Protein phosphorylation maintains the normal function of cloned human Cav2.3 channels

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R-type currents mediated by native and recombinant Cav2.3 voltage-gated Ca\(^{2+}\) channels (VGCCs) exhibit facilitation (run-up) and subsequent decline (run-down) in whole-cell patch-clamp recordings. A better understanding of the two processes could provide insight into constitutive modulation of the channels in intact cells, but low expression levels and the need for pharmacological isolation have prevented investigations in native systems. Here, to circumvent these limitations, we use conventional and perforated-patch-clamp recordings in a recombinant expression system, which allows us to study the effects of cell dialysis in a reproducible manner. We show that the decline of currents carried by human Cav2.3+β3 channel subunits during run-down is related to adenosine triphosphate (ATP) depletion, which reduces the number of functional channels and leads to a progressive shift of voltage-dependent gating to more negative potentials. Both effects can be counteracted by hydrolysable ATP, whose protective action is almost completely prevented by inhibition of serine/threonine but not tyrosine or lipid kinases. Protein kinase inhibition also mimics the effects of run-down in intact cells, reduces the peak current density, and hyperpolarizes the voltage dependence of gating. Together, our findings indicate that ATP promotes phosphorylation of either the channel or an associated protein, whereas dephosphorylation during cell dialysis results in run-down. These data also distinguish the effects of ATP on Cav2.3 channels from those on other VGCCs because neither direct nucleotide binding nor PIP\(_2\) synthesis is required for protection from run-down. We conclude that protein phosphorylation is required for Cav2.3 channel function and could directly influence the normal features of current carried by these channels. Curiously, some of our findings also point to a role for leupeptin-sensitive proteases in run-up and possibly ATP protection from run-down. As such, the present study provides a reliable baseline for further studies on Cav2.3 channel regulation by protein kinases, phosphatases, and possibly proteases.
two processes in native cells. The human embryonic kidney (HEK-293) cell line is widely used for heterologous expression of recombinant ion channels and receptors because it contains few endogenous channels, whereas most signaling pathways for regulation and posttranslational processing are operational (Toth et al., 1996; Thomas and Smart, 2005; Clare, 2006). Apart from circumventing the need for R-type current isolation, HEK-293 cells have a simple and uniform shape, which facilitates reproducible manipulation of their intracellular milieu. We therefore used conventional and perforated-patch-clamp recordings together with different inhibitors and cytosolic factors to study the effects of cell dialysis in a stably transfected HEK-293 cell line expressing human Cav_{2.3+β}\_3 channel subunits. Our findings show that the decline of macroscopic currents during run-down can partly be accounted for by changes in channel voltage dependence and that it can be prevented or slowed down by provision of intracellular ATP and in perforated-patch recordings. Protection from run-down depended on ATP hydrolysis and was not related to lipid kinase-mediated PIP\_2 synthesis or phosphorylation of tyrosine residues but was sensitive to inhibition of serine/threonine kinases. Protein kinase inhibition in intact cells also reduced peak current densities and reproduced the effects of run-down on channel voltage-dependence. Together, these findings indicate that run-down involves constitutive dephosphorylation of sites on the channels themselves or an associated protein and that ATP promotes phosphorylation of these sites by one or more endogenous kinases. Interestingly, our findings also indicate that the current facilitation during run-up could involve activation of leupeptin (Leu)-sensitive proteases, which may also influence the protective action of ATP.

Materials and methods

**Cell culture**

Human embryonic kidney (HEK-293) cells stably transfected with human Cav_{2.3} and β\_3 Ca\+_\_ channel subunits (Nakashima et al., 1998) were cultured under normal growth conditions (37°C and 5% CO\_2) in Dulbecco’s modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% FCS and antibiotics (1% penicillin–streptomycin and selection markers: 1 mg/ml gentamicin [G–418] and 200 µg/ml hygromycin B). Cells were routinely passaged twice a week by using 0.05% trypsin/0.02% EDTA. For electrophysiological recordings, cells were seeded on nitril acid–washed glass coverslips and used within 24–48 h after plating.

**Electrophysiological recordings**

Cells were voltage-clamped by using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Pipettes were prepared from thick-walled borosilicate glass capillaries (1.5/0.84-mm-outside/inside diameter; World Precision Instruments) by using a P97 Micropipette puller (Sutter Instruments). Resistance of the resulting electrodes was between 1.5 and 6.5 MΩ (mean = 3.8 ± 0.1 MΩ in 339 recordings) when filled with standard internal solution. The bath was connected to ground via 140 mM sodium chloride agar bridges. Currents were sampled at 20 or 50 kHz and filtered at 10 kHz by using an EPC9 amplifier (HEKA) controlled with HEKA’s Pulse software. Leak and capacitive currents were subtracted online by use of a −P/5 protocol. Recordings obtained with voltage ramps were leak-corrected by a linear fit to the current recorded between −80 and −50 mV, which was extrapolated to +60 mV and subtracted from the whole recording. Series resistance (R\_s) was compensated electronically by up to 90% (mean R\_s after compensation = 2.7 ± 0.1 MΩ) and continuously monitored throughout the measurements. The charging time-constant after compensation was always ≤100 µs (mean = 38 ± 1 µs), and the maximum uncompensated R\_s-error at the time of peak current was ≤2.5 mV (mean = 0.82 ± 0.04 µs). All experiments were performed at room temperature, from a holding potential of −80 mV and, unless noted otherwise, in cells with a whole-cell capacitance (C\_w) between 5 and 25 pF (mean = 15 ± 1 pF), as estimated from the slow capacitance compensation of the amplifier. Perforated patch recordings were performed by using β-escin, which was dissolved in type I ultrapure water to prepare a 25 mM stock solution, protected from light and stored at −20°C for up to 2 wk. Before the recordings, aliquots of the stock solution were diluted in standard internal solution and vortex-mixed for 1 min to give a final escin concentration of 40 µM. After patch formation, gradual perforation was observed as a progressive increase in the speed and amplitude of capacitive transients during 5-ms voltage-steps to −75 mV. Cells showing signs of spontaneous patch rupture (i.e., sudden increase in the amplitude and speed of capacitive transients) were omitted or used as control recordings to exclude that β-escin itself affected the channel properties or the time course of changes in I\_Ba.

