Temperature-dependent Modulation of Ca$_{v}$$3$ T-type Calcium Channels by Protein Kinases C and A in Mammalian Cells\textsuperscript{*}$^{1}$

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Jean Chemin$^{1}$, Alexandre Mezghrani$^{3}$, Isabelle Bidaud$^{2,3}$, Sebastien Dupasquier$^{3,7}$, Fabrice Marger$^{3,4}$, Christian Barrière$^{3}$, Joël Nargeot$^{3}$, and Philippe Lory$^{3}$

From the$^{4}$Département de Physiologie and the$^{6}$Département d’Oncologie Moléculaire et Cellulaire, Institut de Génomique Fonctionnelle, CNRS UMR 5203, INSERM U661, Universités de Montpellier 1 et 2, Institut Fédératif de Recherche 3, Montpellier 34094, France

Modulation of low voltage-activated Ca$_{v}$$3$ T-type calcium channels remains poorly characterized compared with high voltage-activated Ca$_{v}$$1$ and Ca$_{v}$$2$ calcium channels. Notably, it is yet unresolved whether Ca$_{v}$$3$ channels are modulated by protein kinases in mammalian cells. In this study, we demonstrate that protein kinase A (PKA) and PKC (but not PKG) activation induces a potent increase in Ca$_{v}$$3.1$, Ca$_{v}$$3.2$, and Ca$_{v}$$3.3$ currents in various mammalian cell lines. Notably, we show that protein kinase effects occur at physiological temperature ($\sim 30$–$37^\circ C$) but not at room temperature ($\sim 22$–$27^\circ C$). This temperature dependence could involve kinase translocation, which is impaired at room temperature. A similar temperature dependence was observed for PKC-mediated increase in high voltage-activated Ca$_{v}$$2.3$ currents. We also report that neither Ca$_{v}$$3$ surface expression nor T-current macroscopic properties are modified upon kinase activation. In addition, we provide evidence for the direct phosphorylation of Ca$_{v}$$3.2$ channels by PKA in in vitro assays. Overall, our results clearly establish the role of PKA and PKC in the modulation of Ca$_{v}$$3$ T-channels and further highlight the key role of the physiological temperature in the effects described.

Voltage-gated Ca$^{2+}$ channels (VGCCs)$^{5}$ are unique among voltage-gated ion channels because the permeant Ca$^{2+}$ ion also acts as an intracellular second messenger, triggering diverse cellular functions (1, 2). VGCCs are therefore implicated in neuronal and cardiac excitability as well as in muscle contraction, neurotransmitter release or hormone secretion, and gene expression (1–6). Thus, the modulation of VGCCs plays a pivotal role in the control of cardiac and brain activities.

VGCCs are divided into three families: the L-type channels (Ca$_{v}$$1$ family); the neuronal N-, P/Q-, and R-type channels (Ca$_{v}$$2$ family); and the T-type channels (Ca$_{v}$$3$ family) (7). Although the molecular mechanisms implicated in the modulation of high voltage-activated Ca$^{2+}$ channels of the Ca$_{v}$$1$ and Ca$_{v}$$2$ families are beginning to be unraveled (mainly, protein kinases for Ca$_{v}$$1$ channels and $\beta$-$\gamma$-subunits of G proteins and protein kinase C (PKC) for Ca$_{v}$$2$ channels) (6, 8), those implicated in low voltage-activated Ca$_{v}$$3$ T-type channel regulation remain debated (9). Some transduction pathways mediate either decreases or increases in native T-currents, depending on the tissues/species studied and/or on the recording conditions (9). This apparent complexity could be explained by the existence of three T-channels (Ca$_{v}$$3.1$ or $\alpha_{1G}$, Ca$_{v}$$3.2$ or $\alpha_{1H}$, and Ca$_{v}$$3.3$ or $\alpha_{1I}$), which include different splice variant isoforms with specific tissue patterns and developmental expression (10). Additional regulatory subunits of T-channels might also be involved in their modulation, but the native composition of T-channels remains unknown (10). Therefore, molecular studies on recombinant T-channels are required to clarify their modulation.

