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Short Communication

Pharmacological studies of $Ca_V3.1$ T-type calcium channels using automated patch-clamp techniques

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Abstract. T-type calcium channels are involved in a variety of physiological and pathophysiological processes, and thus could be therapeutic targets. However, there is no T-type channel selective blocker for use in clinical practice, demanding a need for the development of novel drugs where a higher-throughput screening system is required. Here we present pharmacological studies on $Ca_V3.1$ T-type channels using automated patch-clamp. The IC_{50} values obtained from automated patch-clamp and conventional one showed a good correlation (correlation coefficient of 0.82), suggesting that the automated patch-clamp is an efficient and reliable method for ranking the drug potencies for T-type channels.

Key words: T-type calcium channel — $Ca_V3.1$ — Automated patch-clamp — Drug discovery

T-type calcium channels are present in nerve tissues, heart, smooth and skeletal muscles, and endocrine tissues, involved in neuronal excitability, cardiac pacemaker activity, smooth muscle contraction and hormone secretion (Perez-Reyes 2003). Neuronal T-type calcium channels in particular have been suggested to play a vital role in regulation of sleep (Anderson et al. 2005), nociception (Kim et al. 2003), epilepsy (Tsakiridou et al. 1995) and neuropathic pain (McCallum et al. 2003), thus becoming an attractive target for such disorders. Indeed, T-type calcium channel blockers have been demonstrated to be effective for treatment of cerebral diseases such as absence epilepsy (Vitko et al. 2005), epilepsy (Khosravani et al. 2005) and neuropathic pain (Flatters 2005). Mibefradil, a specific blocker of T-type calcium channels had been used for therapeutic treatment of hypertension and angina pectoris (Clozel et al. 1990). However, mibefradil is metabolized by cytochromes P450 3A4 and 2D6 with other drugs and interacts with them pharmacokinetically, which results in various adverse effects including irregular heart rhythms (Krayenbuhl et al. 1999). As a result, mibefradil has been withdrawn from the market so that a selective blocker for T-type calcium channels for clinical use is not available, waiting for the development of T-type calcium channel specific drugs.

For ion channel drug discovery, patch-clamp measurements have been the gold-standard assay. However, the conventional patch-clamp is not suitable for effective screening in early stages of drug discovery due to its time-consuming and labor-intensive nature. Recent developments of automated patch-clamp devices are rapidly utilized for ion channel drug discovery as well as safety pharmacology such as cardiac toxicity tests using hERG channels (Dunlop et al. 2008). Numerous research papers on assessments of cardiac hERG channel liability with various automated patch-clamp devices have been reported (Bridgland-Taylor et al. 2006; Ly et al. 2007), revealing that the automated patch-clamp is reliable for ranking the potencies of drugs. However, T-type calcium channels have not been rigorously tested using automated patch-clamp except for a brief case study on $Ca_V3.2$ T-type calcium channels (Mathes et al. 2009). Thus, pharmacological studies on T-type calcium channels with automated patch-clamp would be valuable due to urgent needs for developments of T-type calcium channel specific drugs for clinical use. In the present work, recombinant $Ca_V3.1$ T-type calcium channels were studied using auto-

Electronic supplementary material. Detailed experimental procedures, additional results, discussion, Figures S1–S8 and Table S1. The online version of this article (doi:10.4149/gpb_2011_01_100) contains supplementary material, which is available to authorized users.

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mated patch-clamp, where four independent patch-clamp measurements can be conducted in parallel.

To characterize pharmacological properties of $Ca_v3.1$ T-type calcium channels, whole-cell currents were recorded from HEK293 cells stably expressing $Ca_v3.1$ channels with automated patch-clamp. Mibefradil, the best-known T-type calcium channel specific blocker (Martin et al. 2000) was initially tested. The effects of mibefradil on $Ca_v3.1$ chan-

