude evoked from a holding potential of −100 mV. Mean normalized current amplitudes (○, $n = 3$) are plotted against the prepulse holding potential. The continuous line represents the best fit to a modified Boltzmann equation as determined by least-squares non-linear regression. The half-inactivation potential was −38 mV with a slope factor of 11 mV. *B*, superimposed *I–V* relationships from a single cell in the presence of 10 mM Ba^{2+} (○) and in the presence of 10 mM Ca^{2+} (●). *C*, *I–V* curves in the presence (○) and absence (●) of 25 μM Cd^{2+}. *D*, superimposed current traces from the same cell shown in *C* for a voltage step to −3 mV in the presence and absence of 25 μM Cd^{2+}.

the P-type channel was investigated by using the funnel-web spider peptide toxin ω-agatoxin IVA (ω-Aga IVA), which has been shown to block P-type Ca^{2+} channels in cerebellar Purkinje neurons (Mintz, Adams & Bean, 1992). ω-Aga IVA (0·2 μM in the presence of 0·1% cytochrome *c*) had no effect on the voltage-dependent Ca^{2+} currents in type I cells from the adult rat carotid body. In the presence of 0·2 μM ω-Aga IVA the peak Ca^{2+} current was changed by $-1·7 \pm 4·0\%$ ($n = 6$). By this criterion, type I cells from the adult rat carotid body do not have P-type Ca^{2+} channels.

ω-Conotoxin MVIIC (ω-CgTX MVIIC) is a synthetic peptide that inhibits a ω-CgTX GVIA-resistant Ca^{2+} current in CA1 hippocampal neurones (Hillyard *et al.* 1992), a Q-type Ca^{2+} channel in cerebellar granule neurons (Randall, Wendland, Schweizer, Miljanich, Adams & Tsien, 1993; Zhang *et al.* 1993) and α_{1A} Ca^{2+} channels expressed in *Xenopus* oocytes (Sather, Tanabe, Zhang, Mori, Adams & Tsien, 1993). ω-CgTX MVIIC inhibited the Ca^{2+} current of type I cells by $38 \pm 5\%$ ($n = 6$) at a test potential of −3 mV (data not shown). However, since ω-CgTX MVIIC can inhibit N-type Ca^{2+} channels (Hillyard *et al.* 1992), we applied 1 μM ω-CgTX MVIIC after pretreatment with 10 μM ω-CgTX GVIA to determine if the inhibition produced by ω-CgTX MVIIC was due to a block of the N-type Ca^{2+} channel. Application of ω-CgTX MVIIC failed to block Ca^{2+} channels that were insensitive to ω-CgTX GVIA (Fig. 4*C* and *D*). ω-CgTX MVIIC increased the voltage-dependent Ca^{2+} current by $11 \pm 2\%$ ($n = 4$) after pretreatment with ω-CgTX GVIA. This increase in Ca^{2+} current amplitude is probably due to a relief of block by ω-CgTX GVIA because ω-CgTX MVIIC is able to displace high affinity ω-CgTX GVIA binding (Hillyard *et al.* 1992). Consequently, type I cells do not have a Q-type current that is sensitive to ω-CgTX MVIIC and insensitive to ω-CgTX GVIA.

Relative contributions of L- and N-type Ca^{2+} channels to the whole-cell Ca^{2+} current

Type I cells have at least two types of Ca^{2+} channels that can be distinguished by pharmacological criteria: a nimodipine-sensitive L-type and a ω-CgTX GVIA-sensitive N-type. To assay the relative contributions and the voltage ranges of these two Ca^{2+} channel types to the