**Recording solutions and drugs**

All solutions for electrophysiological experiments were prepared by using type I ultrapure water (Milli-Q by Millipore Corporation or Purelab Flex 2 by ELGA Labwater) and, unless noted otherwise, reagents purchased from Sigma-Aldrich. During the recordings, cells were constantly perfused with external solution containing (mM) 120 NaCl, 5 BaCl\_2 or CaCl\_2, 5 KCl, 1 MgCl\_2, 20 TEA chloride, 10 glucose, 10 HEPES, and 0.1 BaEDTA with the pH adjusted to 7.4 by using NaOH and osmolality of 300–320 mOsm. The solution was filtered through 0.2-µm polyethersulfone membranes and applied to cells at a rate of ~2–4 ml/min by using a gravity-driven perfusion system controlled by manual precision flow regulators (Sarstedt). The standard intracellular solution was composed of (mM) 130 CsCl, 5 oxaloacetic acid, 5 creatine, 5 pyruvic acid, 10 EGTA, and 0.1 HEPES with the pH adjusted to 7.3 by using CsOH and osmolality of 275–295 mOsm. It was stored at −20°C, thawed on the day of the experiments, filtered through 0.2-µm surfactant-free cellulose acetate membranes (Corning), and kept on ice between the recordings. The liquid junction potential between internal and external solution (calculated by using the JPCalc algorithm in pClamp 10; Molecular Devices) was ~5.5 mV. Because no correction for the liquid junction potential was done, all voltages shown were actually 5.5 mV more negative.

Nucleotide triphosphates (NaATP, MgATP, and MgGTP) were dissolved in 10 mM HEPES to prepare 100 mM stock solutions (pH = 7.3), stored at −20°C and diluted in standard internal solution immediately before the recordings. Calmodulin (CaM) was dissolved in type I ultrapure water to prepare a 100 µM stock solution and stored at −20°C. AMP-PNP was used as the lithium
salt and dissolved directly in the standard internal solution. Del-
tamethrin, wortmannin, U73122, genistein, and staurosporine
(stau) were dissolved in DMSO to prepare 2–50 mM stock
solutions, protected from light and stored at −20°C. Before the
experiments, they were diluted in standard internal solution,
and the same amount of DMSO (0.1–0.25% vol/vol) without
drug was added to the internal solution used for parallel control
recordings. To account for the short half-life in aqueous media,
all solutions containing wortmannin were discarded after a max-
umum of 20 min after its dilution into standard internal or exter-
nal solution, respectively. Cytosolic extract was prepared on the
day of the experiments by freeze/thaw lysis of ~2.1 × 10⁷ cells in
1.5 ml ice-cold pipette solution. After centrifugation at 0°C for
5 min, the supernatant was filtered through a 0.2-µm surfac-
tant-free cellulose membrane (Corning), an aliquot was removed
for quantification of the protein concentration, and the rest was
immediately used for the recordings.

Whole-cell protocols

Unless noted otherwise, time-course recordings were performed
by repetitive application at 0.03 Hz of a 30-ms test pulse to +10
mV followed by 10-ms repolarization at −50 mV to record well
resolved tail-currents. In some experiments, a 50-ms voltage
ramp from −80 mV to +60 mV (2.8 V/s) was used instead of the
voltage steps to monitor changes in the quasi steady-state voltage
dependence. Because recordings with drugs added to the internal
solution could not be used as their own control, we always per-
formed parallel control recordings with standard internal solu-
tion. To study changes in current immediately after establishing
the whole-cell configuration, cells were clamped at the holding
potential of −80 mV before disruption of the patch. After patch
rupture, capacitance was quickly compensated by using the auto-
function of the amplifier, and stimulation started after a delay
of no more than 10 s. Because initial current amplitudes were
small and several seconds were often required for Rs to stabilize
completely, the first response was always recorded without Rs
compensation. We then stopped stimulation, adjusted the Rs
compensation of the amplifier to achieve maximal compensa-
tion without ringing, and restarted the stimulation protocol. To
reduce variation introduced by differences in the time required
for stabilization of Rs, initial values (Istart) for statistical com-
parison were always calculated as the average of the first two
responses, which also provided a compromise between the lack
of Rs compensation during acquisition of the first response and
the time that had already evolved when the second response was
recorded. To construct steady-state current-voltage (IV) rela-
tionships, peak currents recorded with a protocol consisting of 25-ms
test pulses to potentials between −80 mV and +60 mV (10-mV inc-
ements at 0.1 Hz) were normalized by the maximum current
amplitude at time 0 and plotted as a function of the test-pulse
potential. Instantaneous IV (IIV) relationships were obtained
with a protocol consisting of a fixed 25-ms prepulse to +60 mV
followed by 40-ms test pulses to potentials between −80 mV and
+60 mV (10-mV increments at 0.1 Hz). Instantaneous (tail) cur-
rent amplitudes recorded during the test pulses were normalized
by the maximum tail current amplitude at time 0 and plotted as
a function of the prepulse potential. The voltage dependence of
activation was assessed by a protocol consisting of 25-ms pre-
pulses to potentials between −80 mV and +60 mV (10-mV inc-
ements at 0.1 Hz) followed by a fixed 10-ms test pulse to −50 mV.
Tail-current amplitudes recorded during the test pulse were
normalized to the maximum tail-current amplitude and plotted
as a function of the prepulse potential to construct isochronous
activation curves. The fraction of channels available for activa-
tion from different holding potentials was assessed by a protocol
consisting of 3-s conditioning prepaulses at potentials between
−120 mV and +10 mV (10-mV increments at 0.1 Hz) followed by
a fixed 35-ms test pulse to +10 mV. To construct prepulse inacti-
vation curves, peak current amplitudes recorded during the test
pulse were normalized by the maximum amplitude and plotted
as a function of the prepulse potential.

Data analysis and statistics

Leak-subtracted current traces were directly analyzed with PulseFit (HEKA) or exported for further processing with Micro-
soft Excel 2010 and OriginLab Pro (version 9; OriginLab).

Time-course recordings were quantified in terms of the
current increase during run-up, the duration of run-up, and
the residual current after 7 min of run-down, as detailed in the
results section. To compare the effect of various test solutions,
each of these parameters was also normalized by the mean value
observed in parallel control recordings according to Eq. 1:

\[ Δp_i = p_i − p_{\text{mean}} \]

where \( p_i \) is the value of parameter \( p \) measured in a cell with test
solution \( t \), \( p_{\text{mean}} \) is the mean value of \( p \) in parallel control recordings,
and \( Δp_i \) is the normalized value of \( p \), which should provide an
estimate for the amount of change over control.

IV relationships were fitted with a combined Ohm-Boltzmann
equation (Eq. 2):

\[ I = \left( V_m - V_{\text{rev}} \right) g_0 / \left( 1 + \exp \left( (V_m - V_{0.5}) / k \right) \right) \]

where \( I \) is the (normalized) peak current density measured at
the test potential \( V_m \), \( V_{\text{rev}} \) is the apparent reversal potential, \( g_0 \)
is the maximum slope conductance, \( V_{0.5} \) is the voltage eliciting
half-maximal inward currents, and \( k \) is the slope factor. Isochro-
nous activation and prepulse inactivation curves were fitted with
single Boltzmann equations (Eq. 3):

\[ I/I_{\text{max}} = A_2 + (A_1 - A_2) / \left( 1 + \exp \left( (V_m - V_{0.5}) / k \right) \right) \]

