Hypoxia-elicited catecholamine release is controlled by L-type as well as N/PQ types of calcium channels in rat embryo chromaffin cells

José-Carlos Fernández-Morales,1,2 Juan-Fernando Padín,1,2 Juan-Alberto Arranz-Tagarro,1,2 Stefan Vestring,4 Antonio G. García,1,2,3 and Antonio Miguel G. de Diego1,2

1Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain; 2Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain; 3Servicio de Farmacología Clínica, Instituto de Investigación Sanitaria, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain; and 4Medizinische Fakultät Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

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During fetal life, at birth, and for days after delivery the fetus and the infant often suffer of hypoxia stress. Survival potential of the fetus or the newborn (23, 33, 35). At this early life period, the neonate must undergo drastic changes to adapt to its new environment. For instance, at birth cardiovascular changes include alterations in the circulatory pattern and vascular resistance; respiratory changes are needed for the onset of continuous breathing and metabolic changes are also required to regulate temperature in the cold extrauterine environment (24). These changes, as well as the adaptation to hypoxia during fetal life, mostly depend on the release of adrenaline from adrenal chromaffin cells (23, 35).

That the hypoxia-induced catecholamine release at early life (the HIS response) depends on Ca2+ entry through voltage-activated calcium channels (VACCs) of chromaffin cells, is proven by the fact cadmium causes its blockade (1, 14). These channels could be opened through a modulatory action exerted by hypoxia on several types of potassium channels that leads to depolarization of the cell membrane (9, 21, 40). Hypoxic stress at early life may last minutes, and thus VACCs undergo inactivation during those long periods of depolarization. In fact, the various subtypes of VACCs expressed by adult, mature chromaffin cells are known to inactivate under sustained depolarization; such inactivation, however, is fast for N (α1B, Cav2.2) and PQ channels (α1A, Cav2.1) while L channels (α1D, Cav1.3) inactivate at a substantially lower rate (18, 42).

The pharmacological dissection of the various subcomponents of the whole cell calcium (ICa) or barium currents (IBa) has defined the fraction of high-VACCs expressed by chromaffin cells of various adult mammalian species, including humans (16, 27). Relevant to the present study is the report showing that in voltage-clamped chromaffin cells from adult rats using 5 mM external Ca2+ as charge carrier, L channels contributed 50% to ICa and non-L channels (N and PQ) contributed the remaining 50% (13).

To our knowledge, ICa carried by high-VACCs in chromaffin cells at early life has been explored only in three reports. In an early study it was found that ICa of rat embryo chromaffin cells (ECCs) has two subcomponents: one is carried by high-VACCs and the other by low-VACCs (T channels, Cav3.1); whether the high-VACC subcomponent was carried out by L, N, or PQ channels was unexplored in this study (9). The second study was performed in ovine ECCs using 60 mM Ca2+ as charge carrier; ICa was dissected out with specific blockers and it was concluded that L, N, and PQ channels equally contrib-
uted to \( I_{Ca} \), about 30–35% each (20). A third study has monitored \( I_{Ca} \) in 5 mM external Ca\(^{2+}\) in chromaffin cells from neonatal mice (12–15 days old); 50% of \( I_{Ca} \) was carried by L channels and 37% by PQ channels (6); at this age, however, chromaffin cells have likely matured and are innervated. Pharmacological experiments included in three reports have determined that L channels dominate the control of the HIS response. Arrival to this conclusion came from the fact nifedipine blocks the HIS response at early life in the perfused sheep adrenal gland (1), in neonatal rat chromaffin cells (39), and in neonatal rat adrenal slices (38). Whether N/PQ channels contribute to such response is unknown.

The present investigation was executed to define the relative contribution of L- and non-L (N/PQ) subtypes of high-VACCs to the following three functions of rat ECCs: 1) whole cell \( I_{Ca} \) in 2 and 5 mM external Ca\(^{2+}\); 2) elevations of cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{i}\)] in fura 2-loaded cells exposed to hypoxia; and 3) the HIS response. We found that in rat ECCs: 1) 60% of \( I_{Ca} \) was carried by L channels and 30% by N/PQ channels; the remaining 10% of \( I_{Ca} \) was resistant to blockers; 2) L as well as N/PQ channels contributed to the hypoxia-elicted [Ca\(^{2+}\)\(_{i}\)] elevations; and 3) L and N/PQ channels contributed to the regulation of the HIS response: initially, burst secretion may be controlled by L as well as PQ channels; later on, L channels dominate such response.

**METHODS**

**Ethical approval.** All experimental procedures with animals have been carried out in accordance with the Declaration of Helsinki and were performed following the rules approved by the Ethical Committee for the care and use of animals of the Medical School, Autonomous University of Madrid, Spain, in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and with the Spanish Real Decreto of October 10, 2005 (RD 1201/2005). All efforts were made to minimize animal suffering.

