Relevance of the proximal domain in the amino-terminus of HERG channels for regulation by a phospholipase C-coupled hormone receptor

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Abstract **We used *Xenopus* oocytes co-expressing thyrotropin-releasing hormone (TRH) receptors and human ether-a-go-go-related gene (HERG) K^+ channel variants carrying different amino-terminal modifications to check the relevance of the proximal domain for hormonal regulation of the channel. Deletion of the whole proximal domain (Δ138–373) eliminates TRH-induced modifications in activation and deactivation parameters. TRH effects on activation are also suppressed with channels lacking the second half of the proximal domain or only residues 326–373. However, normal responses to TRH are obtained with Δ346–373 channels. Thus, whereas residues 326–345 are required for the hormonal modulation of HERG activation, different proximal domain sequences contribute to set HERG gating characteristics and its regulation by TRH.**

Key words: Human ether-a-go-go-related gene; K^+ channel; Thyrotropin-releasing hormone receptor; Hormonal regulation; Gating; Amino-terminus

1. Introduction

The human-ether-a-go-go related gene (HERG) encodes a potassium channel that mediates the cardiac repolarising current I_{Kr} [1,2]. ERG currents also play a key role setting the electrical behaviour of a variety of cell types including neurones, glial cells, pancreatic B cells, adenohypophyseal cells and several tumour cell lines [3–10]. Undoubtedly, the relevance of HERG channels makes them a primary target for physiological regulation. However, unlike the relatively well known molecular and kinetic characteristics of HERG, the mechanisms of regulation by different physiological agents or the structural determinants of such a regulation are largely unknown. We showed recently that the proximal domain located between the conserved initial amino-terminus and the first transmembrane helix is an important determinant of HERG gating properties [11,12]. By co-expressing in *Xenopus* oocytes the phospholipase C-coupled thyrotropin-releasing hormone receptor (TRH-R) and HERG we also showed that TRH modifies channel gating slowing activation, shifting the activation voltage dependence in the depolarising direction and accelerating deactivation [13]. In this report the relevance of proximal domain sequences for TRH-induced regulation of HERG is explored. Our results demonstrate that selective deletion of different proximal domain segments ranging from a stretch of 47 amino acid residues (Δ326–373) to the whole proximal domain (Δ138–373) abolishes TRH effects on activation parameters. However, normal responses to TRH are observed with Δ346–373 and Δ355–373 channels. Furthermore, the TRH-induced effects on deactivation are totally recovered in channels lacking residues 284–373. Thus the proximal domain, particularly residues 326–345, is necessary for the regulatory influence of TRH on HERG activation. Among previous results [11], this indicates that different sequences in the proximal domain contribute to set the gating characteristics of HERG and its regulation by the phospholipase C-coupled TRH-R.

2. Materials and methods

Isolation of the TRH-R cDNA has been described previously [14]. The plasmid containing the HERG cDNA was provided by Dr E. Wanke (University of Milan, Italy). Procedures for generation of the Δ138–373, Δ223–373, Δ355–373, S620T and HA-HERG variants have been detailed elsewhere [11,12]. To construct the Δ284–373 variant a forward polymerase chain reaction (PCR) primer containing a *Hin*dIII site and covering the coding sequence for the first six residues of HERG (5′-TTG AAG CTT CTC AGG ATG CCG GTG CGG AGG GGC-3′) was synthesised and used in PCR reactions with a reverse primer containing 30 bp of coding sequence corresponding to amino acids 274–283, followed by a 9-bp sequence corresponding to amino acids 374–376 and covering a unique *Bst*EII site (5′-CCG GTG GAC CGA GGC GCG GCG CAC GCT GGC GCA GCT TTC-3′). Variant Δ326–373 was generated in a similar way but using a PCR reverse primer containing HERG coding sequence corresponding to residues 319–325 followed by the 9-bp sequence carrying the unique *Bst*EII site (5′-CTG GGT GAC CAC GAG GTC GGA GTC CGA GGT-3′). The point mutant S283A was created by site-directed mutagenesis using the PCR-based overlap extension method as previously described [11]. The resulting PCR products were digested with *Hin*dIII/*Bst*EII, gel-purified and ligated into *Hin*dIII/*Bst*EII-digested wild-type HERG in the psP64A+ vector. All constructs were sequenced to confirm the mutations and to ensure the absence of frame errors. Plasmids were linearised and capped cRNA was in vitro synthesised from the linear cDNA templates by standard methods using SP6 RNA polymerase as described [14].