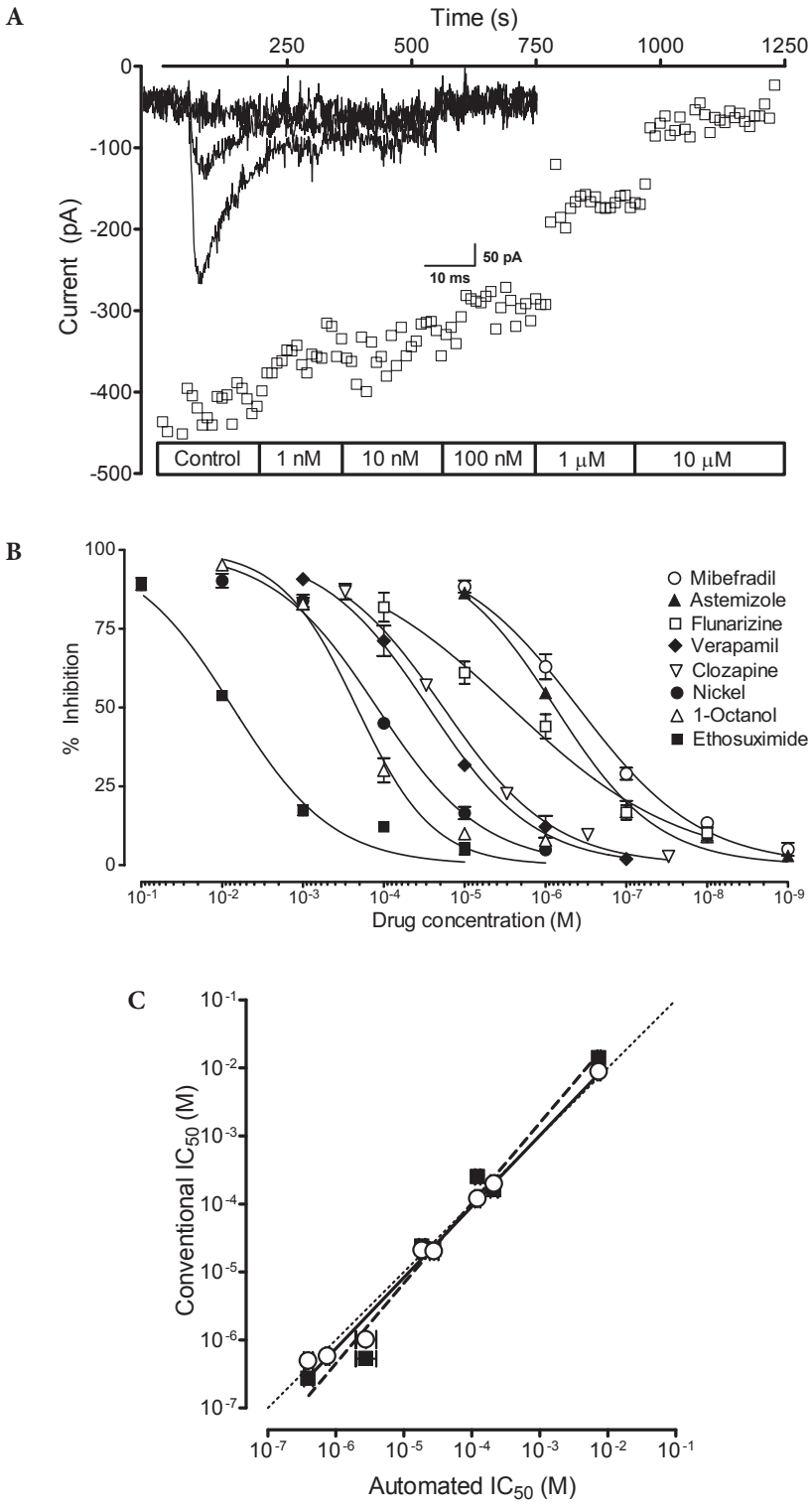

Figure 1. Pharmacological characterization of $Ca_v3.1$ channels with automated patch-clamp. **A.** Mibefradil inhibition of $Ca_v3.1$ channels was tested in automated patch-clamp. Time course of the amplitude of the peak $Ca_v3.1$ currents in response to increasing concentrations of mibefradil of 1 nM, 10 nM, 100 nM, 1 μM and 10 μM as shown at the bottom. Insert: A representative whole-cell recording of $Ca_v3.1$ channels evoked by 50-ms test pulses to –20 mV at a holding potential of –100 mV. Whole-cell currents of $Ca_v3.1$ channels were blocked by ~60% on average upon applying 1 μM mibefradil to the bath solution (control, 1 μM and 10 μM of mibefradil applied). **B.** Dose-response curves of drug inhibition of the peak $Ca_v3.1$ currents presented as percent inhibition (% inhibition) as a function of drug concentration: mibefradil (○), astemizole (▲), flunarizine (□), verapamil (◆), clozapine (▽), nickel (●), 1-octanol (△) and ethosuximide (■). Each point is pooled from at least five recordings. The curves were fitted to a Hill equation to obtain the IC_{50} values, summarized in Table 1. Error bars represent S.E.M. **C.** Comparison of the potencies of T-type calcium channel blockers obtained with automated and conventional patch-clamps. The IC_{50} values determined in automated patch-clamp were plotted against those obtained with conventional patch-clamp in the present study (open circles) as well as in the literature (filled squares). The solid line indicates a linear regression with the values measured in the present experiments (slope = 0.96 ± 0.05, correlation coefficient, $R = 0.82$), and the dashed line indicates a linear regression with the literature values (slope = 0.85 ± 0.09, $R = 0.70$). Unity is represented as a dotted line. Error bars represent S.E.M.