where \( I/I_{\text{max}} \) is the normalized current at the prepulse poten-
tial \( V_m \), \( V_{0.5} \) is the voltage of half-maximal activation \( V_{0.5,\text{act}} \)
or inactivation \( V_{0.5,\text{inact}} \), \( k \) is the activation \( (k_\text{act}) \) or inactivation
\( (k_{\text{inact}}) \) slope factor, and \( A_1 \) and \( A_2 \) are the initial and final values,
respectively. All fits were performed by using the Leven-
berg-Marquardt least-squares algorithm, and the goodness of
fit was judged based on residual plots and adjusted \( \chi^2 \) values.
Smooth curves in the figures represent fits to average data
whereas values given in the text are average data from fits to
individual measurements. Values in the text and figures are
expressed as mean ± SEM based on the number of independent
experiments. Statistical significance was assessed with Origin-
Lab Pro 9 by using a repeated-measures ANOVA followed by Bon-
ferroni’s post hoc analysis when comparing mean values from
the same cells or a one-way ANOVA followed by Bonferroni’s post hoc analysis when comparing multiple independent mean values. Homogeneity of variances between groups was tested by using Levene’s test for equality of variances on the squared deviations. In the case of heteroscedastic data (P < 0.05 in Levene’s test and ratio of largest-to-smallest variance ≥4), statistical significance was assessed with Minitab (version 17; Minitab Inc.) by using Welch’s ANOVA and the Games-Howell multiple-comparison method. The secondary Ca\(_{\text{2.3}}\) channel structure in Fig. 14 was visualized by using the web-based tool Protter for interactive protein feature visualization (available at http://wlab.ethz.ch/protter/start/; Omasits et al., 2014). Phosphorylation sites in the same figure were predicted at the highest threshold with the Group-based Prediction Software for Prediction of Kinase-specific Phosphorylation Sites 3.0 (available at http://gps.biocuckoo.org; Xue et al., 2008).

**Results**

**Evolution and run-down of cloned Ca\(_{\text{2.3}}\) channel currents**

Fig. 1 summarizes changes over time in macroscopic Ca\(_{\text{2.3}}\) channel currents carried by 5 mM Ba\(^{2+}\) and evoked by voltage-steps to 10 mV in dialyzed HEK-293 cells. Peak current amplitudes recorded immediately after establishing the whole-cell configuration (I\(_{\text{start}}\)) were small and tended to increase for several minutes (run-up) until they reached a maximum value (I\(_{\text{max}}\)). This was invariably followed by a progressive but somewhat slower current decline (run-down), so that the response that remained 7 min after complete run-up (I\(_{\text{7min}}\)) amounted to approximately half of the maximum current amplitude (Fig. 1, A and B).

We quantified the time course of changes by estimating (1) the time required after patch rupture for currents to reach I\(_{\text{max}}\) (= time to I\(_{\text{max}}\)), (2) the ratio between I\(_{\text{max}}\) and I\(_{\text{start}}\) (= increase), and (3) the ratio between I\(_{\text{min}}\) and I\(_{\text{max}}\) (= remaining I\(_{\text{Ba}}\)). Under standard recording conditions (Fig. 1), with mean R\(_{\text{s}}\) and C\(_{\text{s}}\) values of 6 ± 1 M\(\Omega\) and 14 ± 1 pF, respectively (n = 10), run-up took 4 ± 1 min and was associated with a 2.7 ± 0.2-fold increase of peak I\(_{\text{Ba}}\) at 10 mV, whereas the residual current amplitude after partial run-down amounted to 42 ± 4% of its maximum value.

As illustrated by inspection of scaled current traces (Fig. 1C), the initial increase of currents was paralleled by a decrease of fractional inactivation during the 30-ms test-pulses from 53 ± 4% immediately after establishing the whole-cell configuration to 40 ± 4% (P < 0.001) after complete run-up. On the other hand, run-down tended to increase inactivation, although quantification of this effect was often confounded by the reduced signal-to-noise ratio due to current decline (but see also Fig. 13 E).

The general time course of changes was very similar among cells with different peak current densities, not attributable to voltage loss or R\(_{\text{s}}\) variation (Fig. 1B) and unaffected by inclusion of two 3-min resting periods without stimulation (not depicted).

**Factors involved in run-up and run-down**

Increased mechanical tension associated with fluid flow has been shown to cause run-up and functional alterations in some other VGCCs (Peng et al., 2005; Park et al., 2007), but the time-dependent
Figure 2. Dependence of run-up and run-down on cell dialysis, Ca$^{2+}$, and voltage. (A) Comparison of time-course recordings performed under standard recording conditions (Ctrl, with $R_s = 6 \pm 1$ MΩ and $C_s = 14 \pm 1$ pF; same data as in Fig. 1) or with high-resistance pipettes ($R_s = 12 \pm 1$ MΩ) in large cells ($C_s = 29 \pm 6$ pF) to retard cytosolic dilution (high $C_s$, $n = 6$). (B) Comparison of the run-up duration, determined as the time required for currents to reach their maximum amplitude (same cells as in A). (C) Comparison of the residual current after partial run-down, determined as the ratio between current amplitudes after 7 min of run-down and at time 0 (same cells as in A). (D) Comparison of the current increase during run-up, determined as the ratio between maximum and initial current amplitude (same cells as in A). (E) Comparison of time-course recordings performed with 5 mM Ba$^{2+}$ ($n = 5$) or Ca$^{2+}$ ($n = 5$) as the charge carrier. Dotted lines are extrapolated single exponential fits to the evolution of currents during run-up, which yielded the time-constant indicated above. (F) Comparison of the run-up duration observed with Ba$^{2+}$ or Ca$^{2+}$ as the charge carrier (same cells as in E). (G) Mean current traces evoked by a 50-ms voltage ramp from −80 mV to 60 mV immediately after establishing the whole-cell configuration ($I_{\text{start}}$, blue solid line), at the time of maximum $I_{\text{Ba}}$ ($I_{\text{max}}$, black solid line) and after 7 min of run-down ($I_{7\text{min}}$, orange dotted line; $n = 7$). Inset: Same current traces but scaled to their maximum value to illustrate the lack of changes in position and shape during run-up and subsequent left shift of the curve during run-down. **, $P < 0.01$; ***, $P < 0.001$ vs. Ctrl in B and C or vs. Ba$^{2+}$ in F (one-way ANOVA with Bonferroni post-hoc correction). Values are expressed as mean ± SEM.
Run-down involves separable changes in gating and maximum conductance