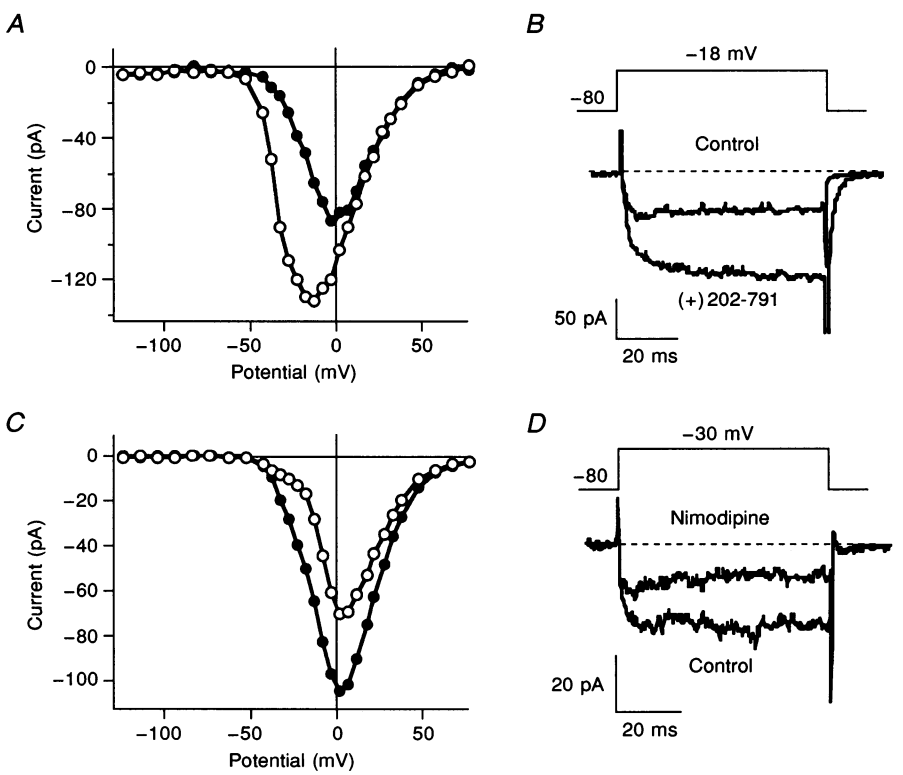

Figure 3. Effects of a dihydropyridine agonist and an antagonist on the Ca^{2+} current of isolated type I cells

A, superimposed *I–V* curves in the presence (○) and absence (●) of 1 μM (+)202-791, a dihydropyridine agonist. (+)202-791 increased the current amplitude and shifted the peak of the *I–V* relationship from −3 to −13 mV. *B*, superimposed current traces from the cell shown in *A* elicited by a voltage step to −18 mV from a holding potential of −80 mV in the presence and absence of 1 μM (+)202-791. The tail current in the presence of (+)202-791 was prolonged. *C*, *I–V* curves from a holding potential of −80 mV in the presence (○) and absence (●) of 3 μM nimodipine. *D*, superimposed current traces in the presence and absence of nimodipine, a dihydropyridine antagonist, elicited by depolarizing voltage steps to −30 mV, which corresponds to the shoulder on the *I–V* curve in *C*.

whole-cell currents, we performed experiments using a combination of nimodipine and ω-CgTX GVIA. Figure 5*A* illustrates the combined effect of 3 μM nimodipine plus 10 μM ω-CgTX GVIA on the *I–V* curves in a single cell. The *I–V* curve in the presence of nimodipine (3 μM) and ω-CgTX GVIA (10 μM) was not blocked completely (Fig. 5*A*, ○). This could be due to an incomplete block of the L-type current by nimodipine at the holding potential of −80 mV or to an additional Ca^{2+} current type. After washout of the drugs, the *I–V* curve did not recover completely (Fig. 5*A*, ▲) because of the irreversible block by ω-CgTX GVIA. Current traces for a voltage step to −8 mV from a holding potential of −80 mV under control, nimodipine plus ω-CgTX GVIA and wash conditions are shown in Fig. 5*B*. Unlike the whole-cell Ca^{2+} current, which was non-inactivating during the 70 ms voltage step, the current in the presence of nimodipine plus ω-CgTX GVIA had a transient component which inactivated with a time constant of 25·5 ± 2 ms ($n = 5$), in addition to a sustained component. N- and L-type currents, obtained by subtraction, showed characteristic *I–V* curve maximums (Fig. 5*C*). In this cell, the L-type current peaked at a membrane potential of −13 mV and the N-type current at +2 mV. In summary, the L-type current peaked at −12·4 ± 1·8 mV ($n = 8$) and the N-type current at +2·0 ± 1·4 mV ($n = 8$).