Procedures for frog anaesthesia and surgery, obtaining oocytes, and microinjection have been detailed elsewhere [11–14]. Oocytes were maintained in OR-2 medium (in mM: 82.5 NaCl, 2 KCl, 2 $CaCl_2$,

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Abbreviations: HERG, human ether-a-go-go-related gene; TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; PKC, protein kinase C; PKA, protein kinase A

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2 $MgCl_2$, 1 Na_2HPO_4, 10 HEPES, at pH 7.4). Electrophysiological recordings in oocytes were made at room temperature using the two-electrode voltage-clamp method with a Turbo TEC01C (NPI, Tamm, Germany) as described previously [11–14]. Unless otherwise indicated, recordings were obtained in extracellular high-K^+ OR-2 medium in which 50 mM KCl was substituted for an equivalent amount of NaCl. Functional expression was assessed 2–3 days after microinjection. Membrane potential was typically clamped at −80 mV, and at −100 or −110 mV for proximal domain-deleted constructs to compensate for the left shift in voltage dependence of activation caused by the deletions [11,12]. Kinetic parameters of activation and deactivation were obtained as previously described [11–13], using the voltage protocols shown on the graphs. To compensate for the shift in voltage dependence of activation, depolarisation voltages more negative than those used with wild-type channels were applied to proximal domain-deleted channels to monitor the activation time course. TRH data collection started 2 min after adding the hormone. Stimulation and data acquisition were controlled with Pulse+PulseFit software (HEKA Elektronic, Lambrecht, Germany) running on Macintosh computers. Data analysis was performed with the programs PulseFit (HEKA Elektronic) and Igor-Pro (WaveMetrics, Lake Oswego, OR, USA).

For generation of permanently transfected cell clones monolayer cultures of HEK293 (ATCC CRL-1573) cells were transfected using Lipofectamine (Gibco) with plasmid pcDNA3.1/Hygro(+) (Invitrogen) containing the TRH-R cDNA. Three days after transfection, cells were trypsinised and cultured in medium containing 150 μg/ml hygromycin B until cell colonies were visible. Individual colonies were picked with cloning cylinders and tested for TRH-induced calcium responses. A clone named TRH-R2 was selected for further transfection. Cells of this clone were transfected with HERG channel cDNAs subcloned into the pcDNA3 vector (Invitrogen). In this case, both full-length and Δ138–373-deleted channels carrying also a point mutation from Ser to Thr in residue 620 (S620T) that abolished inactivation [11,12], but showing analogous activation and deactivation properties as those of wild-type channels [11,12], were used. Geneticin sulphate (1 mg/ml) and hygromycin B were used to select individual clones co-expressing TRH-Rs and HERG channels that showed robust HERG currents under voltage clamp, and reproducible calcium responses when perfused with TRH after loading the cells with the fluorescent Ca^{2+} indicator Fura-2 [15].

For patch-clamp recordings transfected cells were plated in 35-mm-diameter tissue culture plastic dishes containing sterile glass coverslips coated with poly-L-lysine and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium contained Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DME/F12 1:1 mixture, Sigma) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% foetal bovine serum. Cells were maintained in the presence of geneticin and hygromycin B. Experiments were performed in extracellular saline containing (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The pipette solution contained (mM): 65 KCl, 30 K_2SO_4, 10 NaCl, 1 $MgCl_2$, 50 sucrose and 10 HEPES (pH 7.4 with KOH). Current recordings were performed at room temperature with the perforated-patch variant of the patch-clamp technique as described previously [3,4,15].

Data are presented as means ± S.E.M. with the number of cells and frogs indicated by *n* and *N*, respectively. The significance level after Student's *t*-test is indicated by *P*.

3. Results and discussion

To test the relevance of the HERG channel proximal domain on TRH-induced regulation of activation kinetics we initially compared the hormonal effects in oocytes expressing either wild-type channels or a Δ138–373 mutant in which the domain was completely removed (Fig. 1). The effect of the deletion on TRH-induced slowing of activation is illustrated in Fig. 2. Depolarisation voltages of 0 and −50 mV were used for wild-type and proximal domain-deleted channels, respectively. In both cases, only small depolarisation-induced currents were obtained due to overlap of relatively slow activation and very fast inactivation, followed by big deactivation tail currents after return to negative voltages. For this reason, we monitored the time course of transitions from closed to open states using an indirect envelope of tail currents, following the increase of tail current magnitude as previously described [2,11,13]. As shown in Fig. 2A,B, the time necessary to attain a half-maximal tail current magnitude was increased by TRH more than twofold in wild-type channels. However, the hormonal effect was abolished in Δ138–373 channels.