Recent studies on recombinant T-channels have highlighted the complexity of their regulation (9). On one hand, as for Ca$_{v}$$2$ channels, Ca$_{v}$$3.2$ currents are inhibited by $\beta$-$\gamma$-subunits of G proteins in mammalian cells (11, 12). In contrast with Ca$_{v}$$2$ currents, this mechanism cannot be generalized because this modulation is restricted to Ca$_{v}$$3.2$ and specifically occurs with a G$\beta$-$\gamma$ dimer containing $\beta_{2}$-subunits (11, 12). On the other hand, as observed with Ca$_{v}$$1$ channels, Ca$_{v}$$3$ currents are increased by protein kinase activation (13–18). Yet again, kinase effects on T-currents appear complex (9) because protein kinase A (PKA)- and PKC-mediated T-current increase has been observed in Xenopus oocytes but not in mammalian cells expressing Ca$_{v}$$3$ currents (11, 16–19). In this context, it is interesting to note that protein kinase effects on native VGCCs were first described in heart frog cells and appear more robust in these cells compared with those observed in mammalian tissues (20–23). Thus, we reasoned that these differences in kinase modulation could involve the temperature at which the experiments were performed, i.e. mostly at room temperature. Although room temperature is within the physiological range for amphibian cells, it is far below that required by mammalian cells. In this study, we provide new data on PKA and PKC activation in the function of temperature that clearly establish the role of these two kinases, but...
not of protein kinase G (PKG), in the modulation of Ca\textsubscript{v}3 T-currents in various mammalian cell lines.

**MATERIALS AND METHODS**

**Cell Culture and Transfection Protocols**—tsA-201 cells and a Chinese hamster ovary (CHO) cell line stably expressing Ca\textsubscript{v}3.2 channels (CHO-Ca\textsubscript{v}3.2; a generous gift from Dr. Emmanuel Bourinet) were cultivated in Dulbecco's modified Eagle's medium and Ham's F-12 medium, respectively, supplemented with GlutaMAX and 10% fetal bovine serum (Invitrogen). Neomycin (0.6 mg/ml; Invitrogen) was added to the CHO-Ca\textsubscript{v}3.2 cell medium. tsA-201 cell transfection was performed using jetPEI (Qbiogen, Inc.) according to the manufacturer's protocol (4 µl of jetPEI for 1.5 µg of DNA/35-mm Petri dish) with a DNA mixture containing 0.5% of a green fluorescent protein (GFP) plasmid and 99.5% of one of the pcDNA3 plasmid constructs that code for the human Ca\textsubscript{v}3.1a, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 T-channel isoforms (24). Ca\textsubscript{v}2.1-hemagglutinin (HA) (25) and Ca\textsubscript{v}2.3 (rat brain E-II) (26) were transfected under the same conditions with a mixture containing the α\textsubscript{2}δ\textsubscript{1} and β\textsubscript{4} subunits at a 2:1:1 ratio. Two days after, cells were then dissociated with Versen (Invitrogen) and plated at \(35 \times 10^3\) cells/35-mm Petri dish. Electrophysiological recordings were performed the following day.

**Electrophysiological Recordings**—Macroscopic currents were recorded at room temperature (\(~22^\circ\text{C}\)) by the whole-cell patch clamp technique using an Axopatch 200B amplifier (Axon Instruments). The extracellular solution contained 135 mM NaCl, 20 mM tetraethylammonium chloride, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH adjusted to 7.4 with KOH, \(~330\) mosm). Borosilicate glass pipettes have a typical resistance of 1.5–2.5 megohms when filled with an internal solution containing 140 mM CsCl, 20 mM tetraethylammonium chloride, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH adjusted to 7.4 with KOH, \(~315\) mosm). Recordings were filtered at 2 kHz. Data were analyzed using pCLAMP9 (Axon Instruments) and GraphPad Prism software. Student’s t-test or one-way analysis of variance combined with a Newman-Keuls post-test were used to compare the different values, which were considered significant at \(p < 0.05\). Results are presented as the means ± S.E., and \(n\) is the number of cells used.