Table 1. Summary of the pharmacological studies of $Ca_v3.1$ channels obtained with automated and conventional patch-clamp

Blocker	Chemical structure	IC_{50} conventional (μM)	IC_{50} automated (μM)	IC_{50} literature (μM)	Reference
Mibefradil	phenylalkylamine	0.50 ± 0.05	0.41 ± 0.06	0.27 ± 0.03	(Martin et al. 2000)
Verapamil	phenylalkylamine	20.2 ± 1.0	28.7 ± 3.8	21.4	(Freeze et al. 2006)
Flunarizine	diphenylpiperazine	1.02 ± 0.10	3.11 ± 0.95	0.53 ± 0.11	(Santi et al. 2002)
Ethosuximide	pyrrolidine	8.83 ± 0.43 ($\times 10^3$)	7.45 ± 0.42 ($\times 10^3$)	14 ± 3.7 ($\times 10^3$)	(Todorovic et al. 2000)
Clozapine	dibenzodiazepine	20.9 ± 0.7	18.8 ± 2.1	23.7 ± 1.3	(Choi and Rhim 2010)
1-Octanol	aliphatic alcohol	198 ± 10	217 ± 25	160 ± 13	(Todorovic et al. 2000)
Astemizole	piperadine	0.59 ± 0.07	0.76 ± 0.05	N/A	N/A
Nickel	divalent metal	121 ± 11	124 ± 11	250 ± 22	(Lee et al. 1999)[a]

All reference studies were performed in HEK293 cells. [a] 10 mM Ba^{2+} was used as a charge carrier in Lee et al. 2 mM Ca^{2+} was used in all other references.

nels were studied from whole-cell currents evoked by the test pulses of –20 mV for 50 ms at a holding potential of –100 mV. On average, 1 μM mibefradil inhibited the peak currents of $Ca_v3.1$ channels by 63 ± 4% (n = 5). Five concentrations of mibefradil (0.001 μM to 10 μM) were applied to the patched cells in a cumulative fashion as shown in the representative time trace of the peak currents of $Ca_v3.1$ channels in Fig. 1A.

Further pharmacological studies on $Ca_v3.1$ channels were carried out using the chemically diverse drugs known to block T-type calcium channels (see Supplementary material, Fig. S1). The inhibition of $Ca_v3.1$ peak currents by all of the examined blockers was concentration-dependent (Fig. 1B). Normalized peak current amplitudes in the presence of each drug were plotted as a function of drug concentration and fitted to a Hill equation. The concentration required for 50% inhibition (IC_{50}) was summarized in Table 1. For comparison, the IC_{50} values of each drug for $Ca_v3.1$ channels were also obtained using conventional patch-clamp (see Supplementary material, Fig. S2), which exhibited the similar values to those obtained with automated patch-clamp (Table 1). Overall, pharmacological data showed a good agreement between the two methods; a correlation coefficient (R) of 0.82 and a slope of 0.96 ± 0.05 were determined by a linear regression (open circles and solid line in Fig. 1C). A decent correlation was also observed in the comparison between the automated patch-clamp results and the reported values; R of 0.70 and a slope of 0.85 ± 0.09 (filled squares and dashed line in Fig. 1C).

For pharmacological studies of $Ca_v3.1$ channels with automated patch-clamp, the sealing rate was good (>80%); gigaseals onto the cell were mostly formed once the cells were attached (Fig. 2A). However, the rate for obtaining the whole-cell configuration was reduced to 43% with the mean initial membrane resistance (R_{wc}) of 1.66 ± 0.12 GΩ (median value of 710 MΩ, n = 390, Fig. 2B). The overall success rate of getting usable recordings was further dropped to 16% partly due to lack of functional channel expression, which is evident in the mean peak amplitude of $Ca_v3.1$ channel currents of –169 ± 7 pA (median value of –130 pA, n = 395), ranged from –20 to –1160 pA (Fig. 2C).