The macroscopic conductance of a uniform population of voltage-gated channels can be approximated by the product of (1) the voltage- and time-dependent channel open probability \( P_o \), (2) the holding potential-dependent availability for activation \( P_f \), and (3) the voltage-independent maximum macroscopic conductance \( G_{\text{max}} \), which depends on single-channel conductance \( \gamma \), the total number of channels \( N \), and the maximum values of \( P_o \) \( (P_{0,\text{max}}) \) and \( P_f \) \( (P_{f,\text{max}}) \). In this framework, run-down could involve reduced activation, increased inactivation, and/or an intrinsically voltage-dependent decrease of \( G_{\text{max}} \). In an attempt to distinguish between these possibilities, we compared steady-state \( (IV) \) and instantaneous \( (IIV) \) current-voltage relationships, determined at the onset of run-down and in 5-min intervals thereafter (Fig. 3, A–C). Because IIV currents were recorded immediately after a brief prepulse to open most channels available for activation, they should be little affected by changes in the voltage-dependence of \( P_o \). Fig. 3 D plots the fraction of IV and IIV currents that remained after partial run-down for 5 min as a function of the test potential. The decline of steady-state currents increased with voltage but reached voltage-independent values at positive test potentials (where \( P_o \) approaches \( P_{0,\text{max}} \)), which is consistent with the shift of ramp-evoked currents (Fig. 2 G). When measured with the IIV protocol on the other hand, run-down was about the same at different test potentials and similar to IV-current decline at depolarized voltages (Fig. 3 D). The same pattern was observed at the later time points examined, so that the kinetics of IIV-current run-down were well represented by the exponential decline in slope conductance between −80 and 0 mV (Fig. 3 E). The time course of IV-current decline was markedly delayed during weak depolarization but similar when it was measured at sufficiently positive test potentials (\( \tau = 9 \pm 1 \text{ min} \)) or in terms of the maximum slope conductance (\( \tau = 10 \pm 2 \text{ min} \)).
Based on these findings, the decrease of macroscopic conductance by itself was independent of the test-pulse potential and had an approximately exponential time course, so the apparent voltage-dependence must have derived from changes in the voltage dependence of $P_0$, which (partly) countered IV-current decline and even caused some transient stimulation at negative test potentials.

Run-down is paralleled by changes in channel voltage dependence

To assess how changes in the voltage dependence of $P_0$ and $P_f$ are involved in current decline, we compared IV, activation, and prepulse inactivation curves (for details on the voltage protocols see Materials and methods), determined at different time points after the onset of run-down. Fig. 4 summarizes the results...
obtained in a total of 49 cells (Cslow = 29 ± 2 pF), from which meaningful currents could be recorded for at least 21 min. The shape of IV current lost to run-down (Fig. 4A, inset), was essentially identical to the shape at time 0 and contained a prominent ON-gating current component, suggesting that run-down reduced the total number of active channels (i.e., P_F at −80 mV, P_F,max, and/or N). It was paralleled by progressive but unequal hyperpolarizing shifts of activation and prepulse inactivation, which are illustrated in Fig. 4 (C and D). The half-activation voltage (V0.5act) significantly decreased from 0 ± 1 mV at time 0 to −6 ± 1 mV (P < 0.001) after 21 min of run-down, corresponding to a shift by roughly −6 mV. In the same time, the half-inactivation voltage (V0.5inact) significantly decreased from −52 ± 1 mV to −69 ± 1 mV (P < 0.001), corresponding to a shift by roughly −17 mV. In both cases, the rate of shift in individual recordings could be well described by linear fits to the changes in half-point as a function of time, with slopes of −0.33 ± 0.02 mV/min (adjusted R² = 0.90 ± 0.02) for activation and −0.82 ± 0.03 mV/min (adjusted R² = 0.95 ± 0.01) for inactivation. Based on these findings, P_F at −80 mV was reduced by ∼5, 12, and 19% after 7, 14, and 21 min of run-down, respectively (Fig. 4C), which corresponds to a constant rate of roughly 1%/min. In the same time, g_IV declined by ∼25, 47, and 61%, so that reduced availability for activation alone could only explain part of the IV-current decline at positive test potentials (Fig. 4B). Together with a complete loss of gating currents during run-down, these findings suggested that the decline in conductance involves additional (holding potential–independent) changes in the number of active channels (i.e., N and/or P_F,max). This was also evident in time-course recordings where the holding potential after partial run-down was made more negative, which led to considerable but incomplete recovery of the current at 10 mV (not depicted).

In addition to the shift, run-down reduced the steepness of both curves in Fig. 4C, which was reflected in a gradual increase of the activation and inactivation slope factors (k_act and k_inact) from 9.0 ± 0.2 and 9.2 ± 0.1 mV/e-fold change at time 0 to 10.5 ± 0.3 (P < 0.001) and 11.1 ± 0.3 (P < 0.001) mV/e-fold change after 21 min of run-down, respectively (Fig. 4E).

Hydrolysable ATP provides protection from run-down
Because one of the most common reasons for “washout” of ionic currents is a decrease in the level of intracellular high-energy

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Figure 5. Hydrolysable ATP provides protection from Ca_{2.3} channel run-down. Comparison between time-course recordings performed with the indicated test substances and parallel control recordings with standard internal solution. (A) Mean data from 5 recordings with 1 mM MgATP (orange squares), 8 recordings with 5 mM MgATP and (orange circles), and 13 parallel control recordings (open squares). (B) Comparison of the residual I_{Ba} after 7 min of run-down under control conditions and with 1 or 5 mM MgATP in the pipette solution (same cells as in A). (C) Comparison of the run-up duration under control conditions and with 1 or 5 mM MgATP in the pipette solution (same cells as in A). (D) Comparison of the current increase under control conditions and with 1–5 mM MgATP in the pipette solution (same cells as in A). (E) Mean data from five recordings with 1 mM MgCl_{2} (orange squares) and seven parallel control recordings (open squares). (F) Mean data from eight recordings with 5 mM AMP-PNP (orange squares) and seven parallel control recordings (open squares). **, P < 0.01; ***, P < 0.001 vs. Ctrl (one-way ANOVA with Bonferroni post hoc correction). Values are expressed as mean ± SEM.
addition of 1 mM MgCl₂

transfer of the phosphate group.
tide binding and indicate a requirement for ATP-hydrolysis and
Together, these findings argue against a role of direct nucleo-

Fig. 7). On the other hand affected neither run-up nor run-down (

5 mM of the nonhydrolyzable ATP-analogue AMP-PNP (Fig. 5 G)
or

nucleotide was combined with 3 µM CaM (Fig. 7 C). Supplemen-

tation (open squares). All recordings were performed with 40 µM β-escin as the perforating agent.

recordings with 5 mM MgATP (orange circles), and 3 perforated-patch recordings with standard internal solution in which the patch ruptured during perfora-
tion (open squares). All recordings were performed with 40 µM β-escin as the perforating agent.

neither run-up nor run-down (Fig. 5 E). Moreover, with a pipette solution containing 5 mM NaATP and no added free Mg²⁺, there was still a significant slowing of run-up and a significant increase of the current fraction remaining after 7 min of run-down compared with parallel control recordings (Fig. 5, A and B) and produced a concentration-dependent slowing of run-up (Fig. 5, A and C). These effects were not attributable to changes in intracellular free Mg²⁺ (~0.4 mM with 5 mM MgATP) because addition of 1 mM MgCl₂ alone (~0.5 mM free Mg²⁺) to the standard internal solution affected neither run-up nor run-down (Fig. 5 E). Moreover, with a pipette solution containing 5 mM NaATP and no added free Mg²⁺, there was still a significant slowing of run-up and a significant increase of the current fraction remaining after 7 min of run-down (Fig. 7, A and C).