**Culture of rat ECCs.** Rats were housed individually under controlled temperature and lighting conditions with food and water provided ad libitum. Chromaffin cells were obtained from 18-day-old (E18) rat embryos by a protocol previously described for mice (36) with some modifications. The pregnant Wistar rat was killed by decapitation, and the fetuses were rapidly extracted and immediately decapitated. Adrenal glands were then rapidly removed from the embryos, fat-trimmed, and introduced in 1 ml of an enzymatic solution of identical composition to the one described by Sorensen et al. (36). Papain (20 U/ml) was added for tissue digestion. Adrenals were digested for 20 min; 1 ml of another solution aimed at stopping the enzymatic reaction was then added for 15 s. This solution was centrifuged for 4 min at 400 rpm. The supernatant was removed, and 0.8 to 1.5 ml of DMEM were added depending on the final desired cell density. A 100- to 120-µl drop of cell-containing solution was plated on poly-L-lysine-coated coverslips of one 12-well plates (for secretion experiments) or on 6-well plates (for Ca\(^{2+}\) transient experiments). After 1 h in an incubator, DMEM (1 ml for 12-well plates and 2 ml for 6-well plates) supplemented with 4% fetal bovine serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin was added to each well. Experiments were carried out in cells that were kept in a water-saturated incubator at 37°C and a 5% CO\(_2\) atmosphere for 1 to 2 days.

**Recording of whole cell currents through VACCs.** For patch-clamp recording of whole cell Ca\(^{2+}\) currents (\( I_{Ca}\)), the perforated-patch mode of the patch-clamp technique was used (19, 26), using amphotericin B as the permeating agent (2, 31). Tight seals (>5 GΩ) were achieved in a standard Tyrode solution composed of the following (in mM): 137 NaCl, 1 MgCl\(_2\), 2 or 5 CaCl\(_2\), 5.3 KCl, 10 glucose, and 10 HEPES; tetrodotoxin was added at 1 µM to block Na\(^{+}\) currents. The intracellular solution had the following composition (in mM): 100 CsCl, 14 EGTA, 20 TEA-Cl, 10 NaCl, 5 Mg-ATP, 0.3 Na-GTP, and 20 HEPES/ CsOH, pH 7.3. \( I_{Ca} \) were recorded at room temperature (22–25°C) using an EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by PULSE software running on a personal computer. The access resistance (\( R_{a}\)) was monitored until it decreased to < 20 MΩ. \( R_{a} \) averaged 8.8 ± 0.3 MΩ in ECCs (\( n = 97 \)) and was always compensated by 95%. In all recordings, the holding potential was −80 mV. \( I_{Ca} \) were activated in response to 50-ms depolarizing pulses from −80 to −10 mV. Test voltage protocol was applied every 10 s. Current signals were filtered at 5 kHz, digitized at 50 kHz, and on-line leak subtracted via a P/4 protocol.

**Immunoblotting and image analysis of the expression of VACC subtypes.** Adrenal glands were removed from the rat embryo (E18), and membrane proteins were extracted using the MEM-PER Membrane Protein Extraction Kit (Thermo Scientific) following the manufacturer’s instructions. At all times, the proteins were in the presence of protease inhibitors (Halt protease Inhibitor Cocktail; Thermo Scientific). Proteins were quantified using the Bicinchoninic acid protein assay (Bioscience). Proteins (20 µg) were resolved by SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore). Membranes were blocked in Tris-buffered saline with 0.05% Tween 20 containing 4% bovine serum albumin and incubated for 2 h at room temperature with primary antibodies anti-α1D (L-type VACC; 1:200), anti-α1B (N-type VACC; 1:200), and anti-α1A (PQ-type VACC; 1:200), all of them from Santa Cruz Biotechnology, and with anti-α-tubulin (1:10,000; Sigma-Aldrich, Madrid, Spain) as loading control, and then for 45 min with secondary antibodies conjugated with peroxidase (1:10,000; Santa Cruz Biotechnology). The membrane was developed using the enhanced chemiluminescence reagent (ECL Select Western blotting detection reagent; GE Healthcare). Different band intensities corresponding to immunoblot detection of protein samples were quantified using Scion Image Alpha 4.0.3.2 program (Scion). Data are represented as means ± SE of eight experiments. Comparisons between groups were performed by one-way ANOVA followed by the Newman-Keuls post hoc test. The level of statistical significant difference was accepted when \( P \leq 0.05 \).