For the past five years, the developments of automated patch-clamp electrophysiology have been widely adapted in drug discovery (for reviews see Dunlop et al. 2008). The number of papers on ion channel studies using automated patch-clamp have been rapidly accumulated, where most studies compare the potencies of blockers and/or agonists obtained from automated patch-clamp to the values measured with conventional one, showing a good agreement.

T-type calcium channel specific drugs for clinical use need to be developed in a timely fashion, in which higher-throughput screening with automated patch-clamp techniques is necessary. The automated patch-clamp studies on T-type calcium channels would thus provide the valuable data for usage of an automated patch-clamp platform for such drug discovery. T-type calcium channels were previously studied with recombinant $Ca_v3.2$ T-type calcium channels using automated patch-clamp for the basic biophysical properties (Mathes et al. 2009), but this study did not provide pharmacological data. To rigorously characterize pharmacological properties of T-type calcium channels with automated patch-clamp, we focused on $Ca_v3.1$ channels, one of the three isoforms of T-type calcium channels. Various blockers with diverse chemical structures were tested against $Ca_v3.1$ channels to validate the ability of automated patch-clamp for ranking the drug potencies. The IC_{50} values obtained with automated patch-clamp are in good agreement with those determined with conventional patch-clamp (R of 0.82) as well as the published data (R of 0.70). A better correlation was observed with the values obtained in the present study than with the previously reported values, which is probably due to the differences in experimental conditions such as recording solutions and voltage protocols. The underestimated

IC_{50} values previously often observed due to the limited time for solution exchange and the adsorption of lipophilic test compounds (Guo and Guthrie 2005; Sorota et al. 2005) were not observed in the present work. Also, the range of the IC_{50} values extends over six orders of magnitude of concentrations with a slope of 0.96 determined by a linear regression, close to the unity line, indicating that the dynamic range of this method is broad enough for most of the test compounds to fall into this range. Thus, our pharmacological data with various drugs suggest that T-type calcium channels could be well studied using automated patch-clamp in a fast and reliable fashion.

The experimental throughput of an automated patch-clamp method has been reported to be increased by 5- to 45-fold over conventional patch-clamp (Brown 2009; Friis et al. 2009; Mathes et al. 2009). In the present work, the automated patch-clamp increases throughput of roughly 4-fold compared to conventional patch-clamp mainly due to the relatively low overall success rate of getting usable patches in $Ca_v3.1$ channel recordings, which could be a potential drawback for screening purposes. In contrast to conventional patch-clamp, where cells are manually selected for patch-clamping, cell selection is random in automated patch-clamp recordings. The low overall success rate may thus be partly caused by the small size of cells randomly selected, leading to the reduced current amplitudes. This problem could be solved by generating improved recombinant cell lines. Other cell lines such as Chinese hamster ovary (CHO) cells rather than HEK293 cells may express sufficiently measurable currents, thus being more compatible and robust with automated patch-clamp experiments (Clare et al. 2009). Indeed, the overall success rates in automated patch-clamp mainly depend on the combination of the cell line chosen and ion channels expressed, and CHO cells have been suggested to be a better choice than HEK293 cells to obtain the high success rates in automated patch-clamp experiments (Dunlop et al. 2008; Mathes et al. 2009). Another way to maximize the current amplitude is to optimize the cell preparation conditions and/or to change the composition of the patch solutions (Balasubramanian et al. 2009; Mathes et al. 2009). Using non-enzymatic cell dissociation solutions instead of trypsin for cell detachment may be less harsh on cells, thus