ATP protection from run-down was about the same with dif-
ferent charge carriers (i.e., Ba²⁺ vs. Ca²⁺) and not altered when the nucleotide was combined with 3 µM CaM (Fig. 7 C). Supplemen-
tation of the pipette solution with 0.3 mM MgGTP (Fig. 5 F) or 5 mM of the nonhydrolyzable ATP-analogue AMP-PNP (Fig. 5 G) on the other hand affected neither run-up nor run-down (Fig. 7). Together, these findings argue against a role of direct nucleo-
tide binding and indicate a requirement for ATP-hydrolysis and transfer of the phosphate group.

Note also that (with Ba²⁺ as the charge carrier) none of the manipulations tested significantly altered the magnitude of run-up (Figs. 5 B and 7 B) or the absolute values of Iₘₐₓ (not depicted), suggesting that ATP can slow but not prevent the pro-
cesses underlying run-up. The apparent increase in the magni-
tude of run-up by ATP observed with Ca²⁺ as the charge carrier (Fig. 7 B) was also not related to a more pronounced up-regula-
tion of Iₐₓ per se because absolute values of Iₘₐₓ were the same as in parallel control recordings (not depicted). Although there was a tendency for Iₘₐₓ to be smaller in recordings with ATP, this effect could hardly be ascribed to an action of the nucleotide. Because the faster run-up kinetics in Ca²⁺ hampered accurate determination of Iₘₐₓ, it might instead have resulted from small differences between groups in the time required for Rs to stabil-
ize after patch-rupture.

ATP protection is reproduced by perforated-patch recordings
In principle, exogenous ATP might counteract run-down by pre-
venting a decrease of cytosolic nucleotide levels or by increasing them above the normal value in intact cells, thereby stimulating the channels through an unrelated mechanism. To distinguish between these two possibilities, we performed perforated-
patch recordings with β-escin, diluted into the same inter-
nal recording solution as in ruptured-patch experiments. ATP-diffusion through β-escin pores has been demonstrated, but perforating efficiency is concentration and time depen-
dent, so washout should be considerably slower than in rupt-
tured-patch recordings (Arnould et al., 1996; Fan and Palade, 1998; Fu et al., 2003). As illustrated in Fig. 6 A and summa-
rized in Fig. 7, the time course of changes during perforated recordings was very similar to that observed in ruptured recordings with ATP. Importantly, and consistent with a role of ATP-depletion for the current decline, perforated recordings were almost as effective in reducing run-down as provi-
sion of ATP in ruptured recordings. Moreover, the protective
effects of perforated recordings and ATP were not additive, so that provision of 5 mM MgATP in perforated recordings produced the same effects as in ruptured recordings (Figs. 6 B and 7 C). Collectively, these findings indicate that both, perforated recording and/or provision of ATP reduced run-down by preventing depletion of the nucleotide during the recordings. This is in contrast to the effects on run-up duration, which were much more pronounced in perforated recordings and further increased in the presence of ATP (Figs. 6 C and 7 A). Moreover, because our measurements were started only after $R_s$ had reached values $\leq 15 \text{M}\Omega$, we most likely missed part of the run-up process, so these results may still underestimate the true slowing of run-up in perforated recordings. Thus, the initial but not maximum peak current density was significantly larger in perforated recordings (not depicted), and this was reflected in a reduced magnitude of run-up (Figs. 6 D and 7 B). Together, these observations point to the involvement of additional cytosolic factors during run-up, which are more effectively retained in perforated-patch recordings. However, a cytosolic extract prepared in standard internal solution (4.32 mg protein/ml) was ineffective in altering run-up or run-down (Fig. 7), possibly because these factors depend on the additional presence of ATP.

**ATP stabilizes channel gating and maximum conductance**

All the above findings supported the assumption that Ca$_{2.3}$ channel run-down and, to some extent, run-up in dialyzed cells are related to a depletion of cytosolic ATP. Because it was of interest how ATP affects the different alterations associated with run-down, we reexamined the changes in channel voltage dependence during current decline with a pipette solution supplemented with 5 mM MgATP. As illustrated in Fig. 8 A, inclusion of ATP completely abolished the early phase of current decline, so that on average, $g_V$ after 7 min of run-down amounted to 97 ± 7% of its initial value ($n = 6$). In addition, the stabilizing action of ATP was associated with an almost complete prevention of time-dependent changes in channel voltage dependence and sensitivity (Fig. 8, B–E), suggesting that basal ATP-dependent modulation alters channel-gating behavior and is required for maintaining it in a functional state.

**The effects of ATP are not related to lipid kinase-mediated PIP$_2$ synthesis**

Because run-down of several neuronal VGCCs and its reversal by MgATP have been linked to depletion and resynthesis of membrane PIP$_2$, respectively (Wu et al., 2002; Suh et al., 2010), we next examined the effects of wortmannin (WM), a potent and
irreversible lipid kinase inhibitor (Wipf and Halter, 2005). When MgATP in the pipette solution was combined with 10 µM WM (ATP+WM in Fig. 9, A–D), it still significantly increased the current fraction remaining after 7 min of run-down (Fig. 9 B) and markedly slowed run-up (Fig. 9 C). Relative to recordings with MgATP alone, WM actually tended to enhance the protective effects (Fig. 10 C), suggesting that it increased ATP availability because of reduced consumption by lipid kinases. Likewise, pre-treatment of cells by incubation in extracellular solution containing 10 µM WM for 15 min (ATP+WM PT in Fig. 9, A–D) did not significantly impair the stabilizing action of MgATP (Fig. 9 B) nor did it alter the time course of changes observed in parallel control recordings. Unlike acute treatment (i.e., ATP+WM) however, WM pre-treatment markedly enhanced the ATP-induced slowing of run-up in a subset of cells (Fig. 9 C), so that on average, the latter effect was stronger but also more variable (Fig. 10 A). A similar modification of ATP-induced changes in the duration of run-up was observed in only one of seven recordings with ATP+WM (Fig. 9 C), suggesting that PIP2 depletion rather than acute inhibition of its resynthesis may be required to modify ATP-effects on run-up. Brief application of WM inhibits PIP2 replenishment without affecting PIP2 hydrolysis (Zhang et al., 2003), so the variable effectiveness of WM pre-treatment might have been related to differences in lipid turnover and degree of actual PIP2 depletion between cells. Regardless of the exact effects on run-up however, the protection by ATP against run-down was clearly unaffected by WM, suggesting that it was not related to lipid kinase–mediated mechanisms and arguing against a role of PIP2 depletion for Ca,2.3 channel run-down in our system.

Another line of reasoning has been that not PIP2 depletion itself, but rather accumulation of one of its fatty acid cleavage products, arachidonic acid (AA), is responsible for rundown of some neuronal VGCCs (Liu et al., 2001, 2006; Liu and Rittenhouse, 2003). However, neither 1 mg/ml of the AA-scavenger BSA (Fig. 9 E) nor 5 µM of the phospholipase C inhibitor U73122 (Fig. 9 F) affected the time course of run-up or run-down when they were added to the (ATP-free) internal solution (Fig. 10, A–C).

