Ca^{2+} SENSITIZING TROPONIN T MUTATIONS LINKED TO HYPERTROPHIC CARDIOMYOPATHY INCREASE APPARENT CYTOSOLIC Ca^{2+} BINDING

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Dr. Oleksii Hryshchenko *started working in the Department of General Physiology of Nervous System in 1983 as a senior student of Moscow Institute of Physics and Technology. Having graduated in 1985 he continued working in the Department. Studying biophysical properties of excided cells' membranes, he employed various methodological approaches, namely, the patch-clamp, artificial membranes, optical methods, etc. In 1997-1998 Oleksii Hryshchenko worked in Germany where he mastered the stem cells derived cardiomyocytes, which allowed studying early cardiomyocytes. In 2000-2001 he worked in Texas Tech University (USA) studying sino-artrial cardiomyocytes in normal and pathology conditions, in particular, in ischemia condition. Late in 2001 he came back to Kyiv to work in Platon Kostyuk Department where he studied the properties of neurons in pathology in Nana Voitenko's team. Within scientific research collaboration Oleksiy Gryshchenko visits the best world laboratories. In 2004-2007 in Professor Ole Petersen and Professor Oleksiy Tepikin labs he studied electro-physiological properties of acenar pancreatic cells considered as non-excitable though proving to be quite attractive for electro-physiological research. In 2008 he was invited to collaborate with Vanderbilt University (Nashville, USA) aimed at studying the roll of calsequestrin in cardiomyocyte functioning.*

The photo shows a student of P.G. Kostyuk, Dr. O. Hryschenko.

In intact contracting myocytes, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) varies dynamically and is determined by the sarcolemmal and trans-sarcoplasmic reticulum Ca^{2+} fluxes, the binding kinetics to Ca^{2+} ligands in the cytosol (Ca^{2+} buffering) and, to a lesser extend, by mitochondrial Ca^{2+} fluxes (Shannon and Bers, 2004; Bers, 2001). Major Ca^{2+} binding ligands are the myofilament protein Troponin C (TnC; about 50% of fast Ca^{2+} binding during a typical heart beat), the

sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) and binding sites along the sarcolemmal membrane which remain to be identified (Bers, 2001; Fabiato, 1983). Additional Ca^{2+} is bound by slow Ca^{2+} buffering sites (Shannon and Bers, 2004). However, these high affinity Ca^{2+}/Mg^{2+} binding sites are largely saturated with Mg^{2+} during diastole (Robertson et al., 1981). Other high-capacity low-affinity buffers (e.g. ATP, creatine phosphate) in aggregate also significantly contribute to Ca^{2+} buffering (Bers, 2001).

Increased myofilament Ca^{2+} sensitivity is a common finding among mutations in thin filament proteins (e.g., cardiac troponin T (TnT)) which cause FHC and carry a high risk for sudden cardiac death from ventricular arrhythmias (Knollmann, 2001). The degree of Ca^{2+} sensitization correlates with the risk for ventricular arrhythmias in troponin T (TnT) mutant mice, likely as the result of increased conduction velocity (CV) dispersion and action potential (AP) alternans (Baudenbacher et al, 2008). How myofilament sensitization causes AP alternans and CV dispersion remains unknown. The effect of TnT mutation on myofilament Ca^{2+} sensitivity is likely due to the indirect action of TnT on Ca^{2+} binding by TnC, which is possible as TnT, TnC, and troponin I (TnI) form a trimeric protein complex (Solaro, 1995). Considering that Ca^{2+} binding to the troponin complex is responsible for approximately 50% of Ca^{2+} buffering, we hypothesized that Ca^{2+} sensitizing TnT mutants changes cytosolic Ca^{2+} buffering sufficiently to alter global cytosolic Ca^{2+} transients during a normal heart beat, a concept previously proposed on the basis of our modeling studies (Knollmann et al, 2001).

To test this hypothesis, we measured cytosolic Ca^{2+} buffering in ventricular myocytes from transgenic mice expressing WT and different mutant TnT associated with FHC (Knollmann et al, 2001, Hernandez et al, 2005). Two mutations, TnT-F110I and TnT-I79N, increased Ca^{2+} sensitivity of force development in skinned fibers, while TnT-R278C has no significant effect (Baudenbacher et al, 2008, Hernandez et al, 2005). Cytosolic Ca^{2+} buffering was measured as illustrated in Fig. 1.

