Selective pressure modulation of synaptic voltage-dependent calcium channels— involvement in HPNS mechanism

Ben Aviner a, *, Gideon Gradwohl b, Alice Bliznyuk a, Yoram Grossman a

a Department of Physiology and Neurobiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel
b Department of Physics, Jerusalem College of Technology, Jerusalem, Israel

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Abstract

Exposure to hyperbaric pressure (HP) exceeding 100 msw (1.1 MPa) is known to cause a constellation of motor and cognitive impairments named high-pressure neurological syndrome (HPNS), considered to be the result of synaptic transmission alteration. Long periods of repetitive HP exposure could be an occupational risk for professional deep-sea divers. Previous studies have indicated the modulation of presynaptic Ca2+ currents based on synaptic activity modified by HP. We have recently demonstrated that currents in genetically identified cellular voltage-dependent Ca2+ channels (VDCCs), CaV1.2 and CaV3.2 are selectively affected by HP. This work further elucidates the HPNS mechanism by examining HP effect on Ca2+ currents in neuronal VDCCs, CaV2.2 and CaV2.1, which are prevalent in presynaptic terminals, expressed in Xenopus oocytes. HP augmented the CaV2.2 current amplitude, much less so in a channel variation containing an additional modulatory subunit, and had almost no effect on the CaV2.1 currents. HP differentially affected the channels’ kinetics. It is, therefore, suggested that HPNS signs and symptoms arise, at least in part, from pressure modulation of various VDCCs.

Keywords: hyperbaric pressure • voltage-dependent calcium channel • high-pressure neurological syndrome

Introduction

Humans, as most terrestrial mammals, are sensitive to hyperbaric pressure (HP). Pressure is a thermodynamic variable affecting the kinetics and steady-state equilibrium of biological processes. Membrane phospholipids fluidity, ion channels, receptors, enzymes and other proteins functions are all potential targets for HP effects [for review, see (1)]. Exposure of humans to HP (usually above 1.0 MPa) causes a constellation of signs and symptoms known as the high-pressure neurological syndrome (HPNS). HPNS is the major problem associated with HP environment, as it occurs due to the effects of pressure per se [2, 3]. Divers at depth above 90 msw may exhibit various symptoms, such as dizziness, nausea, tremors, vision and auditory disturbances, decrements in locomotion [4, 5] and cognitive performance [3, 6–9], changes in electroencephalography (EEG) and sleep disorders [10], myoclonus [5], convulsions and a loss of consciousness (for review, see [11]). Alteration in synaptic transmission is a plausible explanation for the HPNS (for review, see [12]). Indeed, HP suppressed synaptic activity in most preparations. This suppression may occur via modulation of postsynaptic ionotropic receptors activity [13, 14], decreased AP amplitude [15], slowed kinetics [16, 17], depression of neurotransmitter release [18–21] and modulation of its quantal release mechanism [22–24] and decreased vesicle fusion [13, 19]. Most of these synaptic processes are known to be Ca2+ dependent. Earlier studies on crustacean neuromuscular synapses that examined the relationship between [Ca2+]o, excitatory post synaptic potential (EPSC) amplitude and facilitation [25–27] have suggested that pressure depresses Ca2+ influx rather than intracellular removal of Ca2+. Further support to this notion was the observations that low [Ca2+]o partially mimics the effects of HP [20, 27] and high [Ca2+]o can antagonize to some extent HP depression of current amplitude [15, 25, 28]. In fact, modulation of presynaptic Ca2+ currents at HP has been already suggested [15, 29, 30]. We, therefore, postulated that the major mechanism by which HP alters synaptic transmission is the modulation of Ca2+ influx into the presynaptic terminals through voltage-dependent Ca2+ channels (VDCCs).

Various VDCC subfamilies are known, characterized by their electrophysiological and pharmacological traits: CaV1.1-4 (L-Types), CaV2.1 (PQ-type), CaV2.2 (N-type), CaV2.3 (R-type) and CaV3.1-3 (T-types), comprising the a1, a2δ, β and γ subunits [31, 32]. The major difference between the channels results from the variation in the a1 subunit, which holds the ion conducting pore, the voltage sensor, the channel gating section and the known sites of channel regulation by second messengers, drugs and toxins [32]. The a2δ, β and γ subunits

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have a modulatory effect on the ionic flux via $\alpha_1$ (for review, [33, 34]), including its kinetic properties and voltage dependence. For example, the $\beta_3$ subunit slows channel inactivation in many subunit combinations. On the other hand, the coexpression of $\gamma_0$ subunits [35, 36] and $\gamma$ subunits [37] has a smaller functional effect. Lately, it has been suggested that the $\gamma_0$ subunit is regulating the CaV2.2 indirectly by counteracting Gs$\gamma$-mediated effects such as slowing of activation and voltage-dependent inactivation [38]. Notwithstanding, a functional recombinant channel does not always require expression of all subunits.

Early findings of HP effects on VDCC currents were indirectly obtained (for review, see [39]) from various preparations [27, 40–44]. The sensitivity of the CaV2.2 channel to HP [40, 41] was suggested, while the CaV2.1 channel was rendered HP resistant [13, 17]. Furthermore, Talpalar et al. [28] have postulated, based on mathematical modelling of experimental synaptic depression at HP, that rat dentate gyrus synapse is composed of pressure-sensitive (probably CaV2.2-dependent) and pressure-resistant (probably CaV2.1-dependent) independent modules of releasable vesicles pools.

In another attempt to study the HP selectivity of real currents, we have lately recorded extracellularly two components of Ca$^{2+}$ currents in frog presynaptic terminals [15]. Partial pharmacologic identification has suggested that a fast component is N-type like and a slow component is probably one of the L-type channels. Hyperbaric pressure differentially affected the currents; the fast Ca$^{2+}$ currents being highly depressed, while the slow Ca$^{2+}$ currents were much less inhibited.