The effects of ATP depend on protein kinase–mediated phosphorylation

We next examined the role of changes in protein phosphorylation and dephosphorylation, which may result from ATP-depletion and have been implicated in the run-down of L-type high-voltage-activated Ca2+ channels (Armstrong and Eckert, 1987; Hilgemann, 1997). As summarized in Fig. 10 (A–C), the effects of MgATP were well preserved and again even somewhat enhanced when it was combined with 100 µM of the protein tyrosine kinase inhibitor genistein (GS). Combination with 10 µM of the broad-spectrum serine/threonine kinase inhibitor stauro on the other hand completely abolished MgATP effects on the duration of run-up, so the time to I_{max} with MgATP+stauro was not significantly different from the time course in parallel control recordings (Fig. 11, A–C; and Fig. 10 A). Moreover, although MgATP in the presence of stauro still significantly increased the fraction of current remaining after 7 min of run-down relative to parallel control recordings (Fig. 11, A and B), the net effect was significantly reduced when compared with that observed in other recordings with MgATP. Thus, expressed relative to the corresponding control recordings, MgATP+stauro was significantly less effective in increasing the current fraction after 7 min of run-down than MgATP alone, MgATP+WM, or MgATP+GS (Fig. 10 C).

There was no evident effect of stauro in the absence of ATP (Fig. 11, A–C), indicating that it acted by reducing ATP-protection from run-down. From these findings it follows that the ATP-induced slowing of run-up and most but not all the protection from run-down must have involved protein kinase–mediated phosphorylation of serine/threonine residues. On the other hand, run-down in the absence of ATP might reflect a progressive...
decrease of channel phosphorylation because of changes in the balance between constitutive de- and rephosphorylation. To test this assumption, we examined the effects of a sustained (30–60-min) incubation of cells in extracellular solution containing 1 µM stauro, which should mimic run-down by reducing basal protein phosphorylation. Consistent with (partial) run-down of the channels before establishment of the recordings, stauro pretreatment increased the number of cells lacking macroscopic currents and significantly reduced both $I_{\text{start}}$ and $I_{\text{max}}$ in the remaining cells (Fig. 11, D–F). Interestingly, it also significantly increased the magnitude and duration of run-up (Fig. 11, G–I), although the latter effect may have been related to the appearance of a plateau phase with little change in current amplitudes for several minutes. Subsequently, run-down proceeded with a normal time course, so the decrease of currents after 7 min was essentially the same as in parallel control recordings.

To assess how these effects were related to the ATP-sensitive gating changes, we also examined the effects of stauro pretreatment on channel voltage dependence (Fig. 12). As illustrated in Fig. 12 (B and C), activation and prepulse inactivation curves recorded in pretreated cells before the onset of run-down showed a significant shift to more negative test potentials when compared with the results obtained in untreated cells, although the voltage-sensitivity (i.e., $k_{\text{act}}$ and $k_{\text{inact}}$) was similar (not depicted).

In addition, stauro pretreatment tended to accelerate the voltage shifts during run-down (Fig. 12, D and E), although comparison of the exact time course is confounded by differences in cell size between the experiments, so no firm conclusions can be drawn from this finding. Interestingly however, it also effectively prevented the decrease in activation voltage sensitivity during run-down (Fig. 12 F) without altering the changes in inactivation voltage sensitivity (not depicted), possibly pointing to the involvement of multiple mechanisms at distinct sites.

Because the serine/threonine phosphatase calcineurin has been shown to interact with neuronal VGCCs (Chad and Eckert, 1986; Fomina and Levitan, 1997) and implicated in the run-down of certain K+ channels (Horváth et al., 2002), we also tested the effects of pretreating cells with a 60-min incubation in extracellular solution containing 10 µM of the membrane-permeable calcineurin-inhibitor deltamethrin (DM). However, the time course of changes in $I_{\text{ba}}$ observed in DM-pretreated cells (recorded with ATP-free pipette solution) was not significantly different from that in parallel control recordings (Fig. 10).
Run-up may involve activation of Leu-sensitive proteases
Finally, we performed experiments with Leu, a protease inhibitor that has been shown to prevent an irreversible ATP-resistant component of run-down in L-type VGCCs (Chad and Eckert, 1986; Elhamdani et al., 1994). Unexpectedly, and in contrast to all other manipulations tested, MgATP+100 µM Leu significantly decreased the magnitude of run-up (Fig. 13 A), so that Istart was similar but Imax was approximately half of the value observed in parallel control recordings. In addition, Leu not only abolished the MgATP-dependent slowing of run-up but actually produced a significant decrease of the time to Imax when it was combined with ATP (Fig. 13 D). Apart from altering the time course and net increase of currents, ATP+Leu reduced cell-to-cell variability in the magnitude of run-up (variance = 0.89 in control recordings vs. 0.16 with MgATP+Leu, P < 0.05) and diminished the initial slowing of inactivation (Fig. 13 C), indicating that both processes may be related to activation of Leu-sensitive proteases and influenced by variable endogenous protease and/or protease inhibitor levels.

Curiously, Leu also suppressed the protective effect of MgATP against run-down (Fig. 13 A), so that expressed relative to the corresponding control recordings, MgATP+Leu was significantly less effective against rundown than MgATP alone, MgATP+WM, MgATP after WM pretreatment, or MgATP+GS (Fig. 10 C). When the same concentration of Leu (100 µM) was used in the absence of MgATP, it had similar but quantitatively much less marked effects on run-up (Fig. 13 A–D) and no effect on the degree of run-down (Fig. 10 C) or the inactivation changes during run-up (Fig. 13 E).

Discussion
In the present study, we used conventional and perforated-patch-clamp recordings in a recombinant expression system to assess changes in Cav2.3 channel currents during cell dialysis. Our findings recapitulate studies about their native counterparts and show that dialysis with ATP-free internal solutions produces a characteristic sequence of run-up and run-down, the time-course of which depends on Rs, cell size, and recording configuration. The exponential decline in conductance during run-down by itself was voltage independent but paralleled by a progressive shift of channel voltage dependence to more negative test potentials. The voltage-shift reduced channel availability at the holding potential (i.e., PF) but proceeded at a constant rate and could only partly account for the complete loss of gating currents during run-down. Therefore, most of the decline in conductance was related to changes in the total number of functional channels (i.e., N and/or PF,max) and possibly to other factors (i.e., decrease of γ or P O,max). Run-down and all the associated
biophysical changes could be slowed or prevented by provision of ATP, and this protective action was almost completely abolished by inhibition of serine/threonine kinases. Protein kinase inhibition also mimicked the effects of run-down in intact cells, so it reduced overall peak current densities and hyperpolarized the voltage dependence of gating relative to untreated cells. The effects of ATP could be replicated neither by a nonhydrolyzable ATP analogue nor by GTP, which argues against a role of direct nucleotide binding or G-protein interactions but is consistent with the reported selectivity of protein kinases for ATP (Becher et al., 2013). Finally, run-down was not influenced by dialysis with a PLC inhibitor or the AA scavenger BSA, and the effects of ATP were unaffected by inhibition of lipid kinases, suggesting that run-down was not related to PIP2 hydrolysis, or accumulation of its cleavage product AA. Based on these findings, we conclude that (1) ATP protects from Ca2+,3 channel run-down by maintaining phosphorylation of serine/threonine residues on the channels themselves or an associated protein, (2) constitutive dephosphorylation of these sites in dialyzed cells affects channel gating and reduces the total number of functional channels, and (3) (de-)phosphorylation of at least some of the sites can also regulate channel function in intact cells. In addition, some of our findings point to a role of Leu-sensitive proteases for Ca2+,3 channel up-regulation during run-up and for the effects of protein phosphorylation on run-down.