The Ca^{2+} current resistant to the combined effects of nimodipine and ω-CgTX GVIA was blocked 82 ± 6% ($n = 3$) by 25 μM Cd^{2+} and 36 ± 3% ($n = 3$) by 200 μM Ni^{2+}. Ba^{2+} substituted for Ca^{2+} as the charge carrier and increased the current resistant to nimodipine and ω-CgTX GVIA by 87 ± 10% ($n = 2$). This current is likely to be composed of L-type Ca^{2+} channels that are incompletely blocked by nimodipine and possibly other Ca^{2+} channel types.

Modulation of Ca^{2+} currents in type I cells

The carotid body contains a variety of neurotransmitters including noradrenaline, dopamine, acetylcholine, 5-HT, vasoactive intestinal peptide (VIP), enkephalin, substance P, tachykinin A, calcitonin-gene related peptide, galanin, neuropeptide Y, bombesin, cholecystokinin and atrial natriuretic peptide located in either cell bodies or nerve terminals (reviewed by González *et al.* 1994). Activation of muscarinic acetylcholine receptors by 10 μM L-(+)-muscarine ($n = 4$), adrenergic receptors by 100 μM

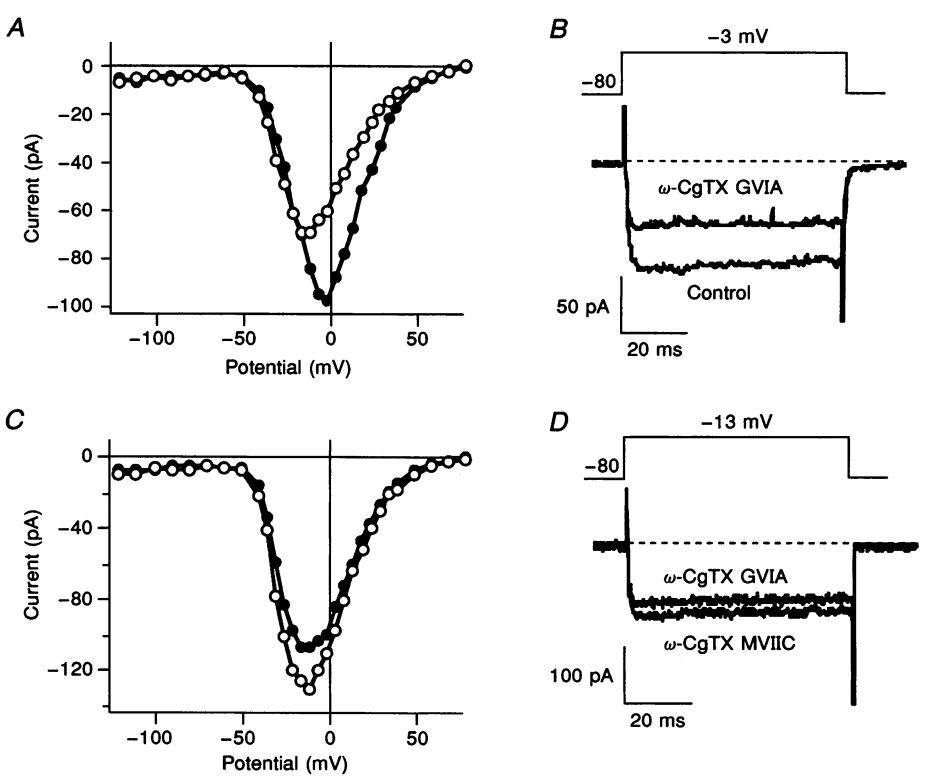

Figure 4. Effect of ω-CgTX GVIA and ω-CgTX MVIIC on the Ca^{2+} currents of isolated type I cells