The difficulty in positively identifying the Ca$^{2+}$ currents in ex vivo experimental tissues, the presence of more than one type of current in each neuron either in the presynaptic terminals or soma and dendrites, the diversity of channels in various preparations and the technical difficulties in performing the pressure experiments have presented us with a major challenge. We have, therefore, embarked on a long-term study that was aimed at overcoming these obstacles: direct measurement of VDCC currents by expressing the genetically identified cRNAs of the channels in frog oocytes under HP conditions. Recently, we have performed such a study for the first time on VDCCs currents of CaV1.2 and CaV3.2 [30], demonstrating selective and sometimes transient HP effects on the channels: CaV1.2 being poten- tiated, while the CaV3.2 is depressed.

In the present report, we extended our study to include two additional VDCCs, CaV2.1 and CaV2.2, which are mainly, but not exclusively, present at the neuronal presynaptic terminals. It is hoped that comprehensive understanding of the behaviour of each VDCC at HP will enable us to refine a model of activity [39] based on known channels spatial distribution along the neurons. This could elucidate the HPNS mechanism and may enable us to reduce or even eliminate its short- and long-term consequences.

Materials and methods

Oocytes extraction and cRNA injection

Oocytes of a *Xenopus laevis* mature female frog were surgically extracted from its ovary and treated with 1.5 mg/ml collagenase for 30–60 min. to remove connecting tissue. Suitable oocytes were sorted out by size, quality and developmental stage (VI), and kept in NDE96 solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 2.5 sodium pyruvate; 50 µg/ml gentamycin; 5 HEPES pH 7.5. Handling of frogs and oocytes extraction procedure were approved by the Ben-Gurion University of the Negev’s ethics committee for the care and the use of animals and are in compliance with international laws and policies.

cRNAs of the subunits of PQ or N-type Ca$^{2+}$ channels (CaV2.1 or CaV2.2, respectively) were synthesized from human, rat, mouse and rabbit cDNA by *in vitro* transcription with T7 or SP6 Amplitak High-Yield Message Maker Kit (Epicentre Technologies, Madison, WI, USA). Oocytes were then injected with the specific cRNA mix (2.5 ng) encoding for the pertinent subunits to express CaV2.1 or CaV2.2 and were kept in an incubator for 4–5 days at 18°C in NDE96 solution. The following subunits were used: $\alpha_{1A} + \beta_1 + \gamma_0$, comprising the CaV2.1; and $\alpha_{1B} + \beta_1 + \gamma_0$ or $\alpha_{1B} + \beta_1 + \gamma_0 + \gamma_2$, comprising the CaV2.2.

Electrophysiological recordings

Four to five days after injection, the oocytes were placed in a specially designed bath, and two-electrode voltage clamp experiments with 10-mV increments and 5-sec. interval between –70 and 40 mV were performed inside a compression chamber, utilizing an AXOCLAMP 2B amplifier (Molecular Devices, Axon Instruments, Inc., CA, USA), WinWCP pulse generating software by Strathclyde University, Axon Instruments DIGIDATA 1322A, and AxoScope 9.2 software. Sharp glass microelectrodes were fabricated using Sutter Instrument P-1000 micropipette puller, filled with 3 M KCl, tip resistance <1.5 MΩ. The oocytes were penetrated by the electrodes, and only then the bath was carefully inserted into the chamber, slid onto an electric socket with preinstalled wires crossing the chamber wall. While in the chamber, each oocyte was continuously perfused with a Ba$^{2+}$ solution containing (in mM): 20–40 Ba(OH)$_2$, 50 NaOH, 2 KOH and 5 HEPES, titrated to pH 7.5 with methanesulfonic acid. Ba$^{2+}$ was used as charge carrier, replacing the Ca$^{2+}$ ions, to avoid Ca$^{2+}$-dependent inactivation and the activation of Ca$^{2+}$-activated Cl$^{-}$ channels (Cl$^{-}_{Ca}$), known to be endogenously expressed in oocyte membrane [45]. We have recently demonstrated in identical experimental system that blocking the Cl$^{-}_{Ca}$ current does not interfere with the HP effect on VDCCs [30]. Both CaV2.1 and CaV2.2 also have higher conductance to Ba$^{2+}$ [46], allowing measurement of minute currents that otherwise would have been unnoticed. The solution, saturated with air at atmospheric pressure, was introduced into the chamber by the use of a high-pressure pump (Minipump; LDC Analytical Inc., Riviera Beach, FL, USA) at room temperature (24–25°C), at a rate of 1.5–2 ml/min. Temperature was constantly monitored throughout the experiments by the use of a thermistor submerged in the solution in the vicinity of the oocyte groove. Deviation of only ±0.5°C was allowed from the control temperature for later measurements. We have also demonstrated in our recent study [30] that the small reversible adiabatic temperature changes are not responsible for the response of the VDCCs to HP. In addition, we have proved that the voltage and currents measurements in our setup are stable along the relatively long duration of compression and decompression. Typical recorded traces are shown in Figure 1. Voltage traces are not ‘command voltages’ but rather the actual recording of the oocyte transmembrane potential. Holding potential was –80 mV (see example in Fig. 1A). The duration of each depolarizing step was 500 msec., which
was preconditioned by a 100-msec. hyperpolarizing step to −90 mV to release the VDCC from partial inactivation. The latter was also used to calculate and monitor the oocytes' instantaneous input resistance for measuring and subtracting the leak currents, which were accounted for at each recorded trace separately, thus unmasking the net VDCC current.