Caffeine was rapidly applied to release Ca^{2+} from the SR (Fig. 1, *a*, upper trace). Integration of the Na-Ca exchanger (NCX) current (Fig. 1, *a*, low trace) yielded the total amount of Ca^{2+} released from the SR (Trafford et al, 1999). SR Ca^{2+} content was reduced to achieve caffeine transients which match the Ca^{2+} transient amplitude during a typical myocyte contraction. We found that the rise in free cytosolic Ca^{2+} was significantly smaller in myocyte expressing the Ca^{2+}-sensitizing TnT-I79N mutant compared to TnT-WT, even though the total amount of Ca^{2+} released from the SR was the same (Fig. 1A). On average, the ratio between the total amount of Ca^{2+} released from the SR and the resulting peak change in $[Ca^{2+}]_{free}$ ($=\Delta[Ca^{2+}]_{total} / \Delta[Ca^{2+}]_{free}$) was significantly higher in TnT-I79N myocytes (Fig. 1, *b-d*), indicating that significantly more Ca^{2+} was bound in TnT-I79N myocytes compared to TnT-WT ones and myocytes expressing the non-sensitizing TnT-R278C mutant. Myocytes expressing the TnT-F110I mutant which has intermediate Ca^{2+} sensitizing effects (6) also exhibited an intermediate effect on

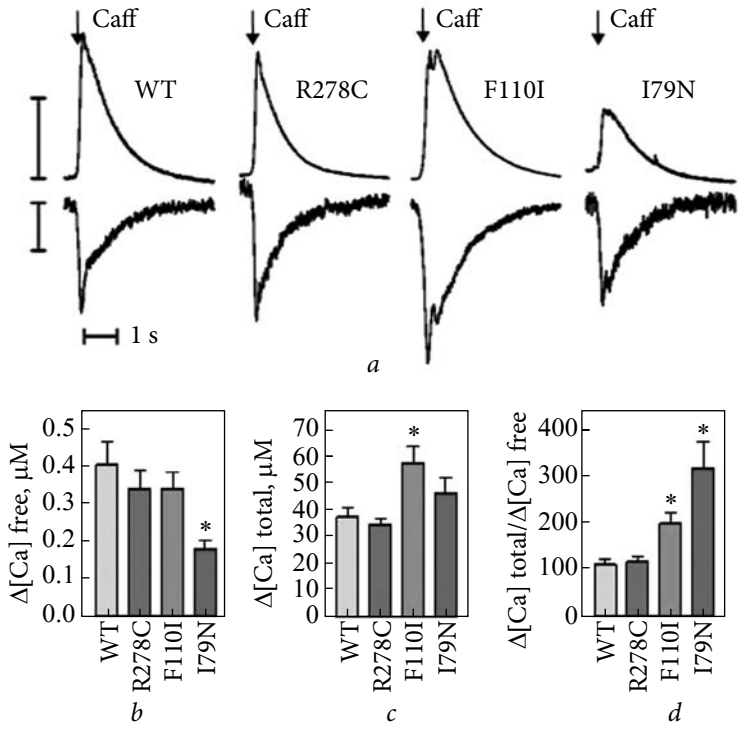

Fig. 1. Ca^{2+} — sensitizing TnT mutants increase cytosolic Ca^{2+} binding: *a* — representative examples of cytosolic Ca^{2+} fluorescence recordings from voltage-clamped myocytes loaded with the fluorescent indicator Fluo-4. To quantify cytosolic Ca^{2+} binding, caffeine (↓, 15 mM) was used to release Ca^{2+} from the SR (upper trace). The rise in $[Ca^{2+}]_{free}$ activated NCX, generating an inward current (lower trace). Integration of NCX currents yielded the total amount of Ca^{2+} released ($[Ca^{2+}]_{total}$)

cytosolic Ca^{2+} binding (Fig. 1, *d*). Importantly, diastolic $[Ca^{2+}]_{free}$ was not significantly different among all four groups (data not shown).

Then, we used the data obtained from the experiments with rapid caffeine application (Fig. 1) to calculate cytosolic Ca^{2+} buffering parameters according to Trafford et al 1999. $\Delta[Ca^{2+}]_{total}$ was plotted as a function of $\Delta[Ca^{2+}]_{free}$, fitted to the modified Michaelis-Menten equation, and B_{max} and K_d were calculated for each myocyte (Fig. 2, *a*).