**Imaging of PKC Translocation**—To study PKC translocation as a function of temperature, we generated a human PKCβ1 construct with the C terminus fused to GFP using the
pEGFP-N1 plasmid (Clontech), PKCβ1-GFP was inserted into pcDNA4 (Invitrogen) and transfected into CHO-CaV3.2 cells. Two days after, cells were plated onto 12-mm glass coverslips. The following day, phorbol 12-myristate 13-acetate (PMA) treatments were performed (100 nM in the culture medium for 10 min at 37 °C or at room temperature), and cells were then fixed for 20 min in 4% paraformaldehyde. Digital images were acquired on a Leica microscope and further analyzed using Adobe Photoshop.

Luminometric Analysis of HA-tagged CaV3.2 Channels—tsA-201 cells were cultured in 24-well plates and transfected with a GFP-CaV3.2-HA construct (27). Two days after transfection, PMA treatments were performed (100 nM in the culture medium for 30 or 60 min at 37 °C), and cells were then rinsed with phosphate-buffered saline (PBS) and fixed for 5 min in 4% paraformaldehyde. After three PBS washes, half of the wells were permeabilized with 0.1% Triton X-100 for 5 min and rinsed three times with PBS. Cells were then incubated for 30 min in blocking solution (PBS plus 1% fetal bovine serum). The GFP-CaV3.2-HA protein was detected using a rat anti-HA monoclonal antibody (1:1000 dilution; clone 3F10, Roche Diagnostics) after incubation for 1 h at room temperature. After four washes with PBS plus 1% fetal bovine serum for 10 min, cells were incubated for 30 min with horseradish peroxidase-conjugated goat anti-rat secondary antibody (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were rinsed four times with PBS for 10 min before addition of SuperSignal enzyme-linked immunosorbent assay Femto maximum sensitivity substrate (Pierce). Luminescence was measured using a VICTOR® luminometer (PerkinElmer Life Sciences), and the protein amount in each well was then measured using the BCA assay (Pierce) to normalize the measurements. All data were normalized to the level of signal obtained for incubation with the control medium (1:10,000 Me2SO). Four independent sets of transfection experiments were performed under each condition, and the results are presented as the means ± S.E.

**RESULTS**

To investigate the effects of protein kinase activation in mammalian cells expressing low voltage-activated T-currents, various protein kinase activators (or the control solution) were incubated with cells at 37 °C or at room temperature (~22 °C) for 10 min before electrophysiological recordings, which were performed at room temperature in either the presence or absence of the activator (Fig. 1, A
and B, insets). Under these conditions, the PKC activator PMA (100 nM) induced a strong increase in the CaV3.1 currents (~100%, \( p < 0.001, n > 70 \)) when preincubated at 37 °C (Fig. 1A). Similar results were obtained for CaV3.2 and CaV3.3 channels because PMA induced an ~105% increase \( (p < 0.001, n > 90) \) in CaV3.2 currents and had a maximal effect on CaV3.3 currents (~145%, \( p < 0.001, n > 100 \)) (Fig. 1C). Interestingly, we found that PMA had no significant effect when incubated at room temperature (up to 1 h of incubation; \( p > 0.05 \) and \( n > 30 \) for each CaV3 current) (Fig. 1, B–D). We then evaluated the PMA effects on CaV3.3 currents during incubation at several intermediate temperatures (27, 30, and 32 °C). We found that the PMA effects gradually developed at 30 and 32 °C (~60 and ~100% increases, respectively; \( p < 0.05; n > 40 \)) (Fig. 2), whereas no effect was observed at 27 °C \( (p > 0.05, n > 34) \) (Fig. 2). The PMA effects as a function of temperature can be described by a sigmoidal Hill equation (Fig. 2), which indicates that the temperature producing PMA half-effects is ~30.5 °C and the Hill slope ~18.9, indicating a strong temperature dependence of PMA effects. We also investigated the temperature dependence of the PMA effects on high voltage-activated CaV2.3 currents, which are increased by PMA at room temperature in *Xenopus* oocytes (26). As observed for CaV3 currents, we found that 100 nM PMA induced a strong increase in CaV2.3 currents in human embryonic kidney 293 cells when incubated at 37 °C (~150%, \( p < 0.001, n > 30 \)) (Fig. 3, A and B), whereas PMA has no effect when incubated at room temperature \( (p > 0.05, n > 35) \) (Fig. 3C).