**Measurements of changes of the cytosolic calcium concentrations.** Chromaffin cells were then placed for 1 h at 37°C in DMEM containing the Ca\(^{2+}\) probe fura-2 AM (10 µM). After this incubation period, the coverslips were mounted in a chamber, and cells were washed and covered with Tyrode solution composed of the following (in mM): 137 NaCl, 1 MgCl\(_2\), 5.3 KCl, 2 CaCl\(_2\), 10 HEPES, and 10 glucose, pH 7.3 with NaOH. The setup for fluorescence recordings was composed of a Leica DMI 4000 B inverted light microscope (Leica Microsystems, Barcelona, Spain) equipped with an oil immersion objective (Leica ×40 Plan Apo; numerical aperture 1.25). Once the cells were placed on the microscope, they were continuously superfused by means of a five-way superfusion system at 1 ml/min with a common outlet 0.28 mm-tube driven by electrically controlled valves with Tyrode’s solution. Fura-2 was excited alternatively at 340 ± 10 and 387 ± 10 nm using a Küber CODIX xenon 8 lamp (Leica). Emitted fluorescence was collected through a 540 ± 20 nm emission filter and measured with an intensified charge coupled device camera (Hamamatsu camera controller C10600 orca R2; Japan). Fluorescence images were generated at 1-s intervals. Images were digitally stored and analyzed using LAS AF software (Leica). A Tyrode external solution was used of the same composition as the one described above, where drugs were added to this solution. Drug concentrations are indicated in the text and figure legends.

**Amperometric recordings of single-vesicle quantic catecholamine release.** Experimental conditions for cell stimulation and recording of single amperometric spike events were as described (12, 44). The coverslips were mounted in a chamber on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) used to localize the target cell, which was continuously superfused by means of a five-way superfu-
sion system with a common outlet driven by electrically controlled valves, with a Tyrode solution, as described above.

Recordings in normoxia (solutions equilibrated with 95% air-5% CO₂) or hypoxia (solutions equilibrated with 95% N₂-5% CO₂) were performed under continuous bubbling of the solutions. A minimum of 30 min of bubbling elapsed before the initiation of the experiment, to allow time enough for cell equilibration with the solution. The percentage of O₂ was measured using an Ecossan Do 6 oximeter (Eutech Instruments, Nijkerk, The Netherlands). Within the reservoir, the solution reached complete anoxia; in the perfusion system outlet, O₂ percentage was ~5%. The perfused solution replaced the surroundings of the cell by this strongly hypoxic solution in ~1 s. At the time of experiment performance, proper amounts of drug stock solutions were freshly dissolved into the normoxic or hypoxic Tyrode solution.

Chemical products. Products to make saline solutions, as well as nimodipine and nifedipine, were purchased to Sigma (Sigma-Aldrich). ω-Conotoxin GVIA and ω-agatoxin IVA were from Peptide Institute (Peptanova). Tetrodotoxin citrate was from Ascent Scientific (Bristol, UK). The following chemicals were used for cell culture: DMEM and penicillin-streptomycin were from GIBCO (Scotland, UK), fetal bovine serum was from PAA Laboratories (Pasching, Austria), and papain was from Worthington (Lakewood, NJ). Stock solutions of ω-conotoxin GVIA and ω-agatoxin IVA were prepared in deionized water, nimodipine, and nifedipine in DMSO at 10⁻² M; solutions of nimodipine and nifedipine were prepared under sodium light and the experiments with these photosensitive dihydropyridines were performed also under sodium light.

Data analysis and statistics. I_on amplitude was measured at the maximum peak current [I(peak)] and at the late of the 50-ms pulse [I(t=50)]. Only the cells that held up the entire protocol (control, blocker, and wash) were included in the statistics. We used a Student's t-test for paired comparisons between normalized values between I_(t=50) and I_(t=0) before and during blocker treatment. Data are expressed as means ± SE of the number of cells and cultures indicated in parentheses in figures.

For amperometric recordings of hypoxia-elicited quantal catecholamine release, data analysis was carried out on a personal computer using Excel (Microsoft, Redmond, WA) and IgorPro (Wavemetrics, Lake Oswego, OR). Amperometric charge (Qamp) was calculated by integrating the amperometric current over time during the stimulus duration with a macro written in IgorPro. The number of spikes >5 pA was manually counted on an extended graph displayed in the computer screen. A ruler was drawn at 5 pA, and only the spikes going above this threshold amplitude were considered. Differences between means of group data fitting a normal distribution were assessed by using either ANOVA or Kruskal-Wallis test for comparison among multiple groups or Student's t-test for comparison between two groups. *P < 0.05 was taken as the limit of significance.

Differences between immunoblot band densities of VACC subtypes were analyzed with Graph Prism Software (GraphPad Software, San Diego, CA). Data are represented as means ± SE. Comparisons between groups were performed by a one-way ANOVA followed by the Newman-Keuls post hoc test. Statistical differences was accepted when P ≤ 0.05.