Comparison with run-down in other voltage-gated Ca2+ channels

Since its first description, Ca2+ channel run-down has been consistently linked to diffusion-controlled dilution of ATP and other cytosolic components, but the underlying processes seem to differ among channels. ATP-induced protection from L-type Ca2+ channel run-down involves several interrelated processes, which may include changes in lipid turnover (Wu et al., 2002; Kaur et al., 2015), direct nucleotide binding (Feng et al., 2014), and phosphorylation of the channels by PKA, CaMKII and possibly other kinases (Wang et al., 2009; Xu et al., 2016). The latter counteracts constitutive dephosphorylation by opposing protein phosphatases and has been proposed to stabilize conformations that can be reprimed by voltage, CaM, or calpastatin and/or are more resistant to proteolytic degradation (Chad et al., 1987; Wang et al., 2009; Sun et al., 2014). This is in contrast to the situation
Figure 12. **Protein kinase inhibition in intact cells reproduces the gating changes during run-down in dialyzed cells.** (A) Voltage dependence of activation (right) and prepulse inactivation (left), determined at the onset of run-down (time 0) and in 7-min intervals thereafter in nine cells pretreated with 1 μM stauro for 30–60 min. (B) Comparison of half-activation voltages at the onset of run-down in cells pretreated with 1 μM stauro (same cells as in A) and under control conditions (same cells as in Fig. 4). (C) Comparison of half-inactivation voltages at the onset of run-down in cells pretreated with 1 μM stauro and under control conditions (same cells as in B). (D) Time-course of changes in half-activation voltages during run-down observed in cells pretreated with 1 μM stauro and under control conditions (same cells as in B). (E) Time-course of changes in half-inactivation voltages during run-down observed in cells pretreated with 1 μM stauro and under control conditions (same cells as in B). ***, P < 0.001 vs. Ctrl (one-way ANOVA with Bonferroni post hoc correction). Values are expressed as mean ± SEM.

Figure 13. **Run-up may involve activation of Leu-sensitive proteases.** (A) Mean data from 10 recordings with 5 mM MgATP and 100 μM Leu (ATP+Leu), 9 recordings with 100 μM Leu only (Leu), and 17 parallel control recordings (Ctrl). (B) Same data as in A but normalized to the initial current amplitude to illustrate differences in the magnitude of run-up. (C) Comparison of the current increase during run-up with ATP+Leu, Leu only, and in parallel control recordings (same cells as in A). **, P < 0.01 (one-way ANOVA with Bonferroni post hoc correction in D or Welch’s ANOVA with the Games-Howell multiple-comparison method in C). Values are expressed as mean ± SEM.
in Ca_{2.1} and Ca_{2.2} channels, where run-down has been linked to constitutive hydrolysis of membrane PIP_{2}, which can be prevented by ATP through lipid kinase–mediated PIP_{2} resynthesis (Wu et al., 2002; Suh et al., 2010). Although we have not directly tested the effects of PIP_{2}, our present findings demonstrate that lipid kinases are not involved in ATP-induced protection from Ca_{2.3} channel run-down and that the current decline is not related to accumulation of AA. Together with a previous study, where PIP_{2}-depletion had no effect on Ca_{2.3} channels (Suh et al., 2010), these findings argue against a major role of PIP_{2} hydrolysis for Ca_{2.3} channel run-down. Instead, ATP appears to maintain Ca_{2.3} channel function through increased phosphorylation of sites on the channels themselves or an associated protein, which may be required to counteract constitutive dephosphorylation. It has been shown that HEK-293 cells contain endogenous kinases and phosphatases, which can regulate the activity of transfected Ca^{2+} channels (Perez-Reyes et al., 1994; Johnson et al., 1997; Fuller et al., 2010; Aita et al., 2011; Blesneac et al., 2015). Although no firm conclusions can be drawn with regard to the exact sites or kinases involved in Ca_{2.3} channel maintenance, our findings provide a reliable baseline for further studies. They also raise the question how protein phosphorylation might be involved in the maintenance Ca_{2.1} and Ca_{2.2} channels, which still exhibit significant rundown in the presence of exogenous PIP_{2} (Gamper et al., 2004) or with mutations that reduce PIP_{2} sensitivity (Zhen et al., 2006). Because there can be cross talk with lipid signaling (Wu et al., 2002) and

Figure 14. **Phosphorylation sites and potential PEST regions in human Ca_{2.3} channels.** The secondary structure was visualized with Protter based on the human protein database entry Q15878, which includes exon 19 and exon 45 encoded stretches of the full-length Ca_{2.3}d-splice variant. Predictions were performed at the highest threshold by using the Group-based System Software for Prediction of Kinase-specific Phosphorylation Sites 3.0 and at a threshold score of +5.0 by using epestfind for detection of potential proteolytic cleavage sites, respectively. CAMKI, Ca^{2+}/Calmodulin-dependent protein kinase I; CAMKII, Ca^{2+}/Calmodulin-dependent protein kinase II; CAMKL, Ca^{2+}/Calmodulin-dependent protein kinase-like kinases; MAPK, mitogen-activated protein kinase; SGK, serum and glucocorticoid-regulated kinase 1; ter, terminal.

Data from Munton et al. 2007; Trinidad et al. 2008; Tweedie-Cullen et al. 2009; Huttlin et al. 2010; Rinschen et al. 2010; Wiśniewski et al. 2010; Goswami et al. 2012; Lundby et al. 2012; Trinidad et al. 2012.
PIP₂-hydrolysis partially inhibited Ca₉,2.3 channels after full activation by PKC (Jeong et al., 2016), it will also be interesting to examine the exact relevance of lipid turnover for this form of regulation.