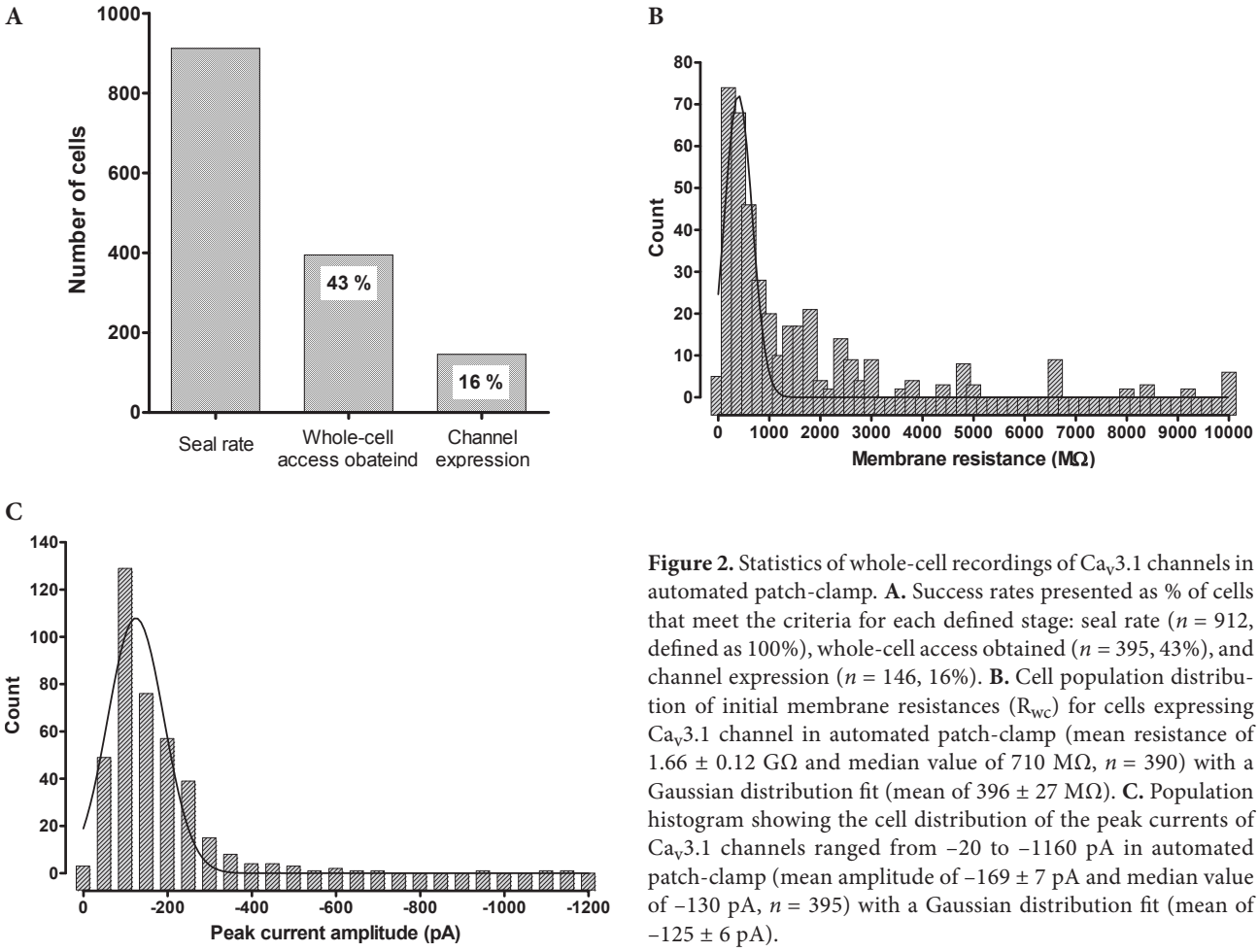

Figure 2. Statistics of whole-cell recordings of $Ca_v3.1$ channels in automated patch-clamp. **A.** Success rates presented as % of cells that meet the criteria for each defined stage: seal rate (n = 912, defined as 100%), whole-cell access obtained (n = 395, 43%), and channel expression (n = 146, 16%). **B.** Cell population distribution of initial membrane resistances (R_{wc}) for cells expressing $Ca_v3.1$ channel in automated patch-clamp (mean resistance of 1.66 ± 0.12 GΩ and median value of 710 MΩ, n = 390) with a Gaussian distribution fit (mean of 396 ± 27 MΩ). **C.** Population histogram showing the cell distribution of the peak currents of $Ca_v3.1$ channels ranged from –20 to –1160 pA in automated patch-clamp (mean amplitude of –169 ± 7 pA and median value of –130 pA, n = 395) with a Gaussian distribution fit (mean of –125 ± 6 pA).

enhancing channel currents since proteases could affect the current amplitudes (Rajamani et al. 2006). Modifying the composition of recording solutions, for example, adding cAMP into the intracellular solution may increase calcium currents because it has been known that PKA (cAMP-dependent protein kinase A)-mediated phosphorylation of $Ca_v3.1$ T-type calcium channels stimulates channel activity (Chemin et al. 2007), thus supposedly inducing enhancement in calcium currents.

In conclusion, the present work demonstrates that blockers of T-type calcium channels can be screened more efficiently and reliably using automated patch-clamp techniques. Drug potencies of T-type calcium channels obtained with both automated and conventional methods were similar, which is an important and useful feature for drug screening. Overall, given the significance of T-type calcium channels as drug targets, automated patch-clamp recordings of $Ca_v3.1$ channels could provide an alternative way for higher-throughput screening for novel drug discovery targeted against T-type calcium channels.

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