RESULTS

Pharmacological dissection of the subcomponents of the whole cell calcium current. In a previous study we found that in adult rat chromaffin cells voltage clamped at ~80 mV, using 5 mM Ca²⁺ as charge carrier, I_on was approximately carried 50% by L channels and 50% by N and PQ channels. That early study was performed with the whole cell configuration of the patch-clamp technique, and the extent of blockade was calculated on the peak amplitude elicited by each blocker (13). We have followed here a similar approach to discern the L and N/PQ subcomponents of I_on in rat ECCs. Hence, we have used the perforated patch configuration of the patch-clamp technique that preserves longer the I_on amplitude along repeated application of depolarizing test pulses. The holding potential was maintained at 0 mV, and 5 mM Ca²⁺ were used as charge carrier; 50-ms test pulses to -10 mV were continuously applied at 10-s intervals. After patch perforation and stabilization of I_on traces, a given blocker was perfused onto the cell surface to elicit current blockade. We used supramaximal concentrations of each blocker that were similar to those used in our previous study in adult rat chromaffin cells (13), namely, 3 μM nimodipine to block L channels and 1 μM each of ω-conotoxin GVIA (GVIA) and ω-agatoxin IVA (Agta) to block N and PQ channels, respectively.

Figure 1A displays a family of I_on current traces obtained before (control), after 2-min of cell perfusion with nimodipine, after blocker washout, and finally during cell perfusion with 100 μM Cd²⁺. Control traces underwent a clear decay that was surely due to Ca²⁺-dependent current inactivation (18). Nimodipine blocked by over 50% both the amplitudes of the initial calcium current peak [I(Ca(p))] and the late current [I(Ca(l))]; this blockade was fully reversible upon nimodipine washout. Given at the end of the experiment, Cd²⁺-elicited the expected I_on suppression.

Because I_on underwent inactivation, we felt it appropriate to calculate the blockade elicited by nimodipine at both traces of I(Cap) and I(Cat). We also calculated the reduction of the total Ca²⁺ entering the cell during the 50-ms test pulse, calculated as the I_on area (Q_on). Blockade of I(Cap) was 50% and that of I(Cat) rose to 64%. Blockade of Q_on was similar, 61% (Fig. 1B). The time course of the blockade elicited by nimodipine is shown in the example cell of Fig. 1C. Note that nimodipine elicited a maximal blockade after only 20 s of cell exposure to the drug; the recovery was initially fast and then a slower recovery period was observed.

Because the L component of I_on was more than half, we were interested in dissecting out the remaining current as a whole, rather than trying to find out the fractions of non-L current carried by N and PQ channels. To this aim we performed experiments similar to those of nimodipine, to find out the degree of blockade of I_on elicited by combined Agta + GVIA (Agta/GVIA). The family of I_on traces exhibited in Fig. 2A shows that Agta/GVIA caused a current blockade of ~35%. This current was only partially reversed upon toxin washout, in agreement with the reported slow washout of these toxins from their binding sites at the channels (16). Quantitative analysis indicated that Agta/GVIA blocked by 33% I(Cap) (Fig. 2B). Figure 2C shows the time course of I(Cap) before, during, and after Agta/GVIA washout. The blockade elicited by the toxins developed slowly to reach a plateau in ~90 s after toxin washout I_on recovered only partially and very slowly.

We also tested the effects of combined Nimo/Agta/GVIA. The family of I_on traces shown in Fig. 3A shows that this combination caused near full blockade of I(Cat) and that the blockade was only partially reversible. However, a small (15%) I(Cap) remained. Of note, the remaining I(Cap) underwent fast inactivation; thus it was plausible that this current was carried by blocker-resistant R-type VACCs (α₁₁,T, Cav2.3) (3–4) or by the low-VACC of the T subtypes that has been described to be present in a fraction of rat ECCs (9) as well as.

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in neonatal rat adrenal slices (25). Pooled data indicated that combined Nimo/Aga/GVIA blocked 79% of $I_{\text{Ca}(p)}$, 91% of $I_{\text{Ca}(l)}$, and 91% of $Q_{\text{Ca}}$ (Fig. 3B). Figure 3C shows the time course of $I_{\text{Ca}}$ blockade elicited by combined Nimo/Aga/GVIA in an example ECC; the initial fast blockade component was surely due to L channel blockade by nimodipine (Fig. 1C) and the ensuing slowly developing blockade to N/PQ channel blockade by Aga/GVIA (Fig. 2C). The fraction of current resistant to Nimo/Aga/GVIA was, however, blocked by Cd$^{2+}$ (Fig. 3C, right).

