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Increase in Ca²⁺ current sustained cAMP levels enhances proliferation rate in GH3 cells

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Abstract

Calcium and cAMP are important intracellular modulators involved with the control of several signaling cascades and functions within cells. A tight control of these molecules is necessary to keep normal cell function. Proliferation is one important function regulated by these second messengers. Here we investigated the effects of long-term (24 and 48h) incubation with forskolin (10μM), an agonist of adenylate cyclase, on voltage-dependent Ca$^{2+}$-channel activity using rat GH3 cells. Patch-Clamp experiments revealed a biphasic effect on whole-cell Ca$^{2+}$ currents. After an initial decrease when applied acutely, prolonged forskolin treatment induced a significant increase in total Ca$^{2+}$ current density. Accordingly, dibutyryl-cAMP incubation (dbcAMP, 200μM) also showed increase in Ca$^{2+}$ current density. However, the maximum effect of dbcAMP occurred only after 72h incubation, whereas forskolin showed maximal effect earlier (at 48h). FRET-experiments confirmed that these results were due to elevated intracellular cAMP with forskolin showing a peak in the first seconds after been applied. In addition, forskolin resulted in a shift in of the activation threshold of the Ca$^{2+}$ current by approximately -7 mV and a fast inactivating component having barium as a charge carrier. Mibefradil (10μM), but not nickel (50μM) inhibited selectively the fast inactivating current component, indicating that the preferentially up-regulated component was T-type Ca$^{2+}$ channels. Further detailed whole-cell Ca$^{2+}$ current analysis also suggested an increase in L-type Ca$^{2+}$ current. We observed that long-term incubation with forskolin significantly increase cell proliferation. We, then, propose
that cAMP chronic modulation of voltage-dependent Ca$^{2+}$ channels promote and
regulate cell proliferation.

Keywords: Patch-Clamp, FRET-imaging, Cell proliferation, cAMP, Forskolin,
Calcium channels
Introduction

Ca$^{2+}$ is a ubiquitous intracellular second messenger and it is critical for the regulation of innumerable cellular processes, including gene expression, apoptosis, cell cycle and proliferation. Perturbations in intracellular Ca$^{2+}$ homeostasis can result in cell malfunctioning (Carruthers & Suntzeff 1944; Marchi & Pinton 2016; Prevarskaya & Skryma & Shuba 2010). Therefore, a tight control of Ca$^{2+}$ mobilization and its signaling pathways are required for cell survival (Reuter 1983; Reuter & Sigel 1991).

Under resting conditions, intracellular Ca$^{2+}$ concentration is maintained at approximately 100nM. Upon stimulation, the intracellular Ca$^{2+}$ concentration can increase dramatically, often reaching micro molar levels (Gutnick et al. 1989; Imredy & Yue 1992; Stern 1992). Such increase occurs via release from intracellular stores and/or influx through voltage-dependent Ca$^{2+}$ channels (Perez-Reyes et al. 1998).

Rapid influx of Ca$^{2+}$ into the cytosol is mediated by voltage-dependent Ca$^{2+}$ channels that are usually divided into two groups: high voltage-activated (HVA) and low voltage-activated (LVA) (Hille 2001). Members of the HVA Ca$^{2+}$ channels (Ca$_v$2.x) sub-family include L, N, P/Q, and R types typically require stronger membrane depolarization (>40 mV) to activate. In contrast, functional LVA Ca$^{2+}$ channels (Ca$_v$3.x), also called T-type Ca$^{2+}$ channels, require less membrane depolarization to activate (~60 mV), exhibiting fast current inactivation, slow deactivation kinetics (Perez-Reyes 2003) and are quite insensitive to the classical organic (dihydropyridines, benzothiazepines and phenyalkylamines,) and inorganic (Co$^{2+}$, Cd$^{2+}$) blockers (Birnbaumer 1994; Dunlap & Luebke & Turner 1995; Striessnig 1998; Yunker & McEnery 2003; Díaz 2005) Molecular biology studies have revealed and
characterized three distinct isoforms: \( \alpha_{1G} (\text{Ca}_{v}3.1) \), \( \alpha_{1H} (\text{Ca}_{v}3.2) \), and \( \alpha_{1I} (\text{Ca}_{v}3.3) \) (Perez Reyes 2003).