Every series of depolarizing pulses was used to construct an I-V curve and repeated at least three times to verify stability of the currents, as was previously described (fig. 1 in [30]). Recorded traces with voltage fluctuation greater than 2 mV during depolarization were disregarded. We studied HP effects on I-V curve, maximal currents, activation and inactivation functions, channel kinetics such as time to peak (TTP) and time constants (τ), and voltage dependency. Maximal currents were measured at the minimal point of the current curve. Inactivation (I/Imax) was measured towards the end of the depolarizing step in comparison to the measured maximal current (as above). A fit was calculated for each decaying section of the current in every recorded trace according to a biexponential equation [47] defining two time constants for decay:

\[
\text{Fit} = -A_1\exp\left(-t/\tau_{\text{fast}}\right) - A_2\exp\left(-t/\tau_{\text{slow}}\right) + C
\]

For the rising phase and the tail currents, a single exponential fit was performed. All fits were calculated between the curves' normalized values of 0.1 and 0.9.

Activation volume (\(\Delta V^i\)) was calculated for time constants of channel activation, inactivation and deactivation under normobaric and hyperbaric conditions, following the known equation [48]:

\[
\Delta V^i = R(TC\ln(\tau/P))\tau
\]
Helium compression

After control measurement taken at 0.1 MPa, compression steps to 0.5, 2.5 and 5.0 MPa were performed by compressed helium. Compression was done manually at a rate of approximately 0.25–0.5 MPa/min. and never exceeded 1.0 MPa/min. Helium was used instead of air due to its inert quality and the need to avoid known nitrogen narcosis and oxygen toxicity-related effects [49]. Principally, compression with helium does not change the other gases (primarily oxygen and nitrogen) partial pressure. Here, the chamber gaseous content was flushed with helium during compression due to the need to drain the excess of physiological solution, and thus the oxygen and nitrogen partial pressure was reduced over time. However, the oocytes were continuously perfused with fresh solution equilibrated with air at 0.1 MPa, and thus the oocytes were exposed to normal partial pressure of oxygen and nitrogen. All pressure units are absolute.

Statistical analysis

The full set of parameters was calculated off-line for each recorded trace separately, considering the instantaneous input resistance and leak currents where appropriate, using a dedicated self-designed MATLAB software program. The data were exported to Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). Repetitive measurements of I-V curves, verifying stability of the measured currents, were averaged and used as a single value for each depolarization step, which in turn was used for averaging with results from other oocytes. The same was done for all other parameters. Each oocyte was used as its own control, and thus values were normalized to 0.1 MPa when needed. When data from more than one oocyte were pooled, binning was performed relative to the voltage generating the maximal current in the I-V curve (Vmax); hence, in figures representing these data (Figs 2–9B, D and F), the X-axis title is AV. The actual X values in all figures were determined by averaging the actual recorded voltages during depolarizing steps. Hence, minor shifts of 1–2 mV from the values indicated in the X-axis may occur. Paired sample t-test was used to analyse the significance of the results: each pooled value was compared with its pertinent pooled value at 0.1 MPa for the same AV. Significant difference (P < 0.05) is represented by asterisks in figures.

Results

Unaffected current in CaV2.1

As expected, the amplitude of Ba2+ currents in CaV2.1 was not affected in oocytes exposed to HP (2.5–5.0 MPa, see example in Fig. 2A). Comparing the normalized maximal currents (negative peak in I-V curve) at Vmax shows that compression to 2.5 and 5.0 MPa did not significantly change the maximal currents (−8 ± 10% and −2 ± 3%, respectively, P > 0.4, n = 7–9, Fig. 2B). Decompression to 0.1 MPa also did not significantly affect the maximal current; it remained slightly depressed by −14 ± 6% (P > 0.3, n = 6). Neither the threshold voltage nor Vmax were affected by HP.

Augmented current in CaV2.2

Surprisingly, Ba2+ currents in CaV2.2 were significantly increased at HP (0.5–5.0 MPa) in a dose-dependent manner (see example in Fig. 2C), in contrast to the expectations based on previous studies (see Introduction). Compression to 2.5 and 5.0 MPa caused a similar augmentation of the maximal currents at Vmax by 132 ± 54% and 123 ± 6% of control values, respectively (Fig. 2D, average ± SEM, P < 0.01, n = 7–9); therefore, lower HP steps to 1.1 MPa and 0.5 MPa were performed in subsequent experiments in order to reveal the threshold for HP effect. However, the maximal current at 1.1 MPa was augmented in a similar manner by 122 ± 56% (P < 0.01, n = 3, data not shown), and only a lower HP perturbations to 0.5 MPa had a weaker effect on the maximal currents at Vmax: a 61 ± 16% augmentation (P < 0.05, n = 12). Neither the threshold voltage nor the Vmax were affected by HP. Decompression to 0.1 MPa only partially recovered the current, which remained augmented by 73 ± 15% (P < 0.05, n = 7).

CaV2.2 expressed including the γ2 subunit

The functionality of a recombinant channel is vastly dependent on the subunits constructing it, their type and isoforms, etc. The unexpected HP-induced current augmentation in the CaV2.2 led us to speculate whether this recombinant channel is affected differently by HP than the native one. In order to elucidate this issue, we have repeated the experiments following expression of the CaV2.2 including the one subunit that was excluded thus far as it is not essential for the channels’ functionality, the γ2. Although identifying it as a classic subunit of this channel is still debatable, its role in modulating it is not [38, 50].

Indeed, the CaV2.2 expressed including the γ2 subunit (CaV2.2,γ2) has reacted differentially to HP perturbation. For example, the augmentation of currents witnessed in the CaV2.2 was substantially subdued (Fig. 2E and F).

Channels’ conductance

The cumulative conductance for the population of the channels (‘input conductance’ of the oocyte) calculated relatively to the membrane potential shows similar results to the general findings in the I-V curves (see examples in Fig. 3A, C and E). High HP (2.5–5.0 MPa) increased the conductance in the CaV2.2 and CaV2.2,γ2 channels (Fig. 3D and F) but did not have a consistent effect in the CaV2.1 channel (Fig. 3B). On average, the change from threshold to maximal normalized response occurred within a 50-mV depolarization range for the CaV2.2 and CaV2.2,γ2 channels and only 30 mV for the CaV2.1 channel.