In another set of experiments, 2 mM Ca$^{2+}$ and a holding potential of −60 mV, conditions closer to physiology, were used. These experiments were not graphed, but the results were

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**Fig. 1.** Blockade by nimodipine (Nimo) of the whole cell calcium current ($I_{\text{Ca}}$) of rat embryo chromaffin cells (ECCs). Cells were voltage clamped at −80 mV and perfused with an extracellular solution containing 5 mM Ca$^{2+}$, under the perforated-patch configuration of the patch-clamp technique. Inward $I_{\text{Ca}}$ were elicited by 50-ms test depolarizing pulses to −10 mV (the usual voltage at which peak $I_{\text{Ca}}$ was obtained), regularly given at 10-s intervals. After patch perforation, $I_{\text{Ca}}$ amplitude stabilized at the 4th-5th test pulse, and then, Nimo was applied and subsequently washed out to allow current recovery. A: current traces obtained from an example cell under control conditions (Ctrl), after the cell perifusion with 3 μM Nimo or 100 μM Cd$^{2+}$, and the current recovery after washout of the compounds (wash traces). B: quantitative data from pooled cells were calculated as area of $I_{\text{Ca}}$ an indication of the total Ca$^{2+}$ entry into the cell during the 50-ms pulse ($Q_{\text{Ca}}$). For each individual cell, the initial Ctrl parameter was normalized to 100%, and the subsequent current traces were normalized as %control. C: time course of the effect of Nimo and Cd$^{2+}$ on $I_{\text{Ca}}$. Data are means ± SE of the number of cells and cultures shown in B. ***P < 0.001 with respect to control.

**Fig. 2.** Blockade by the combination of ω-agatoxin IVA plus ω-conotoxin GVIA (Aga/GVIA) given at 1 μM each of the whole cell calcium current ($I_{\text{Ca}}$) of rat ECCs. Experimental protocol as in Fig. 1 except for the fact here combined Aga/GVIA was used instead of nimodipine. A: example current traces before (Ctrl) and upon treatment with Aga/GVIA or Cd$^{2+}$ (100 μM). B: pooled data on the effects on $Q_{\text{Ca}}$ (ordinate). C: time course of $I_{\text{Ca}}$ peak amplitude obtained upon challenging an example cell with 50-ms depolarizing pulses applied at 10-s intervals before and during cell perfusion with Aga/GVIA or with Cd$^{2+}$ (top horizontal bars). Data are means ± SE of the number of cells and cultures shown in parenthesis in B. ***P < 0.05 with respect to control, Mann-Whitney’s rank-sum test.
as follows. At 3 μM, nimodipine inhibited Q_{Ca} by 60.1 ± 4.7% (15 cells of 3 different cultures). At 2 μM, ω-agatoxin IVA (Aga; 1 μM), and ω-conotoxin GVIA (GVIA; 1 μM) of the whole cell calcium current (I_{Ca}) of rat ECCs. Experimental protocol as in Fig. 1, except for the fact here the 3 blockers were applied in combination. A: example current traces obtained from an ECC before (Ctrl), during treatment with blockers or Cd^{2+}, and after their washout (wash). B: pooled data on the effects of blockers on Q_{Ca}. C: the time course of I_{Ca}, peak amplitude obtained upon challenging an example cell with 50-ms depolarizing pulses applied at 10-s intervals before (Ctrl), with the 3 blockers (top horizontal line) or with Cd^{2+} (100 μM) given at the end of the experiment to test that the cell was functionally viable. Data are means ± SE. ***P < 0.001 with respect to Ctrl current parameters.

Western blot analysis of the relative expression of the subtypes of high-VACCs in rat embryo adrenals. Western blot experiments were made to find out the relative expression of L, N, and PQ VACC-subtypes as membrane proteins extracted from rat embryo adrenals. The whole adrenals were used because it was not possible to dissect out the medulla from the cortex; thus VACC proteins expressed by the adrenal cortex could be contributing to channel density. This possibility was discarded by the fact the rat adrenal cortex mostly express T-type channels while high-VACCs are scarcely expressed if at all (7, 28, 32, 37).

Figure 4A shows an example immunoblot with the three bands corresponding to L, N, and PQ channels and the lower bands to control α-tubulin. Figure 4B shows the relative densities of L, N, and PQ channels that were 55 ± 3, 22 ± 3, and 23 ± 2%, respectively (n = 8 adrenal glands). The greater density of L-channel protein keeps pace with the higher contribution of this channel to I_{Ca}, compared with the lower contribution of N and PQ channels (see Figs. 1 and 2).

Effects of blockers of high-VACC subtypes on the hypoxia-induced elevations of cytosolic calcium concentrations. During cell depolarization Q_{Ca} was double for L channels with respect to non-L (N/PQ) channels; therefore, it was of interest to find out whether a similar contribution was found in the [Ca^{2+}]_{i} elevations triggered by hypoxia. We therefore investigated how nifedipine and Aga/GVIA affected the [Ca^{2+}]_{i} elevations elicited by hypoxia. To this aim we applied two sequential pulses of hypoxia (5% oxygen in the bath, 1-min duration, P1, P2) to each individual cell. This gradually augmented the [Ca^{2+}]_{i} to a peak that decayed slowly but remained elevated beyond the 1-min application of hypoxia.