A number of studies have been done to uncover how these \( \text{Ca}^{2+} \) channels, HVA and LVA, are regulated and how they interfere with cell functions (Bers, Lederer & Berlin 1990; Bers 2002; Lory 2006; Mishra & Hermsmeyer 1994; Scherübl, Hescheler & Riecken 1993; Westenbroek, Ahljanian & Catterall 1990). One of the most common pathways for modulation of \( I_{\text{cal}} \) amplitude is the \( \beta \) adrenergic receptor/cAMP signaling cascade resulting in the phosphorylation of L-type calcium channels by cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) (Asai, Pelzer & McDonald 1996; Bean 1985; Chad & Eckert 1986; Fu et al. 2014; Hescheler et al. 1987; Holz, Rane & Dunlap 1986; Minobe et al. 2014; Patriarchi et al. 2016; Treinys et al. 2016; Xu et al. 2016).

Accordingly, cAMP is involved in cell signalling showing also a large spectrum of biological functions (Calebiro & Maiellaro 2014; Chin et al. 2002; Filadi & Pozzan 2015; Schwartz 2001). Crosstalk between cAMP and \( \text{Ca}^{2+} \) have been demonstrated and indicated (Hofer 2012; Siso-Nadal 2009; Vajanaphanich et al. 1995) homeostasis deregulation affect cell physiology (Déliot & Constantin 2015; Howe 2011) that may result in various diseases (Capiod 2011; Kotturi et al. 2006; Mariot et al. 2002; Taylor et al. 2008).

Contrasting to the overwhelming data supporting the acute modulation of ion channel function by cAMP, little is known about how sustained cAMP levels due to long term exposure affects voltage-dependent \( \text{Ca}^{2+} \) currents and if this would
change cell proliferation rate. We reasoned that exposure time is a very important parameter and hypothesized that sustained intracellular cAMP bioavailability could cause relevant changes in the activity of voltage-dependent Ca\textsuperscript{2+} channels that would impact cell proliferation rate.

Here, by the first time, we show that long-term exposure to either forskolin elicited a substantial increase in L- and T-type Ca\textsuperscript{2+} channel current density and enhanced the proliferation rate in rat GH3 cells.

1. Materials and methods

1.1. Cell Culture

GH3 cells (American Type Culture Collection) were cultured in DMEM-HEPES modification (Sigma-ALDRICH, USA) supplemented with 10% Fetal Bovine Serum (Cultilab, Brazil). The cells were routinely grown as stocks in 75 cm\textsuperscript{2} flasks (Costar, USA) at 37\degree C in a humidified atmosphere. The culture medium was changed three times a week. For electrophysiological recordings the cells were sub-cultured on glass coverslips (Corning, #1, USA) without any pretreatment and plated in 47 mm dishes. Usually they were used 1-2 days after trypsinization. Forskolin was prepared as 25 mM stock solution in dimethyl-sulphoxide (DMSO) and added to the growth medium at a final concentration of 10 μM. Dibutyryl-cAMP (dbcAMP)
was prepared as 1 mM stock solution in water and added to growth medium to a
final concentration of 200 µM. Cells were exposed to dbcAMP (200 µM) up to 72h
or forskolin (10 µM) for 24 and 48 hours.

1.2. Electrophysiology

In all experiments the whole-cell mode of the patch-clamp technique was employed
(Hamill 1981). The electrodes were pulled on a PP-83 two-stage (Narishige,
Japan) puller from both soft (1.5 mm non-heparinized micro hematocrit glass
capillaries, Selecta, Brazil) and borosilicate glass capillaries (1.5 mm diameter,
Clark, UK) to a tip diameter of 1-2 µM. The pipette resistance was 2-5 MΩ when
filled with the appropriate pipette solution.

Membrane currents were recorded with a HEKA-EPC 9 amplifier with Pulse and
Pulse-fit acquisition and analysis software (HEKA, Germany). To minimize space-
clamp errors, only isolated cells were selected for recording. Cells were not
accepted if the initial seal resistance was < 1.5 GΩ. Voltage errors were minimized
using series resistance compensation (generally 60%). Cancellation of the
capacitance transients and leak subtraction was performed using a programmed
P/4 protocol (Bezanilla & Armstrong 1977) delivered at a particular holding-
potential that is appropriate for the measured current. $I_{Ba}$-voltage
data were typically acquired by recording responses to a consecutive series of step
pulses from a holding potential (-80 mV) with increments of +10 mV for 50 ms.
Data acquisition was initiated approximately 3-5 min after establishment of the
whole-cell configuration when membrane currents had stabilized after intracellular
dialysis with the pipette solution. Data were always recorded during continuous
perfusion of the clamped cell with extracellular test solution. There were no
corrections for liquid junction potentials. All the experiments were performed at
room temperature (25-28 °C).
Current traces were acquired at 10 kHz and filtered at 2.5 kHz with a 4-pole low-
pass Bessel filter. Data analysis was carried out off-line using Pulse-Fit programs
(HEKA, Germany), Excel (Microsoft Office suite, USA) and Sigma Plot v5.0 (Jandel
Scientific, USA).