Currents inactivation

We have previously demonstrated that in the absence of Ca2+ ions in the solution, the Ca2+-dependent inactivation of these VDCC is
eliminated [30], leaving only the voltage- and time-dependent inacti-
vation that can be evaluated as the ratio between the remaining cur-
rent at the end of the depolarizing voltage step and its maximal value
(I_{end}/I_{max}; see examples in Fig. 4A, C and E). All channels demon-
strated a greater inactivation at strong depolarizations, as expected
for these VDCCs. For the CaV2.1 channel, HP did not have a consis-
tent or significant effect on inactivation (Fig. 4B). For the CaV2.2
channel, inactivation tended to be stronger when large currents were
evoked (ΔV ~10 to 20 mV) at HP, but was weakened by it around
threshold voltage or towards the reversal potential (e.g. ΔV ~20, 40
mV, respectively). Decompression relieved that effect (Fig. 4D).
For the CaV2.2,γ2 channel, inactivation was weakened at HP of 2.5–
5.0 MPa at the whole voltage range of the channel activity, but only at
a narrower voltage range (ΔV ~10 to V_{Imax}) at 0.5 MPa (Fig. 4F).
Decompression did not recover inactivation to control values.

**Currents kinetics: time to peak**

We have recently demonstrated that HP can affect the kinetics of
VDCC current (Aviner et al.) [30]. If the VDCC kinetic parameters
such as the rates of activation, inactivation and deactivation of the
current are affected by HP, that may change the maximal current
and the total ionic flux through the channel. We have, therefore,
measured the time passing from the stimulating depolarizing step
to the development of I_{max} (TTP). Examples can be seen in Fig-
ure 5A, C and E. Time to peak was not altered by HP in the CaV2.1
channel, excluding a tendency for an increase at V_{Imax} at 0.1 MPa, nor
was it changed by decompression (Fig. 5B). It can be seen that
a barely threshold depolarization led to a longer TTP value due to
the indecisive recruitment of the channels population. For the
CaV2.2 channel, the HP effect on TTP was complex. At 0.5 MPa,
TTP was decreased; at 2.5 MPa, it was decreased for \( V_{\text{Imax}} \) and up to \( \Delta V 20 \text{ mV} \), but increased below \( V_{\text{Imax}} \); and at 5.0 MPa, it was slightly increased below \( V_{\text{Imax}} \) range (Fig. 5D). Decompression recovered TTP to control values. For the Ca\(_{\text{V2.2+}}\) channel, TTP was elongated up to \( \Delta V 20 \text{ mV} \), more clearly at high HP (2.5, 5.0 MPa).

**Currents kinetics: \( \tau_{\text{Rise}} \)**

The time constant of the rising phase of the current, \( \tau_{\text{Rise}} \), is another useful parameter to evaluate the activation of the current (Fig. 6A, C and E). Hyperbaric pressure of 5.0 MPa elongated \( \tau_{\text{Rise}} \) of Ca\(_{\text{V2.1}}\) at a narrow depolarization range (\( \Delta V \) –10 to \( V_{\text{Imax}} \)), whereas at 2.5 MPa, it showed no significant change (Fig. 6B). The maximal increase in \( \tau_{\text{Rise}} \) of Ca\(_{\text{V2.2}}\) was at 2.5 MPa at a wider depolarization range (\( \Delta V \) –10 to 10 mV), whereas a smaller change was observed at 5.0 MPa (Fig. 6D). Hyperbaric pressure had almost no statistically significant effect on \( \tau_{\text{Rise}} \) in the Ca\(_{\text{V2.2+}}\) channel. Decompression recovered \( \tau_{\text{Rise}} \) back to control levels in all channels.

**Currents kinetics: fast \( \tau_{\text{Decay}} \)**

A change in the inactivation value (\( I_{\text{end}}/I_{\text{max}} \)) could originate from an effect on the channels’ rate of decay, as \( I_{\text{end}} \) is measured at the end of
the depolarizing step and not under steady-state conditions. The
decay of VDCCs current is known to have two time constants, fast
and slow, which are commonly attributed to voltage and Ca²⁺ inacti-
vation, respectively. However, even with Ba²⁺ as the charge carrier,
the decaying current could not be fitted satisfactorily using a single
exponent.

For the Caᵥ₂.₁, as expected by the lack of consistent change
in inactivation, HP did not cause a clear change in the fast τ_decay
(τ_decay_fast, Fig. 7A and B). For the Caᵥ₂.₂, a considerable shortening
of τ_decay_fast at HP was observed throughout the activity range of
the channel even at 0.5 MPa (Fig. 7C and D), while decompression gen-
erally relieved this effect. The HP effect was reversed in the Caᵥ₂.₂⁺/₂
channel, where τ_decay_fast was elongated (Fig. 7F).

**Currents kinetics: slow τ_decay**

The slow τ_decay (τ_decay_slow) in all channels was elongated by stronger
depolarizations at 0.1 MPa (Fig. 8A, C and E), similarly to previous
findings in VDCCs [30]. For the Caᵥ₂.₁ channel, the τ_decay_slow was
almost entirely not affected by HP, as may be predicted by inconsis-
tent effect on its inactivation (Fig. 8A and B). For the Caᵥ₂.₂ channel,
τ_decay_slow was shortened by high HP (2.5–5.0 MPa) at suprathresh-
old depolarization (ΔV > 10 mV and above), but compression to
lower HP of 0.5 MPa led to a mixed effect: generally elongating τ_decay
below V_Imax and shortening it above V_Imax. Decompression
eliminated this effect almost entirely (Fig. 8C and D). In the Caᵥ₂.₂⁺/₂
channel, the effect of high HP (2.5–5.0 MPa) was also reversed,
elongating \( \tau_{\text{Decay Slow}} \) above \( V_{\text{max}} \), but low HP (0.5 MPa) had no effect (Fig. 8E and F). Decompression only partially relieved the HP effect.