It has been described that PKC activation is associated with a redistribution of its subcellular localization (28). We asked whether this phenomenon is altered in mammalian cells at a non-physiological temperature (Fig. 4). To this end, we generated a GFP-tagged PKCβ1 construct (PKCβ1-GFP; see “Materials and Methods”) to visualize PMA-induced PKC translocation in transfected mammalian cells (Fig. 4, B and C). As described for PKC of the classical family (28), we found that 100 nM PMA induced PKC translocation to the plasma membrane in mammalian CHO cells when incubated for 10 min at 37 °C (Fig. 4B). However, PMA had no effect on...
PKC translocation when incubated at room temperature (Fig. 4D).

We next investigated whether PMA effects on CaV3 currents are specific to PKC activation (Fig. 5). To assess the involvement of PKC, we used 4α-PMA (100 nm), a PMA analog inactive on PKC, and chelerythrine or Gö 6976 (both at 1 μM), two selective inhibitors of PKC (Fig. 5). We found that 4α-PMA had no significant effect on the three CaV3 currents (p > 0.05 and n > 25 for each CaV3 current), whereas both chelerythrine and Gö 6976 suppressed PMA effects (p > 0.05 and n > 25 for each CaV3 current). In addition, we found no basal effect of chelerythrine on the three CaV3 currents (p > 0.05 and n > 60 for each CaV3 current) (Fig. 5, A–C).

To estimate whether PKC activation-induced increase in T-currents is associated with changes in macroscopic biophysical properties, we next performed steady-state activation and inactivation protocols (Fig. 6, A and B, insets). As shown for CaV3.1 (Fig. 6, A–D), PMA induced no significant change in steady-state activation and inactivation properties. For CaV3.1, current-voltage curves (Fig. 6C) indicated steady-state activation V0.5 values of −48.6 ± 1.4 mV (n = 10) under control conditions and −47.6 ± 1.3 mV (n = 13, p > 0.05) after PMA treatment, whereas slope values were 4.1 ± 0.4 mV (n = 10) under control conditions and 4.3 ± 0.4 mV (n = 13, p > 0.05) after PMA treatment. Similarly, steady-state inactivation properties were not significantly modified by PKC activation (Fig. 6D) because V10 values were −70.7 ± 0.7 mV (n = 10) under control conditions and −71.8 ± 0.6 mV (n = 11, p > 0.05) after PMA treatment, whereas slope values were 4.7 ± 0.2 mV (n = 10) under control conditions and 4.1 ± 0.2 mV (n = 11, p > 0.05) after PMA treatment. In the same way, neither the steady-state activation nor inactivation properties of the CaV3.2 (n > 10 under each condition, p > 0.05) (Fig. 6E) and CaV3.3 (n > 10 under each condition, p > 0.05) (Fig. 6F) currents were significantly affected by PMA treatment.