2.3 FRET measurements

GH3 cells were stably transfected with the EPAC-1 cAMP sensor (Nikolaev et al.
2004) For FRET-measurements, cells were seeded on glass-coverslips 2 days
prior to the measurements. Excitation wavelength for FRET-experiments was 436
± 25 nM, emission was splitted at 455 nm (long pass) using a Dual-View beam
splitter, and recorded at 480 ± 15 and 535 ± 20 nm, respectively, with a Cool Snap
HQ² camera (Photometric). Sample frequency was 0.2 to 0.8 Hz to avoid excessive
bleaching. Data acquisition and initial offline analysis was performed with
MetaFluor Software (Molecular Devices). Calculations, final analysis and
representation was done using Excel (Microsoft) and Graph Pad Prism3 (Graph
Pad Software, Inc.). As the EPAC1 cAMP sensor shows decreasing FRET-ratios
with increasing cAMP-concentrations, for convenience the ratios were calculated
as emission at 480 nm (cyan) divided by the emission at 535 nm (yellow). All
emissions were background subtracted offline. To establish a stable baseline, cells were initially monitored for 5 to 10 minutes. At the indicated time forskolin (final concentration 10 µM) or dbcAMP (final concentration 200 µM) was applied. To evaluate the effect of each drug, the FRET- ratio (Cyan/Yellow) was calculated before and after each drug application and the difference expressed as FRET ratio-change in % normalized to the baseline.

2.4 Proliferation

We use the Real-Time Cell Monitoring (xCELLigence), a highly sensitive system, to measure cell growth. The xCELLigence system Real-Time Cell Analyzer RTCA-SP (ACEA Biosciences, USA) is an electrical impedance-based real-time cell monitoring system for detection of cellular viability (Fig. 2.2). The recording of cell index values (CI), normalizations and the monitoring of Aβ1-42 mediated cytotoxicity was performed using RTCA Software 1.2. The RTCA-SP device was calibrated using RTCA Resistor Plate 96 prior to each experiment and impedance measurements were carried out in 96-well E-plates (ACEA). The impedance readout is expressed as arbitrary cell index values. The normalization of cell index arbitrarily sets cell index to 1 at a desired time point, which is typically the time of adding compounds. The background impedance caused by the media is measured using 100ul in each well prior to seeding of cells and is automatically subtracted by the RTCA software using the following equation: CI – (Zi – Zo)/15 with Zi as the
impedance at any given time point and $Z_0$ being the background signal (Diemert et al. 2012).

GH3 were seeded at a density of 5x10^3/well into 96-well E-plates and left overnight to allow for cell attachment and left until cell index of reached a value of 1 prior to addition of forskolin (10µM) and vehicle controls (DMSO) to ensure consistency of cell number. The resulting data was exported to Microsoft excel using the RTCA software for further analysis.

2.5 Solutions

Patch Clamp: Solutions for whole-cell patch-clamp experiments were designed to eliminate $K^+$ and $Na^+$ currents and to allow stable $Ba^{2+}$ currents to be measured. $Ba^{2+}$ (Bean 1985; Harraz & Welsh 2013; Li et al. 2010; Nikitina et al. 2007; Perez-Reyes 2003) was used as the charge carrier in all experiments. Cells were first bathed in regular tyrode solution. After the whole-cell configuration was established, the external solution was switched to the test solution containing (in mM): 140 NaCl, 5 CsCl, 5 BaCl$_2$, 5 Glucose, and 0.0003 Tetrodotoxin, and 10 Hepes, pH 7.4 (300-306 mOsm). The pipette (intracellular) solution contained (mM): 10 NaCl, 130 CsCl, 20 TEACl, 5 EGTA, and 10 Hepes, pH 7.2 (289-291 mOsm).

For FRET experiments extracellular solution was a modified physiological Tyrode solution containing (in mM): 140 NaCl, 3 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 15 Glucose, 10 Hepes adjusted to pH 7.2 with NaOH.
2.6 Statistics

All values are given as means ± S.E.M. We evaluated statistical differences using Student’s unpaired two-tailed t test. We assumed significance when $P<0.05$.