**Currents kinetics: \( \tau_{\text{Tail}} \)**

The tail current time constant (\( \tau_{\text{Tail}} \)), representing the kinetics of the channels' deactivation, was shortened by increasing depolarization in all channels (see example in Fig. 9A, C and E). Hyperbaric pressure elongated \( \tau_{\text{Tail}} \) in the \( \text{Ca}_{\text{V2.2}} \) and \( \text{Ca}_{\text{V2.2}^+} \) channels almost throughout their activity range (Fig. 9D and F), but only up to \( V_{\text{max}} \) in the \( \text{Ca}_{\text{V2.1}} \) (Fig. 9B). Decompression recovered \( \tau_{\text{Tail}} \) in \( \text{Ca}_{\text{V2.1}} \), but not so much in the \( \text{Ca}_{\text{V2.2}} \); \( \tau_{\text{Tail}} \) remained elongated at depolarizations below \( \Delta V \) 20 mV in the \( \text{Ca}_{\text{V2.2}} \) channel and below \( V_{\text{max}} \) in the \( \text{Ca}_{\text{V2.2}^+} \) channel.

**Activation volume (\( \Delta V^\dagger \))**

\( \Delta V^\dagger \) values were calculated from the change in the rate of processes under hyperbaric conditions compared with control.
pressure, as described in the Materials and methods. \( \Delta V^\text{‡} \) serves as a tool for assessing the sensitivity of a molecule to pressure perturbation, quantifying it in comparable values. Table 1 summarizes \( \Delta V^\text{‡} \) values for the tested VDCCs for 2.5 MPa. Generally, the results correspond to both sensitivity and trend of the changes described above.

A summary of HP effects on these channels is given in Table 2. Overall, \( \text{Ca}_\text{2.1} \) was not significantly affected by HP, whereas the \( \text{Ca}_\text{2.2s} \) channels were HP sensitive. Although \( I_{\text{max}} \) and the conductance showed the same trend, interestingly excluding \( \tau_{\text{Tall}} \), all other parameters measured in \( \text{Ca}_{\text{2.2s}} \) demonstrated an altered response to HP compared with \( \text{Ca}_\text{2.2} \): decreased inactivation value, increased \( \tau_{\text{Decay Fast}} \) and \( \tau_{\text{Decay Slow}} \), and unaffected \( \tau_{\text{Rise}} \) (Table 2).

**Discussion**

**Current activation**

**Currents’ amplitude**

As demonstrated in our previous direct [30] and indirect [15, 20] measurements of currents in VDCCs at HP, pressure effect can be selective. In this study, we report that currents through \( \text{Ca}_\text{2.2} \) are...
increased, whereas currents through the CaV2.1 channel are generally unaffected by HP. Only a partial recovery in the amplitude of the currents in the CaV2.2 was witnessed on return to atmospheric pressure.

The effect of HP found here on the CaV2.1 channel conforms with previous findings [17], whereas the HP effect on the CaV2.2 is in contrast to previous reports that suggested reduction in Ca\(^{2+}\) influx through CaV2.2 [40, 41]. Considering the fact that the channels tested here are recombinant and comprised human and rabbit genetic material (versus native intact lobster and guinea pig preparations), the diversity of VDCCs types and their isoform, the unique HPNS threshold for each animal species and the knowledge that even one amino acid alteration can significantly change the whole protein functionality, this contrast is not necessarily surprising. It, in fact, stresses that the interaction between the channels’ subunits may have an impact on the way the channel will react to HP perturbation. In our experiments, we used Ba\(^{2+}\) and tested only the voltage- and time-dependent inactivation, whereas the previous findings mentioned above were in situ, where Ca\(^{2+}\) was the ion carrying the current. If a Ca\(^{2+}\)-dependent inactivation of the current, known to be stronger than the voltage- and time-dependent one, is increased at HP, the overall effect could be depression of the maximal current, explaining the difference in the HP effect between the present and previous studies.

Fig. 7 Fast time constant of voltage- and time-dependent current inactivation (\(\tau_{\text{decay fast}}\)). (A and B) CaV2.1, (C and D) CaV2.2, (E and F) CaV2.2\(+\gamma2\) channels. (A, C and E) \(\tau_{\text{decay fast}}\) measured in a single oocyte. (B, D and F) Pooled data of the channels, \(n\) as stated in Figure 2. Pressures are colour indicated. Statistical significance for each point on the curve is indicated by corresponding colour asterisks. Holding potential \([\Delta V (mV)]\) is expressed as in Figure 2. Dec indicates decompression.
On the other hand, the increase in the currents’ maximal amplitude in CaV2.2 channel at HP in this work is similar to HP effect found recently in CaV1.2 [30] and reminiscent of the ‘delayed rectifier’ K+ channels (another member of this protein superfamily) in which the non-inactivating currents were greater at steady state during HP exposure in invertebrates such as squid [51–53], snail [54] and lobster [55].

Both CaV2.1 and CaV2.2 channels are mainly expressed at the presynaptic nerve terminals [56, 57] and are involved in neurotransmitters release [58]. However, CaV2.2 channel is also expressed in dendrites and cell bodies of neurons, e.g. in the rat dentate gyrus [59]. In such a case, increased channel activity may augment synaptic release and contribute to ‘dendritic boosting’ (increased transfer function between synaptic inputs and somatic spike generation) previously reported by our laboratory [60]. Such boosting, that conforms well to HPNS hyperexcitability, was attributed also to the CaV1.2 channel that is prevalent in the dendrites [30]. This process is an example of HP influence on neuronal networks that does not act through synaptic transmission. As mentioned above, increased CaV2.2 currents are quite unexpected. However, the current amplitude in the recombinant CaV2.2,\(\gamma\)2 channel, considered to better resemble a native one, was much less affected; the average normalized