A, *I–V* curves in presence (○) and absence (●) of 10 μM ω-CgTX GVIA. *B*, superimposed current traces from the same cell in *A* elicited by 70 ms depolarizing voltage steps to −3 mV from a holding potential of −80 mV in the presence and absence of ω-CgTX GVIA. *C*, *I–V* plots in the presence of 10 μM ω-CgTX GVIA (●) and in the presence of 1 μM ω-CgTX MVIIC after pretreatment with ω-CgTX GVIA (○). *D*, superimposed current traces for a step to −13 mV from the same cell shown in *C* in the presence of ω-CgTX GVIA and in the presence of ω-CgTX MVIIC after pretreatment with ω-CgTX GVIA.

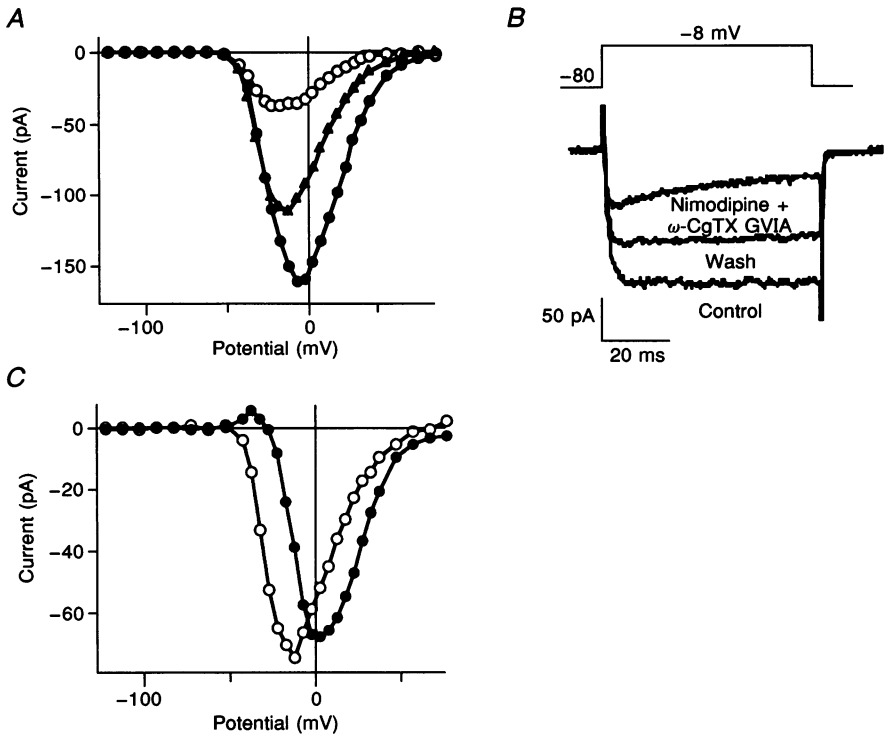

Figure 5. Dissection of L- and N-type Ca^{2+} currents in type I cells

A, superimposed *I–V* curves in the absence of drugs (●), in the presence of a combination of nimodipine (3 μM) and ω-CgTX GVIA (10 μM; ○) and during washout of the drugs (▲). The nimodipine-sensitive current component recovered during the wash. *B*, superimposed current traces from the same cell shown in *A* for a step potential to −8 mV during control and wash conditions and in the presence of a combination of nimodipine and ω-CgTX GVIA. *C*, subtraction of the *I–V* curves in *A* yielded the nimodipine-sensitive L-type (○) and the ω-CgTX GVIA-sensitive N-type (●) Ca^{2+} current components.