The maximal amplitude ([Ca^{2+}]_{i} peak) and area ([Ca^{2+}]_{i} area) were calculated as explained in the example fura 2-loaded ECC of Fig. 5A; the area was calculated in the minute period of hypoxia, the same period taken for the calculation of the
cumulative HIS response (see Effect of blockers of high-VACC subtypes on the hypoxia-induced quantal catecholamine release). For comparative purposes, a [Ca\(^{2+}\)]\(c\) transient elicited by K\(^+\) was given to each individual cell at the beginning of each experiment. Note in Fig. 5A the fast activation of the K\(^+\)-induced [Ca\(^{2+}\)]\(c\) transients and its higher peak amplitude, compared with the slow activation of the hypoxic [Ca\(^{2+}\)]\(c\) transients. Figure 5B shows that the magnitudes of the area and peak [Ca\(^{2+}\)]\(c\), elicited by two sequential exposures to hypoxia (P1, P2), were similar. Figure 5C displays the two [Ca\(^{2+}\)]\(c\) transient traces elicited by hypoxia before (P1) and in the presence of 3 \(\mu\)M nifedipine added 3 min before and present during P2. The normalized pooled data indicated that in 20 cells the nifedipine diminished the [Ca\(^{2+}\)]\(c\) transient peak by 58 ± 2% and the [Ca\(^{2+}\)]\(c\) transient area by 48 ± 1%. Combined Aga/GVIA caused minor changes in the hypoxia [Ca\(^{2+}\)]\(c\) transients, as shown in the example traces of Fig. 5D. Pooled data from 33 cells exposed to Aga/GVIA indicated a 12 ± 4% diminution of [Ca\(^{2+}\)]\(c\) peak and a 11 ± 4% diminution of [Ca\(^{2+}\)]\(c\) area; however, these changes did not reach the level of statistical significance.

Effect of blockers of high-VACC subtypes on the hypoxia-induced quantal catecholamine release. Once the relative contribution of L and N/PQ channels to the whole cell I\(_{Ca}\) was deciphered, we explored how the blockers of these two Ca\(^{2+}\) entry pathways contributed to the hypoxia-induced secretion of catecholamine (the HIS response). To this aim, we first characterized the HIS response in ECCs challenged with a hypoxic solution (5% O\(_2\) in the cell perifusion fluid); this solution was perifused onto the cell surface for 1 min and was repeated two times more at 5-min intervals.

Characteristically, during the first hypoxia pulse (P1) a spike burst was generated 3–5 s after the initiation of hypoxia. This spike burst was then followed by the appearance of more infrequent events that were scattered along the 1-min exposure to hypoxia. On returning to normoxia the cell was promptly silenced. Two more additional hypoxia stimuli elicited similar responses, as documented in the spike traces taken from an
example ECC (Fig. 6A). Total secretion per hypoxia stimulus was calculated by counting the number of spikes and the summatory of spikes areas (Qamp in pC) found along the 1-min hypoxia. Pooled averaged P1 traces from 74 cells had 67 ± 5.9 spikes and 49.2 ± 3 pC. Within each individual cell, data of P2 and P3 were normalized as % of P1. Thus, in control cells subjected to three successive hypoxia challenges P2 was similar to P1; however, a 17% significant decrease of spike number and Qamp was observed in P3 (18 cells, Fig. 6C). At 100 μM, Cd²⁺ blocked the HIS response by 91% suggesting that Ca²⁺ entry through VACCs was responsible for the generation of such response (Fig. 6, B and D).

The next question was whether nimodipine and Aga/GVIA caused a blockade of the HIS response that in relative terms reminded us of the relative blockade of ICa elicited by these agents (Figs. 1 and 2). To explore this question we have recourse to a protocol similar to that used in Fig. 5 namely, the application of three sequential hypoxia pulses before (P1), in the presence of blocker applied 2 min before and during P2, and the application of P3 after 5 min of blocker washout. With this protocol, the effect of a given VACC blocker on the HIS response and its reversibility upon washout can be tested within the same cell. An example of this protocol is shown in Fig. 7A whereby the ECC cell was exposed to 3 μM nimodipine that markedly depressed spike frequency during P2; upon drug washout, spike secretory events in P3 returned to the level of P1. Averaged results from 11 cells indicated that nimodipine caused 88 ± 2% blockade of spike number and 82 ± 3% blockade of Qamp. Nifedipine at 1 μM also elicited 84 ± 7% blockade of spike number and 86 ± 5% blockade of Qamp (n = 8 cells).