Fig. 8 Slow time constant of voltage- and time-dependent current inactivation (\(\tau_{\text{Decay Slow}}\)). (A and B) CaV2.1, (C and D) CaV2.2 and (E and F) CaV2.2,\(\gamma\)2 channels. (A, C and E) \(\tau_{\text{Decay Slow}}\) measured in a single oocyte. (B, D and F) Pooled data of the channels, \(n\) as stated in Figure 2. Pressures are colour indicated. Statistical significance for each point on the curve is indicated by corresponding colour asterisks. Holding potential [\(\Delta V\) (mV)] is expressed as in Figure 2. Dec indicates decompression.
maximal current in $V_{\text{max}}$ was increased by $\sim 20\%$ at 2.5–5.0 MPa, compared with $\sim 125\%$ increase for the same pressures of the CaV2.2. Thus, we may assume that some native CaV2.2 channel would be depressed by HP, similarly to the CaV3.2 channel (Aviner et al.) [30]. At present, we can attribute the synaptic pressure-resistant module (see Introduction) to the CaV2.1 channel activity; however, we cannot safely attribute the pressure-sensitive module (reduction in synaptic release) to the activity of any recombinant CaV2.2 channel that we have tested so far. Yet, as both channels are mainly expressed at the presynaptic nerve terminals and are involved in neurotransmitters release [58] (see Introduction), it may be postulated that the individual relative sensitivity or durability to HPNS development in humans may rise from different spatial distribution and quantitative expression of these channels in somatosensory and motor nerves.

**Table 1** Activation volume values (ml/mole) at 2.5 MPa

| Channel       | $\Delta V^1$ (ml/mole) | $t_{\text{Rise}}$ | $t_{\text{Decay, Fast}}$ | $t_{\text{Decay, Slow}}$ | $t_{\text{Tail}}$ |
|---------------|------------------------|-------------------|--------------------------|--------------------------|------------------|
| CaV2.1        | –111                   | –134              | –223                     | 73                       |
| CaV2.2        | 427                    | –922              | –253                     | 358                      |
| CaV2.2+γ2     | 779                    | 455               | 99                       | 595                      |

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Table 2 General qualitative effect of HP on measured channel characteristics

| Channel | \(I_{\text{max}}\) | Conductance | Inactivation | TTP | \(t_{\text{rise}}\) | \(t_{\text{Decay Fast}}\) | \(t_{\text{Decay Slow}}\) | \(t_{\text{Tail}}\) |
|---------|-----------------|--------------|--------------|-----|----------------|---------------------|---------------------|-----------------|
| CaV2.1  | ↓               | ↓            | =            | =   | =              | =                   | =                   | ↑⇒′            |
| CaV2.2  | ↑               | ↑            | ↑(↓)         | ↓/?|=|=/?(=)        | ↓                   | ↓                   | ↑               |
| CaV2.2+ | ↑               | ↑            | ↓            | ↑   | =              | ↑                   | ↑                   | ↑               |

↑, increase; ↓, decrease; =, no change; ( ), stronger depolarization; /, higher HP.

Channels’ conductance

Generally, the calculated conductance (input conductance) behaviour relative to the membrane potential at HP reflects the changes shown in the I-V curves: unaffected in CaV2.1 and increased in CaV2.2s (Fig. 3B, D and F). However, in the CaV2.2, compression to 0.5 MPa did not increase conductance, despite the augmented current measured. Such a phenomenon could be explained either by altered reversal potential or by changed channel kinetics. A change in the reversal potential, if occurs, will be probably also reflected in the measured conductance at higher HP compressions. This did not happen. However, a faster TTP was measured at 0.5 MPa (see below section), suggesting a mechanism through which elevated total ionic flux could develop without an increase in the steady-state conductance.

Decompression was successful in the CaV2.2+, but only partially recovered conductance in CaV2.2, which was still slightly augmented. Should the conductance remain high for long duration after decompression (presently not tested) in the living organism, that may lead to excitotoxicity of neurons due to high cytosolic [Ca\(^{2+}\)], which could explain the long-term cognitive deficits found in veteran occupational deep divers [61–64].

Currents’ TTP and \(t_{\text{rise}}\)

For the CaV2.1, only a tendency for an elongation of TTP and \(t_{\text{rise}}\) at \(V_{\text{max}}\) at 5.0 MPa was witnessed, whereas in the CaV2.2, there was a mixed response to HP: At low HP (0.5 MPa), TTP decreased, while at high HP (2.5–5.0 MPa), it tended to increase (Fig. 5D). Interestingly, in the CaV2.2+ and 2, both TTP and \(t_{\text{rise}}\) elongate at HP, without recovery after decompression, similarly to the HP effect reported in VDCCs in frog motor nerve (possibly CaV2.2) [15], guinea pig single cerebellar Purkinje cells (probably CaV2.1) [17] and in isolated CaV1.2 expressed in oocytes [30]. The velocity of an action potential was also reduced at HP after a transient increase [16].

Increased measured TTP may also indicate a slower inactivation process, which will make the maximal current appear later. Indeed, the \(t_{\text{Decay Fast}}\) was also elongated in CaV2.2 and 2 at HP (Fig. 7F, see Current inactivation). Overall, greater ionic flux via CaV2.2+ could be generated per given depolarization at HP, due to increased conductance and maximal currents and deceleration of inactivation kinetics.

Current inactivation

A stronger inactivation in the CaV2.2 at HP was also supported by shorter \(t_{\text{Decay Fast}}\) and \(t_{\text{Decay Slow}}\) (Figs 7 and 8). Interestingly, at 5.0 MPa, stronger depolarization (\(\Delta V\) >20 mV) weakened the inactivation, suggesting the HP effect is also dependent on the currents’ driving-force, i.e. membrane potential.

Almost no significant effect of HP on inactivation value was measured in the CaV2.1, excluding some changes at 2.5 MPa, but with marginal \(p\) values, also in \(t_{\text{Decay Slow}}\) (Fig. 8). This seems to be a non-linear HP effect, as was previously found in other VDCCs [30].