We also investigated whether the surface expression of CaV3 channels in mammalian tsA-201 cells is modulated by PKC activation (supplemental Fig. 1). To this end, we used a CaV3.2 channel construct containing an extracellular HA tag, which allowed its surface (non-permeabilized condition) and total expression (permeabilized condition) (supplemental Fig. 1A) to be measured by enzyme-linked
We found that PMA treatment did not induce significant changes in both membrane expression (supplemental Fig. 1B) and total expression (supplemental Fig. 1C) of CaV3.2-HA channels after 30 min or 1 h of treatment at 37 °C. Also, protein kinase activation did not influence the ratio of membrane expression to total expression, which was ~20% under all conditions (supplemental Fig. 1D), further indicating that surface expression of CaV3.2-HA channels is not altered.

We next explored whether, as observed for PKC, cyclic nucleotide-dependent protein kinases modulate T-currents in mammalian cells at physiological temperatures. To investigate the effects of PKA, we used dibutyryl cAMP (dB-cAMP), a membrane-permeant analog of cAMP (Fig. 7). We found that dB-cAMP induced an increase in the three CaV3 T-currents when incubated at 37 °C (Fig. 7, A–C) but had no significant effect when incubated at room temperature (p > 0.05 and n > 30 for each CaV3 current) (Fig. 7, B–D). As observed above with PMA, dB-cAMP produced a stronger effect on CaV3.3 currents (~140% increase, p < 0.001, n > 60) compared with CaV3.2 currents (~70% increase, p < 0.001, n > 80) and CaV3.1 currents (~55% increase, p < 0.001, n > 80) (Fig. 7C). Furthermore, we observed a similar temperature dependence using monobutyryl cAMP (the bioactive product of dB-cAMP acting on PKA). Indeed, monobutyryl cAMP treatment increased CaV3.3 currents at 37 °C (~110% increase, p < 0.01, n > 35) but not at room temperature (p > 0.05, n > 40). These latter results demonstrate that the temperature dependence described here does not involve endogenous esterases or amidases, which convert dB-cAMP into monobutyryl cAMP (for review, see Ref. 29).

We next probed the involvement of PKA activation in the dB-cAMP effects using the specific PKA inhibitor KT5720. KT5720 suppressed dB-cAMP-induced increases in T-currents (p < 0.05 and n > 20 for each CaV3 current) (Fig. 8, A–C) but had no effect on basal T-currents (p > 0.05 and n > 20 for each CaV3 current). It should be noted that in previous experiments, we used the PKA inhibitor H-89, but we observed that although this compound abolished dB-cAMP effects, it had strong direct inhibitory effects at micromolar concentrations on the three CaV3 currents and especially on CaV3.2 (data not shown). We further investigated whether PKA effects would involve a direct phosphorylation of CaV3 channels. To this end, we took advantage of a commercially available PKA catalytic subunit that is constitutively active and allows in vitro kinase assays of immunoprecipitated HA-tagged CaV3.2 channels (Fig. 8D). We found that PKA induced phosphorylation of CaV3.2 channels.

**FIGURE 7.** dB-cAMP induces an increase in CaV3 T-currents in transiently transfected tsA-201 cells at 37 °C but not at room temperature. A and B, effect of 1 mM dB-cAMP (dB-cAMP) on CaV3.1 currents when incubated for 10 min at 37 °C and at room temperature (RT), respectively, before electrophysiological experiments. C and D, summary of the data obtained for CaV3.1, CaV3.2, and CaV3.3 currents upon incubation of dB-cAMP at 37 °C and at room temperature, respectively. Currents were elicited by a −45-mV depolarization (200-ms duration for CaV3.1 and CaV3.2 currents and 450-ms duration for CaV3.3 currents) applied immediately after the whole-cell configuration from a holding potential of −80 mV. Ctrl, control. ***, p < 0.001.
Modulation of CaV3 Currents by PKA and PKC