The resulting K_d values of TnT-WT (582 ± 57 nmol/L, n = 9) were very similar to those reported by Trafford et al. for ferret myocytes (590 ± 170 nmol/L). The Ca^{2+} sensitizing TnT-I79N and TnT-F110I mutants significantly lowered average K_d (Fig. 2, *b*). On the other hand, B_{max} was not significantly different among the four groups tested (Fig. 2, *c*). The lack of change in B_{max} is consistent with the finding that the total TnT protein and therefore TnC protein expression levels are not altered in the transgenic mice studied here (Miller et al, 2001; Wendt et al, 1983). This is also consistent with the result that the SERCA expression level is not affected in TnT-

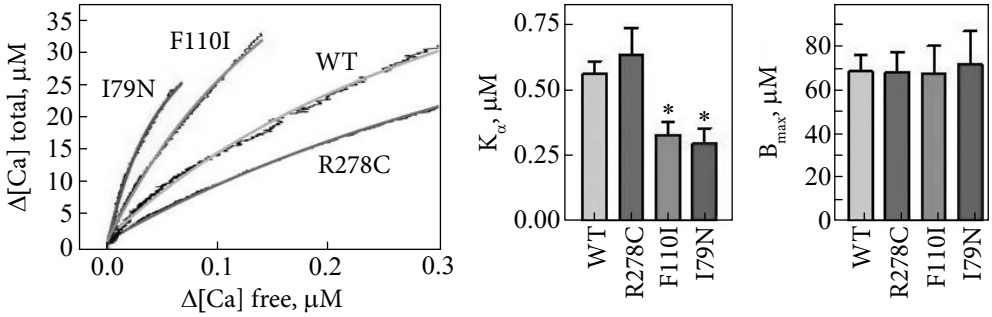

Fig. 2. TnT mutants alter the apparent K_d of cytosolic Ca^{2+} buffering: *a* — representative buffering plots of $\Delta[Ca^{2+}]_{total}$ calculated from the NCX integral as a function of $\Delta[Ca^{2+}]_{free}$ using the data from the caffeine application experiments presented in Fig. 1. Cytosolic buffering parameters (K_d and B_{max}) were determined for each myocyte by fitting curves to a modified Michaelis-Menten equation as described by Trafford et al, 1999; *b* and *c* — comparison of average K_d and B_{max}. n = 9-10 myocytes per group, *p < 0.01 vs R278C and WT.