In the CaV2.2+, inactivation was weaker at HP throughout the activity range of the channel, which correlated with elongation of both \(t_{\text{Decay Fast}}\) and \(t_{\text{Decay Slow}}\) (Fig. 4). This is an opposite finding to the result in the CaV2.2, which may suggest that the \(\gamma\) subunit has a role in the inactivation process of the naïve channel and also the sensitivity of the molecular mechanism controlling the voltage-dependent inactivation to HP. Since the CaV2.2+ may represent a more ‘native’ channel, this result also conforms with the slower inactivation at HP that was reported in Na+ channel in bovine chromaffin cells [13]. The effect of HP on both \(t_{\text{Decays}}\) was only at pressures above 0.5 MPa, which is in agreement with the fact that at least 1.0 MPa is needed in order for the HPNS to develop in humans.

Although generally \(t_{\text{Decay Fast}}\) and \(t_{\text{Decay Slow}}\) were affected similarly by HP for each channel separately (Table 2), both in CaV2.2 and CaV2.2+, \(t_{\text{Decay Slow}}\) was affected differently than \(t_{\text{Decay Fast}}\) at HP for membrane potentials below \(V_{\text{max}}\) (\(\Delta V\) <0 mV). This further supports the well-established concept of different mechanisms for the fast and slow inactivation [65, 66], which can also react differently to external treatment [67]. It was also demonstrated that the molecular structures responsible for these two types of inactivation are differently located in the VDCC’s protein [68] and that the fast inactivation may act similarly to the ‘ball and chain’ mechanism in the K+ channel [69], while the slow inactivation seems to be at least partially dependent on the interaction between \(\alpha\) and \(\beta\) subunits [66]. As the \(\gamma\) subunit is known to affect these mechanisms [37, 70] and to interact with CaV3 subunits, both involved in the channels’ modulation by Gb3 [38, 71], it is not surprising that the HP effect on inactivation is altered by the presence or absence of \(\gamma\). The lack of inactivation recovery to control values after decompression in the CaV2.2+ suggests that either the conformational changes related to inactivation that \(\gamma\) is involved in or the interaction site of \(\gamma\) had been irreversibly altered by HP.

It should be noted that even in the absence of Ca2+, still two components of time constants were necessary in order to fit the voltage- and time-dependent inactivating portion of the current. This leads to the notion that the \(t_{\text{Decay Slow}}\) described here is also voltage dependent that is usually masked by the relatively faster Ca2+-dependent slow inactivation.
Currents deactivation

All channels examined responded to HP by elongation of \( \tau_{\text{tail}} \), whether significantly (CaV2.2 and CaV2.2*,v2) or just by a tendency (CaV2.1), implying a slower deactivation at HP (Fig. 9) for these neuronal channels, in oppose to the CaV1.2 [30]. \( \tau_{\text{tail}} \) is the only kinetic parameter that was similarly affected in CaV2.2 and CaV2.2*,v2 channels, suggesting that the \( \gamma_2 \) subunit is not involved in the regulation of the deactivation mechanism.

Overall, this fits well with the general pressure effect on the CaV2.2 and CaV2.2*,v2 channels witnessed here – an increased flux at HP.

 Activation volume (\( \Delta V^\dagger \))

Excluding \( \tau_{\text{decay Slow}} \) all \( \Delta V^\dagger \) of CaV2.1 are 12–29% of CaV2.2 \( \Delta V^\dagger \) values. This conforms with the weaker, or even non-existent, sensitivity of the channel to HP.

All \( \Delta V^\dagger \) of \( \tau_{\text{tail}} \) are positive values, indicating a deceleration by HP. This suggests that the deactivation process is similar in the examined channels, although less sensitive in CaV2.1, as mentioned above. Interestingly, \( \tau_{\text{tail}} \) \( \Delta V^\dagger \) of CaV1.2 is negative as reported in our recent study [30], suggesting its deactivation mechanism may operate in a different spatial manner.

All \( \Delta V^\dagger \) of CaV2.2*,v2 are positive values, as opposed to the negative \( \tau_{\text{decay Slow}} \) \( \Delta V^\dagger \) in CaV2.1, CaV2.2 and even in CaV1.2 and CaV3.2 as also reported in our previous study [30]. This indicates that \( \gamma_2 \) participates in regulation of the inactivation process, a fact that has been revealed by HP exposure.

Summary

HP hardly affected the behaviour of CaV2.1, but had a major effect in both CaV2.2 and CaV2.2*,v2, albeit HP kinetic effect was generally opposite in all aspects but \( \tau_{\text{tail}} \). These effects may indicate that the conformational changes involved in the channels’ activity are facilitated (e.g. conductance, \( \tau_{\text{decay Fast}} \) and \( \tau_{\text{decay Slow}} \) in CaV2.2) or opposed (e.g. inactivation and deactivation in the CaV2.2*,v2) by an elevated ambient pressure. Indeed, this notion is supported by the calculated activation volumes corresponding to these processes, probably affecting the total ionic flux through the channels at HP.

Some of the effects may indicate a transient or non-linear nature (e.g. TTP and inactivation in CaV2.2, respectively), while other suggested that the HP effect may be reversed by decompression (e.g. inactivation, TTP, \( \tau_{\text{rise}} \), \( \tau_{\text{decay Fast}} \), \( \tau_{\text{decay Slow}} \) in CaV2.2 and \( \tau_{\text{tail}} \) in CaV2.1, but not TTP and \( \tau_{\text{rise}} \) in CaV2.2*,v2). A qualitative summary of the major HP-induced findings is given in Table 2. Among these effects, some were dependent on the membrane potential (e.g. inactivation in CaV2.2, \( \tau_{\text{tail}} \) in CaV2.1) or fluctuated at different HP (e.g. TTP and \( \tau_{\text{rise}} \) in CaV2.2).