**FIGURE 8.** Involvement of PKA in Bt2cAMP-induced increases in the three CaV3 currents and direct phosphorylation of CaV3.2 channels by PKA. A–C, summary of the data obtained for CaV3.1, CaV3.2, and CaV3.3, respectively, after 10 min of incubation at 37 °C of Bt2cAMP (dB-cAMP; 1 μM) and/or KT5720 (a PKA inhibitor; 0.5 μM). KT5720 was applied at least 4 h at 1 μM prior to the 37 °C incubation protocols. Currents were elicited by a −45-mV depolarization (200-ms duration for CaV3.1 and CaV3.2 currents and 450-ms duration for CaV3.3 currents) applied immediately after the whole-cell configuration from a holding potential of −80 mV. Ctrl, control. D, effects of the constitutively active PKA catalytic subunit on immunoprecipitated HA-tagged CaV3.2 channels in in vitro phosphorylation assays. In vitro phosphorylation assays were performed at 30 °C using [γ-32P]ATP, and 32P-labeled proteins were quantified using a PhosphorImager after standard SDS-PAGE. The presence of HA-tagged CaV3.1 and CaV3.2 channels was further confirmed by Western blotting (not shown). **A**, **B**, and **C**, summary of the data obtained for CaV3.1, CaV3.2, and CaV3.3, respectively, after 10 min of incubation at 37 °C of Bt2cAMP (dB-cAMP; 1 μM) and/or KT5720 (a PKA inhibitor; 0.5 μM). KT5720 was applied at least 4 h at 1 μM prior to the 37 °C incubation protocols. Currents were elicited by a −45-mV depolarization (200-ms duration for CaV3.1 and CaV3.2 currents and 450-ms duration for CaV3.3 currents) applied immediately after the whole-cell configuration from a holding potential of −80 mV. Ctrl, control. D, effects of the constitutively active PKA catalytic subunit on immunoprecipitated HA-tagged CaV3.2 channels in in vitro phosphorylation assays. In vitro phosphorylation assays were performed at 30 °C using [γ-32P]ATP, and 32P-labeled proteins were quantified using a PhosphorImager after standard SDS-PAGE. The presence of HA-tagged CaV3.1 and CaV3.2 channels was further confirmed by Western blotting (not shown). ***p < 0.001.

but not of CaV3.1 channels, which are insensitive to PKA (6, 8) and were used here as a negative control (Fig. 8D).

In contrast with the results obtained for PKA activation, the activation at physiological temperature of PKG with dibutyryl cGMP (Bt2cGMP) had no significant effect on the three CaV3 currents (p > 0.05 and n > 40 for each CaV3 current) (supplemental Fig. 2, A and B). Furthermore, we treated cells with the specific PKG inhibitor KT5823 (supplemental Fig. 2B) and found that this compound had no significant effect on the three CaV3 current densities (p > 0.05 and n > 20 for each CaV3 current) (supplemental Fig. 2B).

Finally, we further investigated the effect of PKC, PKA, and PKG activation in a CHO cell line stably expressing CaV3.2 currents (Fig. 9). In these cells, we found that PKA and Bt2cAMP (but not Bt2cGMP) induced an increase in CaV3.2 currents when incubated at physiological temperature (Fig. 9A). As observed above in transiently transfected tsA-201 cells, PMA induced stronger effects on CaV3.2 currents (~125% increase) compared with Bt2cAMP (~50% increase, p < 0.001, and n > 30 under each condition) (Fig. 9B). However, PMA and Bt2cAMP had no significant effect when incubated at room temperature (up to 1 h of incubation; p > 0.05 and n > 24 under each condition) (Fig. 9C).