3. Results

Forskolin has biphasic effects on voltage-dependent Ca2+ channels

We used forskolin, a known adenylate cyclase stimulator (Drust & Sutton & Martin 1982; Drust & Martin 1982; Seamon, Padgett & Daly 1981; Seamon & Daly 1981), in order to increase intracellular cAMP. After 24 or 48 h forskolin incubation, GH3 cells showed significant increase in Ca2+ current density when compared to controls (Fig.1A). $I_{Ba}$ peak were -11.07 ± 1.31 pA/pF for 24h forskolin (72% increase, n=300), - 9.14 ± 1.18 pA/pF for 48h forskolin (41% increase, n=200), and -6.44 ± 0.84 pA/pF for control cells (n=200). Conversely, forskolin (10 µM) provoked a decrease in Ca2+ current density when applied acutely (~1h) to GH3 cells (Data not shown, n=6). No further increase in current density was observed for longer incubation times (96h, data not shown, n=5).

Under control conditions (no extracellular Ca2+), $I_{Ba}$ from control cells inactivates only very slowly (Figure 1Ai). In contrast, $I_{Ba}$ from forskolin-treated cells showed a clear change in inactivation time-course. There was a slight shift (Figure 1B) towards hyperpolarized membrane potentials to reach peak current density in
forskolin-treated cells. These findings suggest a possible involvement of T-type Ca2+ channels.

dbcAMP increases calcium current and mirrors the effect of forskolin

In order to confirm our previous results, we used dbcAMP (a membrane permeable analogue of cAMP) that is thought to elevate intracellular cAMP levels (Swislocki 1970).

Long-term treatment of cells with dbcAMP increases $I_{Ba}$ in a time dependent manner (Figure 2A). Contrasting with forskolin results, this increase in peak current occurred to a greater extent at more positive membrane potentials (> +20 mV) and it was not accompanied by a significant shift in current-voltage relationship (peak $I_{Ba}$ were at $-12.0 \pm 4.9$ mV in control (Figure 2B, n=12), $-10.0 \pm 2.2$ mV in 24 h exposure (Figure 2B, n=17), and $-12.7 \pm 1.4$ mV in 48 h (Figure 2B, n=11)). On average, the peak current density amplitude at -10 mV increased from $3.9 \pm 0.6$ pA/pF (Control, n=5) to $-8.9 \pm 1.1$ pA/pF (after 24 h dbcAMP, $P<0.05$, n=7) and to $-14.1 \pm 1.3$ pA/pF (after 48 h dbcAMP, $P<0.05$, n=11) of its control value (Figure 2C). The observed increase in current density is not related to cell enlargement (data not shown). No effect was observed when forskolin-inactive form (Dideoxi-forskolin) was used (Figure 2D).
Forskolin or dbcAMP increased cAMP in GH3 cells

To confirm if the treatment with forskolin or dbcAMP resulted in intracellular increase in cAMP, we performed FRET experiments. The treatment with both drugs (forskolin or dbcAMP) resulted in increase of cAMP with a clear peak followed by a plateau when forskolin was applied (Figure 3A, black line). The increase in cAMP due to dbcAMP (Figure 3A, gray line) was much slower and gradual differently from forskolin. After plateau has been reached (~2,000 s) no significant differences in cAMP levels were further observed. To exclude unspecific effects, we also performed control experiments incubating GH3 cells with DMSO (used as vehicle) and found no changes in cAMP levels.

L- and T-type voltage-dependent Ca2+ currents are up regulated by cAMP.

So far, our results demonstrate that sustained increase in cAMP levels enhances voltage-dependent Ca2+ currents in GH3 cells. The kinetics of the current decay showed fast and slow inactivating components after 24h and 48h forskolin incubation (Figure 5Ai, ii, and iii). This result strongly suggests the involvement of L- and T-type Ca2+ channels as targets. To investigate this possibility in more detail we combined electrophysiological and pharmacological approaches.