General consideration

Although currents in this study were carried by Ba\(^{2+}\) ions (and not Ca\(^{2+}\)), due to the reasons detailed in Materials and methods, we believe that regarding the main aspect of interest in HP influence on VDCC, i.e. conductance and amplitude of currents, the HP impact on these parameters reflects the modulation of HP when Ca\(^{2+}\) ions are moving through the channels’ pore, as was clearly demonstrated in our previous study [30]. This, however, does not exclude the possibility that HP may additionally affect Ca\(^{2+}\)-dependent mechanisms such as Ca\(^{2+}\)-dependent inactivation.

The fact that HP effect was not always consistent in all membrane potentials suggests that one of the pressure targets is the S4 segment in the transmembrane region of \( \alpha_1 \), holding the positively charged amino acids sequence that serve as a voltage sensor, thus causing any voltage-dependent mechanisms, e.g. activation and inactivation. Hyperbaric pressure interfering with the spatial movement of S4 segment would also cause a change in the gating current. It was indeed demonstrated in the past that a considerable fraction of \( \Delta V^\dagger \) in activation of Na\(^+\) channel is associated with gating current [13, 72].

The non-linear HP effect is reminiscent of a bell-shaped dose–response curve; a certain pressure causes a maximal effect, while lower or higher pressures weaken it. The TTP and \( \tau_{\text{rise}} \) that share this behaviour in the CaV2.2, are different parameters for measuring the channels’ activation, which is dependent on membrane potential and the successful spatial transformation of the same S4 segment. This transformation requires a strong enough electrical field to cross a certain energetic threshold. It seems that HP influences that threshold in a non-linear manner (bell-shaped), suggesting the spatial reorganization to be more complex than one hinge or happening on a single plateau.

Undoubtedly, the changes in both magnitude and kinetics of the response to depolarization at HP would influence these channels’ functionality in the living organism, and hence also its motor and cognitive performance. Indeed, the HPNS constellation of sign and symptoms includes changes in EEG, sleep disorders, decrements in locomotor activity, myoclonus and tremors, which may all be expressed as the manifestation of these HP-induced changes in VDCCs.

We have previously postulated that even a ‘minor’ change made to a section within a subunit [73, 74] or just a single amino acid substitution [75, 76] can significantly alter the VDCC reaction to depolarization, possibly due to a different spatial organization [85], let alone the use of different subunits will have this effect. Naturally, this assumption is supported in the first place by the differential response to HP in the CaV2.1 and CaV2.2, having a different \( \alpha_1 \) subunit comprising the pore and voltage sensor. But further support to this notion is also provided by the differential HP effects in the CaV2.2 and CaV2.2*,v2. The saturation of current augmentation at 0.5–5.0 MPa in the CaV2.2*,v2 versus the dose–response curve of the CaV2.2 may suggest that the \( \gamma_2 \) subunit counteracts the HP effect on the channels’ conductance.

We have recently demonstrated HP effects in VDCC [30] and rat-cultured cortical neurons (unpublished data) already at 0.5 and 0.3 MPa, respectively. Dean & Mulkey (2003) have also reported reversible changes in membrane properties in rat medulla solitary complex upon helium compression to as low as 0.3 MPa. Relatively low HP threshold, 0.5 MPa, was also found here in the CaV2.2 and CaV2.2*,v2.
Since HP can target either the channel (and its subunits) or any external modulator, and although the general impression from this study is that HP affects the channel itself by changing its spatial organization in the active or non-active states, further research is needed to determine whether VDCCs’ modulators are also affected by HP. Notwithstanding, ion channel configuration may also be affected by the membrane characteristics (e.g., fluidity, input resistance, specific capacity), which have been shown to be affected by HP [55, 77–79], even at low HP as well (<0.4 MPa) [80]. Barosensitivity is commonly attributed to be a manifestation of pressure equally exerted in all dimensions, whereas mechanosensitivity is caused by localized shear and strain forces manifested (at HP) by differences in compressibility of adjacent cellular structures [81, 82]. Mechanosensitivity of biological processes has been also demonstrated at relatively low HP (<0.2 MPa) [83], as opposed to barosensitivity of the channel, which usually occurs at high HP (>0.5 and up to 10–40 MPa) [13, 17]. This notion may provide another explanation for the non-linear HP effect: low HP affected the channel via altered membrane traits and perhaps mechanosensitivity, while high HP affected the channels itself as well.

On the other hand, a direct influence of HP on the channel may be supported by crystallographic work that has shown the presence of a hydrophobic cavity within a protein, the ability of gas molecules to penetrate it and a reduction in its volume at HP [84, 85]. Such a cavity has been proposed to have a role in protein flexibility, which in turn is related to functional efficiency [86]. Hence, should a VDCC contain such a cavity, changes in its volume or presence or lack of a gas molecule in it could have a crucial HP-induced influence on the protein functionality. Such a distortion in the spatial organization and/or conformational change of the channel will also undoubtedly interfere with a prompt recovery back to its naïve state and may provide an explanation for the lack of complete recovery of the channel after decompression in general, and specifically within the time frame of our experiments.

Overall, the direct data being accumulated regarding HP-induced effects in several types of VDCCs thus far strongly suggest that the previous concept of uniform influence of HP on certain types of channels should be abandoned. As demonstrated by our group, pressure may augment or depress currents, accelerate or decelerate kinetics or leave some of the channels’ traits unaffected. The actual mechanism(s) underlying this diversity of responses to HP need further elucidation. Yet, we may speculate that the wide spectrum of pressure sensitivity in vertebrates (e.g., tolerance to various levels of HP, while others are obligatory high HP dwellers) is, at least in part, the result of evolutionary differential distribution of these VDCCs throughout neuronal networks, along the single neuron, or structural variations of the same channel in different life forms.

Conclusions

a. HP-selective modulation of various presynaptic VDCCs (in addition to somatic and dendritic channels) probably has an important role in synaptic transmission alteration, which is strongly associated with HPNS.

b. HP selectivity depends on the different α2 subunit comprising the pore and voltage sensor but can also be mediated by other regulatory subunits of the channel protein.

c. Pressure modulation of channels’ kinetics and function is dependent on the membrane potential.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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