**DISCUSSION**

The main findings of our study are that recombinant T-channels are modulated by PKA and PKC (but not by PKG) in different mammalian cells and that these effects are temperature-dependent. Considering the essential role of T-channels in cardiac and neuronal pacemaking (10, 30–32) as well as in slow wave sleep (33, 34), absence epilepsy (35, 36), and pain perception (37–39), the investigation of their modulation at the molecular level is of crucial importance. Three T-channel subunits have been identified: CaV3.1, CaV3.2, and CaV3.3 (7, 10). Among these three subunits, only CaV3.2 is modulated in mammalian cells by βγ-subunits of G proteins and by Ca2+/calmodulin-dependent protein kinase II (11–15). Consequently, the molecular pathways implicated in CaV3.1 and CaV3.3 regulation remain largely unknown (9). In this study, using the PKA activator Bt2cAMP and the PKC activator PMA, we have demonstrated that PKA and PKC activation increases CaV3.1, CaV3.2, and CaV3.3 currents in mammalian tsA-201 cells. Both Bt2cAMP and PMA produced stronger effects on CaV3.3 currents (~150% increase) compared with CaV3.1 and CaV3.2 currents. In addition, CaV3.1 and CaV3.2 currents were more sensitive to PMA (~100% increase) compared with Bt2cAMP (~50% increase). The involvement of PKA in Bt2cAMP effects was further demonstrated with KT5720, a specific PKA inhibitor (40), which suppressed the Bt2cAMP-induced increase in T-currents. In the same way, PKC is implicated in PMA-induced T-current increase, as assessed with 4a-PMA, an analog of PMA inactive on PKC. Furthermore, the PMA effect is abolished by the PKC inhibitors chelerythrine and Go6976 (41, 42). This pharmacological profile of PKC inhibition suggests involvement of the classical PKC family (42), the members of which are recruited by Gq-coupled receptors. In contrast with PKA and PKC activation, we found that PKG activation with Bt2cGMP had no effect on the three CaV3 currents. This lack of effect of PKG activation could be explained by a high basal activity of this kinase in tsA-201 cells. However, basal PKG inhibition with the specific PKG inhibitor KT5823 (40) had no significant effect on the three CaV3 current densities, further indicating that PKG does not modulate T-currents. Because the results described above could be restricted to the cell type studied and/or to transient expression, we investigated the effect of PKC, PKA, and PKG activation in a CHO cell line stably expressing CaV3.2 currents. In these cells, we confirmed that PMA and Bt2cAMP (but not Bt2cGMP) induced an increase in CaV3.2 currents. As observed above in transiently transfected tsA-201 cells, PMA induced stronger effects on CaV3.2 currents compared with Bt2cAMP. Therefore, our results demonstrate
dependence is well described by a sigmoidal Hill function, 27 °C) in both tsA-201 and CHO cell lines. This temperature logical temperature (37 °C) but not at room temperature (22–

Furthermore, we have highlighted here that the physiological properties (43–45) and possibly their regulation. How-

FIGURE 9. Effects of PMA, Bt2cAMP, and dibutyryl cGMP (Bt2cGMP) on CaV3.2 currents stably expressed in CHO cells. A, effects of 100 nM PMA, 1 mM Bt2cAMP and 1 mM Bt2cGMP after 10 min incubation at 37 °C on CaV3.2 currents stably expressed in CHO cells. B, summary of the data obtained for PMA, Bt2cAMP and Bt2cGMP after 10 min incubation at 37 °C. C, summary of the data obtained for PMA, Bt2cAMP, and Bt2cGMP after 10 min incubation at room temperature (−22 °C). Currents were elicited by a −45 mV depolarization of 200 ms duration applied immediately after the whole-cell configuration from a holding potential of −80 mV. **, p < 0.01; ***, p < 0.001.

that protein kinase modulation of T-currents does not depend on the mammalian cell type studied.