To evaluate the contribution of T-type Ca2+ channels in the cells exposed to forskolin we used Ni2+ as a specific inhibitor (Hagiwara & Takahashi 1967; Harraz & Welsh 2013; Huguenard 1996; Jeong 2003; Niwa et al. 2004; Nosal et al. 2013). Figure 4 shows that 50 µM Ni2+ partially inhibited Ca2+ currents in GH3 cells without changes in the inactivation kinetics. It has been demonstrated that some
isoforms of the T-type Ca$^{2+}$ channels are less sensitive to Ni$^{2+}$ blockade (Díaz et al. 2005; Lacinová, Klugbauer & Hofmann 2000; Lee et al. 1999) we, then, used mibefradil as a more specific T-type Ca$^{2+}$ channel organic blocker (Frishman 1997; Lacinová & Klugbauer & Hofmann 2000; Nikitina et al. 2007; Van der Vring et al. 1999). Mibefradil at 10 µM abolished the fast inactivating component of the Ca$^{2+}$ current (Figure 5Aii and 5Aiii, gray area), suggesting that this component is mainly due to the presence of T-type Ca$^{2+}$ channels.

We measured currents at the peak (first 5 ms), where both, L and T-type Ca$^{2+}$ currents were present, and at the end of the test pulse where only L-type Ca$^{2+}$ current was present (Table 1). To calculate the amplitude of the fast inactivating component of the total $I_{Ba}$ current we subtracted the amplitude of the non-inactivating fraction from the peak amplitude. No significant difference was found when this calculated fraction was compared with the amplitude of the mibefradil inhibited current (Table 2). This reinforce that T-type Ca$^{2+}$ channels are responsible for the fast inactivating current component observed in forskolin treated cells.

To investigate the contribution of L-type Ca$^{2+}$ currents during forskolin treatment, we also analysed current amplitudes at the end of the test pulse (Table 3), where only L-type Ca$^{2+}$ channels are present. The data clearly show increased L-type Ca$^{2+}$ current density in cells treated with forskolin or dbcAMP compared to control cells.
Long-term treatment with forskolin increased cell proliferation
A large number of investigations have shown the participation of cAMP or calcium channels in controlling cell proliferation (Lory et al. 2006, Becchetti 2011). As our experimental conditions resulted in an increase of cAMP and Ca2+ currents, we decided to investigate whether cell proliferation were affected. Our results show a significant increase in cell proliferation after forskolin treatment and this were more evident after 24h (Figure 6).

3. Discussion
In this study we have examined the long-term effects of two distinct cAMP producers, namely dbcAMP and forskolin on voltage-dependent L- and T-type Ca2+ channels in GH3 cells. Our study provides a number of important points: 1) dbcAMP and forskolin treatments elevated L- and T-type Ca2+ current densities; 2) forskolin elicits a fast increase in cAMP levels whereas dbcAMP increases it slowly; 3) forskolin augments, preferentially, T-type Ca2+ current density; 4) forskolin treatment elevated Ca2+ currents and stimulated cell proliferation.
The literature about regulation of L-type Ca2+ channels by different protein-kinases including PKA is vast and more consistent than that for T-type Ca2+ channels. Experimental evidences indicate that they could be up or down regulated depending on the cell type and the physiological context where the cells are inserted (27, 29, 34, 36, 43, 50, 53). Our results demonstrated that long-term exposure to cAMP increased L-type Ca2+ current density. Interesting and in
agreement with other studies, acute application leads to an opposite effect, decrease L-type Ca2+ current density. There is no clear consensus about the regulation of T-type Ca2+ channels by PKA, from complete absence to very large effects (Alvarez & Vassort 1992; Bean 1985; Benham & Tsien 1988; Fisher & Johnston 1990; Hagiwara, Irisawa & Kameyama 1988; Hirano, Fozzard & January 1989; Lenglet 2002; Lory, Bidaud & Chemin 2006; Tsieng & Boyden 1989; Tytgat et al. 1988).

The present study demonstrated that forskolin increase T-type Ca2+ current density by 1.7-fold (24h incubation) and by 1.4-fold (48h incubation). These results are partially supported by the study of Giancippoli et al. (2006) and Novara et al. (2004) who found increased activity of T-type but not L-type Ca2+ channels in rat chromafin cells when incubated with cAMP or β-adrenergic receptor (β-AR) agonists for 5 days (120h). In addition to that, Mariot et al. (2002) and Rossier et al. (2003), found an overexpression of T-type Ca2+ channels (specifically the α1H isoform (CaV3.2)) in differentiating prostate cancer cells when they were treated for long periods (3-5 days) with dbcAMP or IBMX (non-specific phosphodiesterases inhibitor). as Choi et al. (2002) demonstrated that T-type Ca2+ currents were markedly up regulated after sustained β-AR stimulation in cultured cardiomyocytes. On contrary, Harazz and Welsh (2013) showed that PKA activators suppressed T-Type Ca2+ currents in rat arterial smooth muscle cells.