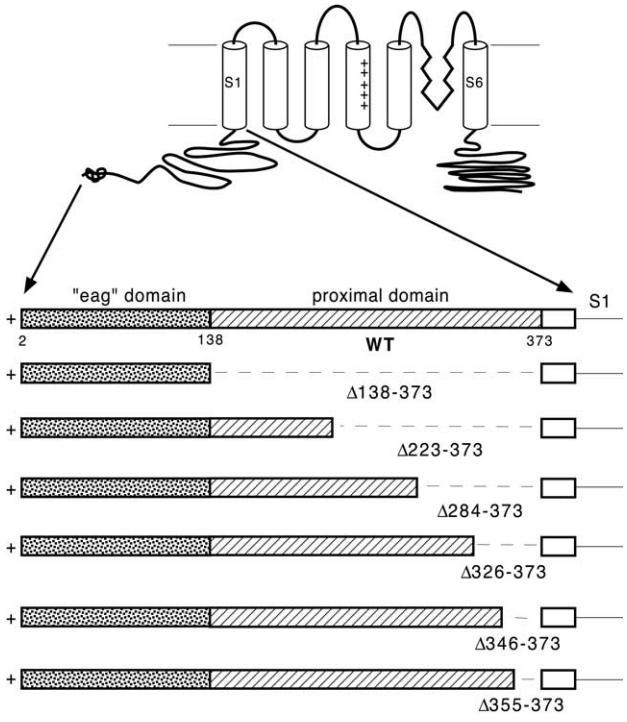

Fig. 1. Topology of a HERG channel a subunit. Transmembrane helices S1–S6 are represented by six cylinders at the top. The positions of the S1 helix, the voltage sensor in S4 and the P region between S5 and S6 are highlighted. Schematic representations of the amino-terminus in wild-type and deletion mutants are shown at the bottom. The eag and the proximal domains are marked by dotted and striped bars, respectively. Deletions are marked by dashed lines. The size of every domain and the lengths of the deletions are represented on a horizontal scale proportional to the total length of the amino-terminus.

In an attempt to further delimit the specific region(s) involved in hormonal regulation of channel properties, other amino-terminally modified variants were also used (Fig. 1). This included the previously described constructs [11,12] lacking almost half of the proximal domain (Δ223–373) or a short stretch of 19 amino acids (Δ355–373). Additionally, we characterised three new variants in which residues 284–373, 326–373 or 346–373 were deleted. As an additional control we monitored the TRH effects in a channel carrying an intact proximal domain, but in which the functionality of the amino-terminal eag/PAS domain had been impaired by introduction of a HA epitope (HA-HERG, see [12]). As shown in Fig. 2B, the increases in the half-maximal activation time induced by TRH were almost abolished for Δ223–373, Δ284–373 and Δ326–373 channels. However, the hormone-induced slowing of activation remained unaltered in Δ346–373, Δ355–373 and HA-HERG channels. This demonstrates that the presence of the proximal domain sequence between residues 325 and 346