Modulation of CaV3 currents by protein kinases has been observed in Xenopus oocytes but not in mammalian cells expressing CaV3 currents (11, 16–19). This absence of kinase modulation in mammalian cells could be explained by the presence or absence of specific protein kinase isoforms, protein kinase-interacting proteins, and/or T-channel auxiliary subunits. In addition, T-channel pore α1-subunits are also subject to alternative splicing, which influences their electrophysiological properties (43–45) and possibly their regulation. However, we have demonstrated in this study that the currents generated by the minimal isotype of each pore α1-subunit (including no additional exon) (46) are modulated by protein kinases when expressed alone (without any auxiliary subunit) in mammalian cells. We have also reported that T-currents are not under the control of basal activation of protein kinase in mammalian cells, as assessed with specific kinase inhibitors. Furthermore, we have highlighted here that the physiological temperature is crucial for protein kinase effects. Indeed, we have shown that both PKA and PKC effects occur near physiological temperature (37 °C) but not at room temperature (22–27 °C) in both tsA-201 and CHO cell lines. This temperature dependence is well described by a sigmoidal Hill function, which indicates that the temperature producing PMA half-effects on CaV3.3 currents is ~30.5 °C and the Hill slope ~18.9, indicating a strong temperature dependence of PMA effects. A similar temperature dependence was observed for the three CaV3 currents as well as for CaV2.3 currents (a high voltage-activated calcium channel), which was strongly increased by PKC at 37 °C in mammalian cells. In addition, we have provided evidence that PMA-induced PKC translocation to the plasma membrane (where T-channels display their activity) is altered in mammalian cells at non-physiological temperatures. It should be noted that contrary to mammalian cells, PKC translocation is fully preserved in Xenopus oocytes at room temperature (47). In this context, it appears that our results on CaV3 currents and CaV3.2 currents are reminiscent of those obtained for modulation of L-type CaV1.2 currents by PKA. Indeed, although the CaV1.2 subunit is directly phosphorylated by PKA in in vitro kinase assays at Ser1928 (8, 48), the phosphorylation and modulation of L-type CaV1.2 currents by PKA activators does not occur in human embryonic kidney 293 cells in the absence of AKAP (A protein kinase-anchoring protein) (8, 49, 50). In fact, AKAP binds and tether PKA to particular sites at the plasma membrane where CaV1.2 channels reside, allowing their phosphorylation at room temperature in mammalian cells and the increase in the current (8, 49–51). Overall, although we cannot completely exclude that kinase activity is involved in the temperature dependence described here, our results strongly suggest that kinase localization (translocation) is impaired at non-physiological temperature. It is interesting to note that PMA effects on T-currents occur near 30 °C, a temperature that is permissive for export from the Golgi network (52, 53), which is implicated in the translocation of PKC (54). Indeed, incubation at 20 °C, which blocks exocytotic vesicle traffic from the Golgi network, abolishes the PMA-induced translocation of PKC to the plasma membrane in mammalian NIH 3T3 cells (54). Therefore, it is attractive to suggest that this phenomenon could be involved in the temperature dependence described in our study (as suggested by our PKC-GFP imaging).

We have also provided data demonstrating that protein kinase effects do not involve changes in T-current macroscopic biophysical properties that could explain the modulation. In addition, the surface expression of CaV3 channels is not altered upon kinase activation, as assessed by enzyme-linked immunosorbent assay/luminometry experiments. Furthermore, the in vitro kinase assays suggest that PKA could act directly on T-channels. Overall, our results are in good agreement with those obtained for the PKA modulation of L-type currents (22). Indeed, the CaV1.2 subunit is directly phosphorylated by PKA, whereas L-type current macroscopic properties are weakly affected by PKA activation. Furthermore, single channel recordings have highlighted an increase in the number of functional channels (resulting from an increase in the proportion of non-blank sweeps), which underlies PKA effects on L-type currents (see Ref. 22 for an insightful review). Therefore, it is tempting to suggest that this mechanism might be conserved for kinase action on VGCCs and that PKA and PKC activation
Modulation of Ca\textsubscript{v}3 Currents by PKA and PKC transforms nonfunctional forms of T-channels to functional forms.

*Xenopus* oocytes and mammalian heterologous expression systems are currently used to study recombinant ion channels. Although these expression systems have provided a lot of important information, especially about electrophysiological ion channel properties, the results obtained in these two systems concerning ion channel modulation are often divergent (9). Our present findings indicate that at least for calcium channels, the physiological temperature is determinant for studying ion channel modulation by protein kinases in mammalian cells. In conclusion, our study clearly establishes the role of PKA and PKC in the modulation of Ca\textsubscript{v}3 T-currents.

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