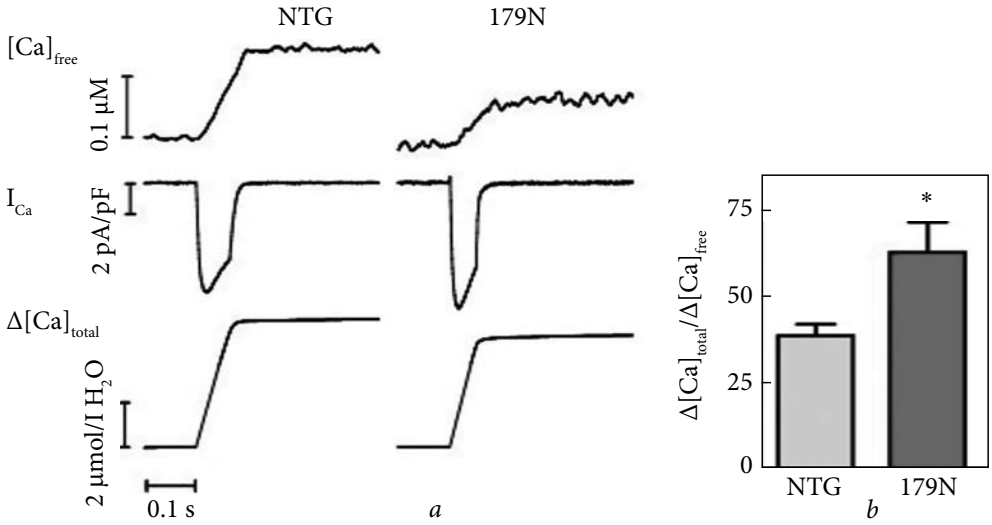

Fig. 3. Caffeine-independent measurement of cytosolic Ca^{2+} binding: *a* — myocytes were voltage clamped, dialyzed with Fluo-4, and cytosolic Ca^{2+} fluorescence recorded ($[Ca]_{free}$). Ca^{2+} current (I_{Ca}) was triggered with a voltage step to 0 mV. $\Delta[Ca]_{total}$ was calculated from the Ca^{2+} current integral as described by Berlin et al.[13] NCX-mediated transmembrane Ca^{2+} flux was prevented by removal of intra- and extracellular Na^+. SERCA-mediated Ca^{2+} uptake into the SR was prevented by incubation with thapsigargin (5 μM). Mitochondrial Ca^{2+} uptake was prevented by Ru360 (10 μM); *b* — average ratio of total and free cytosolic $[Ca^{2+}]$. n = 8-11 myocytes per group from 3-4 different mice, *p < 0.01 vs NTG

I79N mice (91.6 ± 10.3% of TnT-WT normalized to GAPDH, n = 4). Taken together, these data demonstrate that Ca^{2+} sensitizing TnT mutants increases Ca^{2+} buffering by lowering the apparent K_d for cytosolic Ca^{2+} binding (presumably to TnC).

Since caffeine itself can sensitize myofilaments to Ca^{2+}(Wendt et al, 1983), we next used an independent method to confirm the effect of the TnT-I79N mutation on cytosolic Ca^{2+} buffering (Fig. 3) (Berlin et al, 1994). Compared to non-transgenic myocytes (NTG), TnT-I79N myocytes exhibited much smaller rises in $[Ca^{2+}]_{free}$ (Fig. 3A, upper trace) in response to approximately the same amount of total Ca^{2+} influx into the cytosol (Fig. 3, *a*, lower trace), which was calculated by integrating the Ca^{2+} current (Fig. 3, *a*, middle trace).

As before, we calculated the ratio between the increase in $[Ca^{2+}]_{total}$ and the resulting change in $[Ca^{2+}]_{free}$ for each myocyte (Fig. 3, *b*). On average, cytosolic Ca^{2+} binding was significantly higher in TnT-I79N compared to NTG myocytes (Fig. 3, *b*). The use of thapsigargin, which locks SERCA in a Ca^{2+} free state (Kijima et al. 1991), may underestimate cytosolic Ca^{2+} binding and can *at least in part* explain why the values were slightly lower compared to those in case of rapid caffeine application (compare Figs. 1, *c* and 3, *b*). The latter experiments strictly measure rapid Ca^{2+} buffering (<200 ms), while the caffeine application lasts several seconds (Fig. 1) and therefore may also include some slow buffering (Bers, 2001).

Other teams of researchers have shown that increasing maximal Ca^{2+} binding capacity (B_{max}) by Ca^{2+} chelators (Diaz et al, 2001) or cardiac expression of skeletal muscle Ca^{2+} buffering protein (parvalbumin) (Day et al, 2008) can reduce Ca^{2+} transient amplitude. In this study we directly demonstrate, for the first time, that decreasing cytosolic Ca^{2+} binding affinity (K_d) without changing B_{max}, is similarly able to reduce Ca^{2+} transient amplitude. The increased net Ca^{2+} buffering is sufficient to lower systolic $[Ca^{2+}]_{free}$ (Figs 1 and 3) and likely contributes to the decrease in the Ca^{2+} transient amplitude during normal excitation-contraction coupling of TnT-I79N cardiomyocytes (Knollmann et al. 2003). Based on our findings, it is expected that physiological regulatory mechanisms, e.g. TnI phosphorylation (Mope et al, 1980), which alter myofilament Ca^{2+} sensitivity to the same degree or more than the ΔpCa_{50} observed in TnT-F110I and TnT-I79N, will increase the Ca^{2+} transient amplitude.

We have demonstrated that the degree of myofilament Ca^{2+} sensitization directly correlates with the risk for ventricular arrhythmias in mutant TnT mice (Baudenbacher et al, 2008). Here we show that the TnT mutation with the strongest pro-arrhythmic potential (TnT-I79N) also exerts the greatest effect on cytosolic Ca^{2+} buffering. Alterations in cytosolic buffering should be considered as one of the underlying mechanisms responsible for the arrhythmogenic effects of myofilament Ca^{2+} sensitization.

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