noradrenaline ($n = 4$) and opioid receptors with 1 μM Met-enkephalin ($n = 3$), all known to be present on type I cells, had no significant effects on the voltage-dependent Ca^{2+} current of type I cells. VIP, found in sympathetic nerve endings in the carotid body, has been shown to activate chemosensory discharges (reviewed by González *et al.* 1994), but the mechanism of this activation has not been clarified. We therefore tested whether VIP could directly alter type I cell Ca^{2+} currents. VIP (1 μM, $n = 3$) had no significant effect on type I cell Ca^{2+} currents. Many new

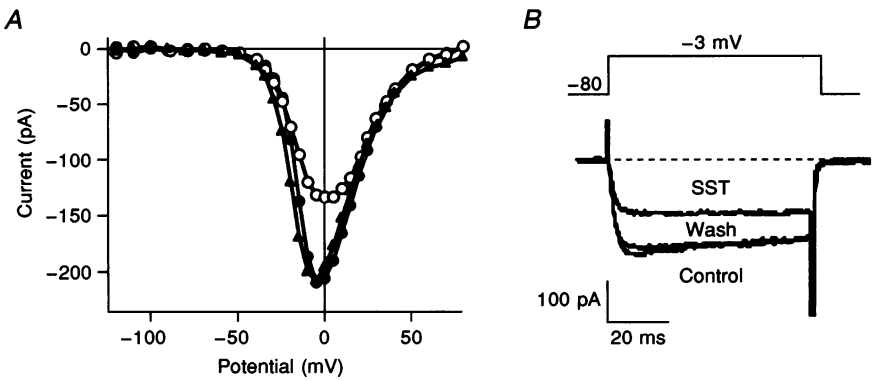

Figure 6. Effect of somatostatin (SST) on the whole-cell Ca^{2+} currents in type I cells

A, *I–V* relationships in the presence (○) and absence (●) of 0·1 μM SST and after washout of SST (▲). *B*, superimposed current traces elicited by voltage steps to −3 mV from a holding potential of −80 mV during control, SST and wash conditions.

5-HT receptor subtypes have recently been cloned but have not yet been mapped in the carotid body. Since 5-HT is present in the carotid body we tested whether the Ca^{2+} currents of type I cells are sensitive to 5-HT. 5-HT (10 μM, $n = 3$) had no significant effect on type I cell Ca^{2+} currents. Substance P (1 μM, $n = 3$) and adenosine (10 μM, $n = 5$) were also without effect. However, two transmitters did modulate the Ca^{2+} current in type I cells. Somatostatin (0·1 μM) reversibly inhibited the peak current by 24 ± 2% ($n = 11$) at a test potential of −3 mV. Figure 6*A* illustrates the reversible effect of somatostatin on the *I–V* curve from a representative cell. The Ca^{2+} current elicited by a voltage step from a holding potential of −80 mV to −3 mV in the presence of somatostatin activated more slowly than the control current (Fig. 6*B*). This slowing of activation is characteristic of a G protein-mediated voltage-dependent inhibition (Bean, 1989; Ikeda, 1991). Dopamine (10 μM) also decreased the peak Ca^{2+} current by 25 ± 0·5% ($n = 2$) in rat type I cells as was previously shown in the rabbit carotid body (Benot & López-Barneo, 1990).

Transmitters present in the blood which could be sensed by the highly vascularized carotid body chemosensory cells include angiotensin II, endothelin and nitric oxide. Nitric oxide could be released from nerve fibres which contain nitric oxide synthase and form an extensive plexus which innervates both the carotid body chemosensory cells and associated blood vessels (Wang, Stensaas, Bredt, Dinger & Fidone, 1994). Neither 1 μM angiotensin II ($n = 3$), 1 μM endothelin-3 ($n = 4$) or the nitric oxide donor *S*-nitroso-*N*-penicillamine (SNAP) ($n = 5$) had an effect on the Ca^{2+} currents of type I cells. Since some of the effects of nitric oxide are thought to be through a cGMP-dependent protein kinase (Southam & Garthwaite, 1991; Vincent, 1994), we tested whether a membrane-permeable cGMP analogue, 8-Br-cGMP, would alter the Ca^{2+} currents in type I cells. Consistent with the lack of an effect by the nitric oxide donor SNAP, 8-Br-cGMP (1 mM) was also without effect ($n = 3$) on the Ca^{2+} currents of type I cells. Activation of protein kinase C by 0·5 μM PMA ($n = 7$) also did not affect the Ca^{2+} currents of type I cells.