**Molecular mechanisms and potential implications**

Very little is known about the molecular and structural mechanisms of Ca²⁺ channel run-down, but possibilities that have been considered include disruption of the linkage between voltage sensors and activation gate, entry into a permanent but existing inactivated state, and spontaneous drops in the total number of channels due to internalization or degradation. Our results do not resolve the exact processes underlying Ca₉,2.3 channel run-down but do argue against a significant decoupling between voltage-sensors and activation gate because run-down abolished both ionic and gating currents. In addition, Leu was ineffective against run-down and actually reduced the protective effects of ATP, suggesting that the current decline was not directly related to increased proteolytic degradation (but see next section). Together, these findings contrast with studies that L-type channel gating currents are not diminished by run-down (Hadley and Lederer, 1991; Costantin et al., 1999) and that Leu prevents an irreversible component of run-down in these channels (Chad et al., 1987). We can only speculate that dephosphorylation of Ca₉,2.3 channels themselves or an interacting protein induces a conformational change that leads to terminal inactivation and/or facilitates their removal from the membrane.

More importantly, our findings reveal that part of the current decline can be attributed to a gradual development of inactivation due to changes in channel voltage dependence. Based on the lack of saturation or concurrent changes in V₉ₑp, these effects may have been related to changes in the ratio of applied membrane voltage sensed by the channels. Interestingly, and in contrast to the exponential decline in conductance, the gating changes occurred at a constant rate, which is difficult to reconcile in terms of a conformational change. An attractive hypothesis that remains to be substantiated is that progressive dephosphorylation could shift channels among their states in a simple manner, such as altering the internal surface charge. It has been quantitatively demonstrated that the bulk electrostatic effects of dephosphorylation are sufficient to produce hyperpolarizing voltage shifts during run-down of other voltage-gated ion channels (Perozo and Bezanilla, 1990, 1991). Regardless of the exact mechanism, our findings document a close relationship between protein phosphorylation and channel voltage dependence, which may influence the basal features of current carried by cloned Ca₉,2.3 channels. Based on previous studies about their native counterparts, it seems reasonable to propose that this is also relevant under physiological conditions. Thus, Ca₉,2.3 channels are the third most extensively phosphorylated ion channels in mouse brain (Cerda et al., 2011; Fig. 14), and depolarization of intact hippocampal slices has been shown to induce bulk changes in their phosphorylation state (Hell et al., 1995).

**Run-up and the role of calpain-like proteases**

Unexpectedly, our findings also indicate that Ca₉,2.3 channel run-up may involve activation of calpain-like proteases (CLPs) and that it can be slowed but not prevented by ATP through increased protein phosphorylation. The process started immediately upon patch rupture, which can hardly be accounted for by ATP depletion but might reflect the loss of small cytosolic protease inhibitors because it was much slower in perforated recordings. In addition, run-up was observed with Ba²⁺ as the charge carrier but significantly faster and more complete with Ca²⁺, which is consistent with studies that show that Ba²⁺ can partially substitute for Ca²⁺ in activating CLPs (DeMartino and Croall, 1985; McDonald et al., 1994; Seydl et al., 1995). That phosphorylation suppresses most CLPs (Shiraha et al., 2002; Smith et al., 2003), while PIP₂ is well known to be required for activation and may considerably lower their Ca²⁺ requirement (Tompa et al., 2001; Leloup et al., 2010), could explain why (1) ATP, but not AMP-PNP or ATP+stauro, slowed run-up and (2) pretreatment with WM (i.e., partial depletion of membrane PIP₂) further increased the ATP-induced slowing in some cells. Dialysis or pretreatment with stauro alone had no effect or even increased run-up, suggesting that the process was not related to proteolytic activation of protein kinases. With this in mind and considering its fast onset, we can only speculate that run-up might involve partial proteolysis of the channels themselves and/or associated Ca₈,β₁ subunits. Both proteins contain several PEST motifs or PEST-like regions in their C and N termini (Fig. 14), and there is convincing evidence for a functional relevance of these sites as potential cleavage sites. For example, deletion of PEST-like regions in the Ca₈,β₁ subunit increased its half-life, stimulated currents mediated by coexpressed Ca₉,2.2 channels, and reduced voltage-dependent inactivation in HEK-293 cells (Sandoval et al., 2006). Likewise, intracellular protease application has been shown to produce strong stimulation of VGCCs and a partial loss of fast voltage-dependent inactivation, which has been linked to C-terminal cleavage of a conserved autoinhibitory region in the pore-forming Ca₉,α₁ subunit (Wei et al., 1994; Klöckner et al., 1995; Gao et al., 2001; Mikala et al., 2003). Given that C-terminal truncation of L-, N- and P/Q-type VGCCs by endogenous proteases has been demonstrated before in HEK-293 cells (Kubodera et al., 2003; Gomez-Ospina et al., 2006) and in vivo (Gerhardstein et al., 2000; Abele and Yang, 2012), it seems not so far off that the structural determinants for Ca₉,2.3 channel run-up could also be located in this region. With regard to the Leu effects on run-down, it may be important that L-type channels can also be cleaved at PEST regions within the core of the Ca₉,α₁ subunit, which has been shown to disrupt channel function (Groth et al., 2014; Michailidis et al., 2014). That Ca₉,2.3 channels lack such regions might account for the inability of Leu to sustain ATP-induced protection. On the other hand, this cannot explain the puzzling finding that Leu actually impaired the protective effects of ATP on run-down. We can only speculate that the latter might be related to differential phosphorylation of long and short forms or changes in the accessibility of certain sites. Because Leu could also alter the effects of ATP in some other way, further studies will clearly be required to delineate the underlying processes. However, our finding that protein kinase inhibition alone replicated most of the gating changes during run-down in intact cells leads us to conclude that proteolysis is either not required for these effects or that it does also occur in intact cells.
Conclusion
In summary, our findings show that run-down of cloned Ca_{2.3} channels in dialyzed cells is associated with progressive changes in channel voltage-dependence and a decrease in the total number of functional channels, which can be prevented by ATP through maintained protein phosphorylation and replicated by protein kinase inhibition in intact cells. These findings distinguish the process from run-down in other Cav2 channels and suggest that one or more sites on the channels themselves or an associated protein must be phosphorylated to maintain them in a functional state. In addition, changes in the phosphorylation of a subset of sites may directly influence channel gating, possibly through bulk electrostatics. Although additional studies will be required to delineate the underlying mechanisms and their relevance in native cells, our results provide a reliable baseline that could stimulate further work on Ca_{2.3} channel regulation by protein kinases, phosphatases, and possibly proteases.

Acknowledgments
We thank Tobias Pook for technical help and supply of software for facilitated analysis and Mrs. Renate Clemens for her excellent and permanent assistance.

This work was financially supported by the Deutsche Forschungsgemeinschaft (SCHN 387/21-1).

The authors declare no competing financial interests.

Author contributions: F. Neumaier contributed to the conception and design of the work, performed the experiments, analyzed and interpreted the data, prepared the figures, wrote the first draft of the manuscript, and coordinated its critical revision. T. Schneider contributed to the conception and design of the work, interpretation of the data, and critical revision of the manuscript. S. Alpdogan and J. Hescheler contributed to analysis and interpretation of the data and drafting of the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. Experiments were carried out in the Institute for Neurophysiology at the University of Cologne, Germany.

Sharon E. Gordon served as editor.

Submitted: 14 August 2017
Revised: 22 December 2017
Accepted: 24 January 2018

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