We reasoned that the opposite results when cAMP is acutely or chronically incubated might be a strategy of GH3 cells to regulate different functions with the same molecule. Supporting this proposal, Zaccolo (2007) demonstrated that the
The final outcome of cAMP signaling cascade could be different even in the same cell depending on the duration and stimulus localization. The difference in reaching the maximum response between forskolin and dbcAMP could be explained by the different time-course in inducing intracellular rise in cAMP. FRET experiments showed that forskolin generated a fast change in intracellular cAMP almost immediately after its application which was in contrast with the very slow change elicited by dbcAMP. Those results reinforce Zaccolo & Pozzan (2002) where kinetics and location is everything when it comes to define which set of components in the cAMP-dependent pathways will be recruited. Intracellular Ca2+ and its signatures are important factors in controlling cell proliferation (Capiod 2011). Voltage-dependent Ca2+ channels, such as T-type Ca2+ channels, are well suited to generate Ca2+ oscillations due to their unique activation/inactivation properties. The treatment with forskolin increased cell proliferation rate. Tierney & Robinson (2002) found that GH3 cells growth was stimulated by cAMP but this was in contrast with Zivadinovic et al. (2005) where cell growth was attenuated with increased levels of cAMP. The participation of L- and T-type Ca2+ channels have been recognized in cell proliferation (Lory, Bidaud & Chemin 2006; Lu et al. 2008, Mariot et al. 2002). A plethora of studies points to a role of T-type Ca2+ channels in cell proliferation in tumors from breast (Artalejo et al. 1990; Reuter & Sigel 1991), brain (Bourinet et al. 1994; Dolphin 1996), colorectal (Eisfeld et al. 1996), gastric (Eisfeld et al. 1996), leukemic (Eisfeld et al. 1996), prostate (Kaspar & Pelzer 1995; Mori et al. 1993), as well as in normal vascular smooth muscle cells (Bezanilla & Armstrong 1977;
Hamill et al. 1981;), fibroblast (Zaccolo & Pozzan 2002), neonate ventricular myocytes (Glassmeier et al. 2001; Yuan & Bers 1995), human myoblasts, and mouse embryos (Klöckner et al. 1999). In retinoblastoma cells (Fomina, Kostyuk & Sedova 1993), low proliferation was accompanied by decreased expression of mRNA for T-type Ca2+ channels and T-type Ca2+ currents. In human myoblasts, T-type Ca2+ currents are expressed just before fusion and subsequent differentiation, and their inhibition suppresses fusion and the increase in intracellular Ca2+ (Fischmeister & Shrier 1989). For Taylor (2008), the fact that cancer cells express T-type Ca2+ channels would provide an altered Ca2+ influx pathway in response to the increasing demand of Ca2+ during rapid proliferation. In contrast, Lory et al. (2006) over-expressing both α1H (Cav3.2) and α1G (Cav3.1) isoforms in neurons observed no evidence of increase in proliferation and Ohkubo & Yamazaki (2012), suppressed the proliferation over-expressing Cav3.1 in MCF-7 cells.

In summary, we have shown that a coordinated modulation of cAMP and L- and T-type Ca2+ currents induces cell proliferation and suggests that these two signal pathways may converge at a downstream point to control mitogenesis.
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Figure #3

(A) Graph showing the effect of forskolin (10 μM) and db-cAMP (200 μM) on FRET change over time.

(B) Bar graph comparing mean FRET change at 510 s for db-cAMP and forskolin.

* denotes statistical significance.
**Figure #4**

(A) Without cAMP/FSK

(i) 

Without cAMP/FSK

(ii) cAMP 24h

10 pA/pF

10 ms

Ni²⁺ (50 μM) → Control

(iii) cAMP 48h

Ni²⁺ (50 μM) → Control

(iv) FSK 24h

Ni²⁺ (50 μM) → Control

(v) FSK 48h

Ni²⁺ (50 μM) → Control

(B) Nickel inhibition (%)

- Control
- 24h
- 48h

![Graph showing nickel inhibition](image)

(C) Nickel inhibition (%)

- Control
- 24h
- 48h

![Graph showing nickel inhibition](image)

- Control
- 24h
- 48h

![Graph showing nickel inhibition](image)
Figure #5

(Ai) Control

(FSK 24h)

(FSK 48h)

(B) Inhibition (%)

Control 24h 48h

*Mibefradil (10 μM)