Compared with dihydropyridines, combined Aga/GVIA produced a milder blockade of the HIS response, namely, 40.4 ± 6.2% of spike number and 32.1 ± 6.5% of Qamp (n = 14 cells) (Fig. 7, B and D). Upon toxin washout, this blockade was only partially reversed. We also explored the effects of toxins given separately. Thus, in 14 ECCs, GVIA reduced spike number by 13.5 ± 5% and Qamp by 19.3 ± 4.7%. On the other hand, in 11 cells Aga caused 14.9 ± 8.3% blockade of spike number and 17.7 ± 7.3% of Qamp. Thus combined Aga/GVIA exhibited additive effects in blocking secretion.

Frequency distribution of secretory spike events along the hypoxia stimulus. Hypoxia causes a mild depolarization in rat ECCs (40) and an enhanced rate of action potential firing (8). Action potentials will surely recruit the three subtypes of high-VACCs to augment Ca²⁺ entry and the ensuing release of catecholamine. At early life during pregnancy, during delivery or neonatal stages, hypoxic conditions may last for minutes or longer. Initially after the hypoxia stress, a greater catecholamine surge may be required for adaptation to the new environment. At later stages, the catecholamine surge may be sustained but at a lower rate. We attempted to find out whether this condition was present in the particular stimulation pattern used in our experiments, that is 1-min exposure to hypoxia of ECCs. We therefore analyzed the number of secretory spike events at 10-s intervals within each 60-s trace, obtained in...
single control cells, in cells treated with Aga/GVIA and in cells treated with nimodipine.

The distribution of secretory amperometric events during exposure of ECCs to hypoxia seemed to differ along the 60-s duration of the stimulus. An initial high-frequency number of spikes developed later on into a lower frequency of secretory events that were scattered along the hypoxia stimulating time period, as illustrated in the original amperometric traces of Figs. 6 and 7. It was therefore of interest to explore whether this frequency distribution followed a given pattern or, rather, it displayed a random distribution. We then counted the number of spikes at 10-s intervals of the HIS response of 74 ECCs from 16 different cell cultures.

The mean total number of spikes produced along the 60-s hypoxic stimulus was 67.1 \pm 5.9. During the first 10-s, a burst of 25.1 \pm 2.3 spikes were produced (Fig. 8D). In other words, \sim 40\% of all secretory events were produced at the spike burst that initiated the HIS response (Fig. 8A). From this time onwards, a gradual time-dependent decay was produced in such a manner that by the end of the stimulus the spike frequency was \sim 10\% (Fig. 8, A and D).

In the presence of Aga/GVIA applied to 14 cells of 4 cultures, the mean total number of spikes produced along the 60-s hypoxic stimulus was 48.5 \pm 3.4, which means a 27\% reduction in the total number of spikes respect to the control hypoxia stimulus (Fig. 8B). During the first 10-s period, a burst of 14.5 \pm 0.9 spikes were produced; this represents a reduction of 42.2\% compared with the control (Fig. 8, B and F). We also analyzed the spikes produced in the presence of nimodipine that in the first 10-s period were only 7 \pm 0.7 spikes (Fig. 8F). Spike number was abruptly decreased in the second time period (10–20 s), from 55\% to 22\% (Fig. 8C), indicating a fast inactivation of the HIS response.

The decay of spike number with time in the three cases was best fitted to a single exponential curve (Fig. 8G). In control cells the time constant (τ) for the decay of secretion was 17.2 \pm 1.1 s (74 cells). In the case of cells exposed to nimodipine the τ decreased to 11.2 \pm 0.8 s (12 cells). Finally, the secretion decay in cells treated with Aga/GVIA exhibited a τ of 20.5 \pm 1.4 s (14 cells). The differences between these three τ values were statistically significant at P < 0.01 for nimodipine vs. control and P < 0.05 for Aga/GVIA vs. control (Fig. 8H). Additionally, the statistical difference between the τ for secretion responses in ECCs treated with nimodipine and Aga/GVIA was highly significant (Fig. 8H). This suggests that secretion mediated by N/PQ channels (cells treated with nimodipine) inactivated considerably faster than the secretion mediated by L channels (cells treated with Aga/GVIA).

DISCUSSION

Using the perforated-patch configuration of the patch-clamp technique, 2 or 5 mM Ca\(^{2+}\) as charge carrier, and selective blockers of high-VACC subtypes, we have found here that rat
ECCs had whole cell Ca\(^{2+}\) currents that was contributed 60% by L channels and 30% by non-L (N/PQ) channels. This proportion differs from a previous study also performed in our laboratory but in adult rat chromaffin cells that showed \(I_{\text{Ca}}\) to be equally contributed by L and N/PQ channels, 50% (13). These differences could be attributed to dissimilar protocols and the use of different concentrations of external Ca\(^{2+}\) or blockers. However, inasmuch as Western blots demonstrated relative protein expression of proteic L channels and N/PQ channels was 55 and 40%, respectively, the conclusion that Ca\(^{2+}\) entry monitored as QCa is near double through L channels with respect N/PQ channels seems to be fairly correct for rat ECCs.