DISCUSSION

Type I cells of the carotid body are thought to sense changes in blood P_{O_2}, P_{CO_2} and pH and to transmit this sensory signal to the afferent sinus nerve by the release of dopamine. Voltage-dependent Ca^{2+} channels of type I cells are thought to play a pivotal role in carotid body neurotransmitter secretion and chemotransduction because the release of dopamine from type I cells depends on external Ca^{2+} and can be reduced by voltage-dependent Ca^{2+} channel blockers (Fidone, González & Yoshizaki, 1982; Obeso, Fidone & González, 1987). Dihydropyridine-sensitive or L-type Ca^{2+} channels in type I cells from both rabbit and rat carotid bodies have been reported previously (Monti-Bloch & Eyzaguirre, 1980; Duchen, Caddy, Kirby, Patterson, Ponte & Biscoe, 1988; Hescheler *et al.* 1989; Fieber & McCleskey, 1993). The present study provides new evidence for an additional type of voltage-activated Ca^{2+} current in type I cells from the adult rat carotid body. We have found that type I cells possess at least two types of voltage-dependent Ca^{2+} channels: a nimodipine-sensitive L-type and an ω-conotoxin GVIA-sensitive N-type. Fieber & McCleskey (1993) reported that ω-CgTX GVIA inhibited the Ca^{2+} current in only one out of four type I cells of the rat carotid body. In contrast, we found that 40% of the peak Ca^{2+} current was irreversibly blocked by ω-CgTX GVIA in every cell tested, suggesting that N-type Ca^{2+} channels make a contribution to the total Ca^{2+} current in type I cells.

Buckler & Vaughan-Jones (1994) suggested that voltage-dependent Ca^{2+} channels other than the L-type may contribute to the rise in intracellular Ca^{2+}. Our results support their hypothesis that additional types of voltage-dependent Ca^{2+} channels are present in type I cells of the rat carotid body which may contribute to the rise in intracellular Ca^{2+} during hypercapnic acidosis. Both L- and N-type Ca^{2+} channels seen in our study may contribute to the rise in intracellular Ca^{2+} during action potentials in type I cells. Further study will be necessary to determine the roles of the L- and N-type Ca^{2+} channels in excitation–secretion coupling in the carotid body.

The Ca^{2+} currents of type I cells were not sensitive to modulation by some neurotransmitters and neuropeptides found within the carotid body which are known to modulate Ca^{2+} currents in other cell types. Only somatostatin and dopamine were found to have an inhibitory effect on the Ca^{2+} currents in type I cells of the rat carotid body. Dopamine has been shown previously to inhibit Ca^{2+} currents in type I cells of the rabbit carotid body (Benot & López-Barneo, 1990) and recently prostaglandin E_2 was also shown to have an inhibitory effect on Ca^{2+} currents in type I cells (Gómez-Niño, López-López, Almaraz & González, 1994). Additionally, neither PMA, 8-Br-cGMP nor a nitric oxide donor had an effect on the Ca^{2+} currents in type I cells, indicating that they are also not modulated by protein kinase C, cGMP-dependent protein kinase or nitric oxide under whole-cell recording conditions. Recording conditions which cause less disruption to intracellular second messenger pathways may uncover other modulatory processes and remain to be investigated.

At least two distinct Ca^{2+} channel types contribute to the whole-cell Ca^{2+} current in adult rat carotid body type I chemoreceptor cells. These Ca^{2+} channel types include dihydropyridine-sensitive L-type and ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels. Both L- and N-type Ca^{2+} channels may contribute to the process of chemotransduction in type I cells of the carotid body. In addition, somatostatin and dopamine may modify the chemotransduction signal by inhibiting Ca^{2+} currents in type I cells.

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