The mild and sustained hypoxia-elicited \(\Delta[\text{Ca}^{2+}]_c\) sharply differed from the fast, large and transient \(\Delta[\text{Ca}^{2+}]_c\) elicited by a pulse of 75 mM K\(^{+}\) that drives the membrane potential of chromaffin cells from −60 mV to 0 mV (30). Although hypoxia also elicits chromaffin cell depolarization at early life, this depolarization is milder than that of K\(^{+}\) and is accompanied by action potential generation (8, 21, 40). This different pattern of cell depolarization could explain the different time courses of \(\Delta[\text{Ca}^{2+}]_c\) elicited by K\(^{+}\) or hypoxia in rat ECCs. It may also explain the sustained elevation of \(\Delta[\text{Ca}^{2+}]_c\) during the 1 min of hypoxia cell exposure that was blocked 50% by nifedipine and 10% by Aga/GVIA. An explanation for the different fractional blockade of \(I_{\text{Ca}}\) and \(\Delta[\text{Ca}^{2+}]_c\) could be...
found in the context of the drastic different protocols used for their monitoring namely, 50-ms depolarizing test pulses applied to voltage-clamped ECCs in the former, and 1-min exposure to hypoxia of cells with their membrane potential free in the latter. In the first case, the $\Delta[Ca^{2+}]_i$ transient is mostly contributed by Ca$^{2+}$ entering through VACCs during the 50-ms duration of the strong depolarizing pulse; in this short time, little inactivation of VACCs and/or Ca$^{2+}$ redistribution in organelles occur. In the second case, however, the mild depolarization taking place during 1-min hypoxia generates $\Delta[Ca^{2+}]_i$ that is the result of Ca$^{2+}$ entry through VACCs as well as its redistribution into and exit from the endoplasmic reticulum store and mitochondria. The sustained [Ca$^{2+}]_i$ elevation must therefore be an end result of the bidirectional Ca$^{2+}$ fluxes between cell Ca$^{2+}$ influx and efflux and the different cell compartments.

This mild but sustained $\Delta[Ca^{2+}]_i$ profile could explain the profile of single-spike secretory events generated during the challenge of ECCs with 1-min hypoxia. It is known that mild [Ca$^{2+}]_i$ elevations favor the transport of secretory vesicles from a reserve pool to subplasmalemmal exocytotic sites of chromaffin cells. This mild [Ca$^{2+}]_i$ elevation will also trigger the exocytotic release of catecholamine from docked vesicles. During the first few seconds of hypoxia, depolarization and superimposed action potentials will open the L and N/PQ VACCs. Later on, N/PQ channels undergo voltage- and Ca$^{2+}$-dependent inactivation while L channels remain open for substantially longer times.

This sequential opening and closing of VACCs is compatible with the HIS response of rat ECCs that trigger secretory spike events at a higher rate during the first 10 s to gradually decay in the next 50 s of cell exposure to hypoxia. In this context, we propose that early after the initiation of a hypoxic stressful conflict at early life, L as well as N/PQ high-VACCs. Later on, when N/PQ channels inactivate, the slow-inactivating L channels maintain such response at a lower rate. These data suggest that a pharmacological intervention on the L-subtype and/or the N/PQ-subtypes of VACCs may have clinical relevance in preventing hypoxia-elicited brain damage during fetal life, at birth, or during early perinatal life. They also suggest that the use of L-type calcium channel blockers during pregnancy, i.e., to treat hypertension of the mother, could impair adaptation to hypoxic stress of the fetus, at birth and during lactation.

In conclusion, with the combination of patch-clamp and Western blot techniques, we have found that rat ECCs express functional high-VACCs of the L-subtype (60%) and N/PQ subtypes (30%). The initial burst HIS response may be controlled by L as well as N/PQ high-VACCs. Later on, when N/PQ channels inactivate, the slow-inactivating L channels maintain such response at a lower rate. These data suggest that a pharmacological intervention on the L-subtype and/or the N/PQ-subtypes of VACCs may have clinical relevance in preventing hypoxia-elicited brain damage during fetal life, at birth, or during early perinatal life. They also suggest that the use of L-type calcium channel blockers during pregnancy, i.e., to treat hypertension of the mother, could impair adaptation to hypoxic stress of the fetus, at birth and during lactation.

**REFERENCES**


**AUTHOR CONTRIBUTIONS**