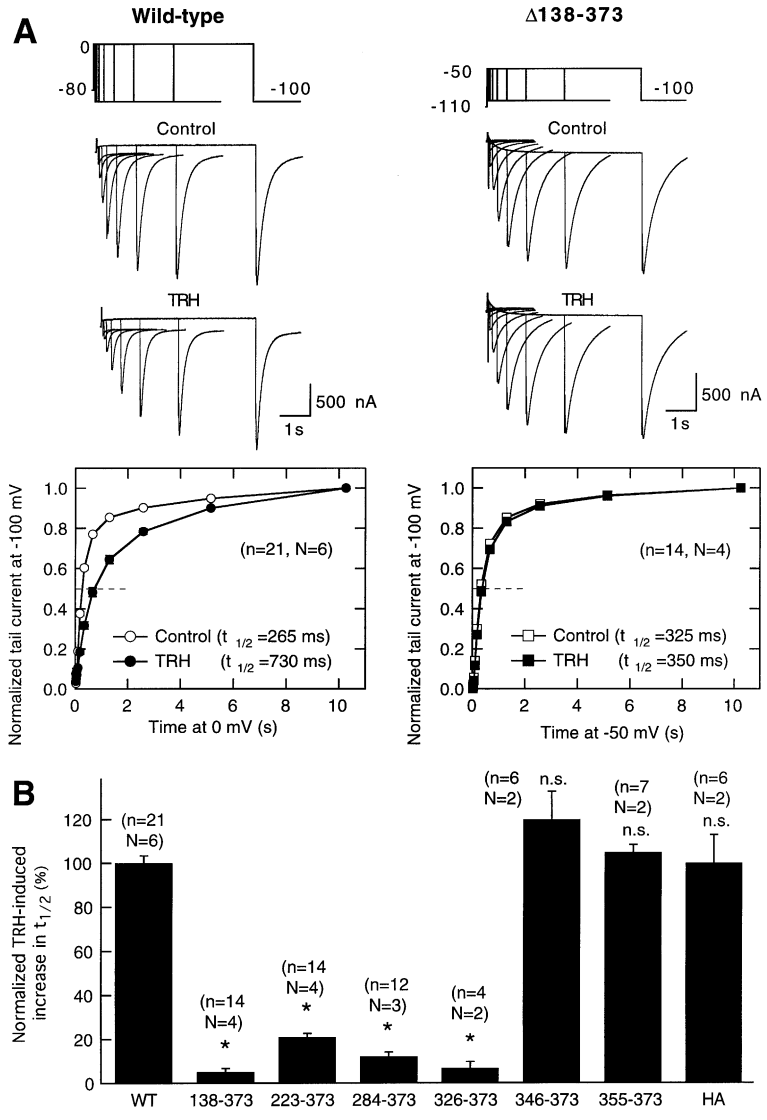

Fig. 2. Effect of amino-terminal modifications on TRH-induced slowing of HERG activation. A: The time course of voltage-dependent activation was studied in the absence (control) or the presence of 1 μM TRH by varying the duration of a depolarising prepulse according to the voltage protocols shown at the top. Data from wild-type and proximal domain-deleted channels are shown. The magnitude of the tail current upon repolarisation normalised to maximum is shown in the lower panels. The time necessary to reach a half-maximal tail current magnitude is indicated. B: Comparison of TRH effects on channels carrying different structural alterations in the amino-terminus. Activation kinetics were studied at −50 mV for Δ138–373, Δ223–373, Δ284–373 and Δ326–373, −30 mV for Δ346–373 and Δ355–373, and 0 mV for HA-HERG and wild-type channels, respectively. $*P < 0.0001$; n.s.: not significant.

is required for the TRH-induced slowing of HERG activation. Interestingly, normal responses to TRH were observed with Δ355–373 channels even though deletion of this 19-amino acid sequence causes around half of the gating alterations induced by deletion of the entire proximal domain [11]. Thus different segments of this domain contribute to set the gating characteristics of HERG channels and its hormonal regulation.

We have previously shown that the TRH-induced modifications in HERG activation properties include a clear shift in the activation voltage dependence [13]. Subsequently, we also compared the dependence of the normalised amplitudes of the maximal available tail currents on the preceding pulse potential using depolarising pulses of 1 s duration. Although this pulse duration is too short to measure the true voltage dependence of steady-state activation [13,16], it is very convenient to record reproducible currents maintaining the viability of the clamped cells and to generate isochronal availability curves that clearly reflect the differences due to the hormonal treatment. Consistent with previous results [13], the addition of TRH to oocytes expressing wild-type channels induced a clear displacement of the *I/V* curves to more positive voltage values (Fig. 3). However, the position of the curves along the voltage axis remained unaltered after adding TRH to oocytes expressing Δ138–373 channels. A similar failure to modify the activation voltage dependence following the hormonal treatment was also observed with Δ223–373, Δ284–373 and Δ326–373 channels. Nevertheless, a TRH-induced displacement of the availability curves equivalent to that observed with wild-type channels was obtained when Δ346–373, Δ355–373 and HA-HERG channels were used. It is important to indicate that failure to detect any shift with some deletions is not due to use of short pulses that would tend to separate the

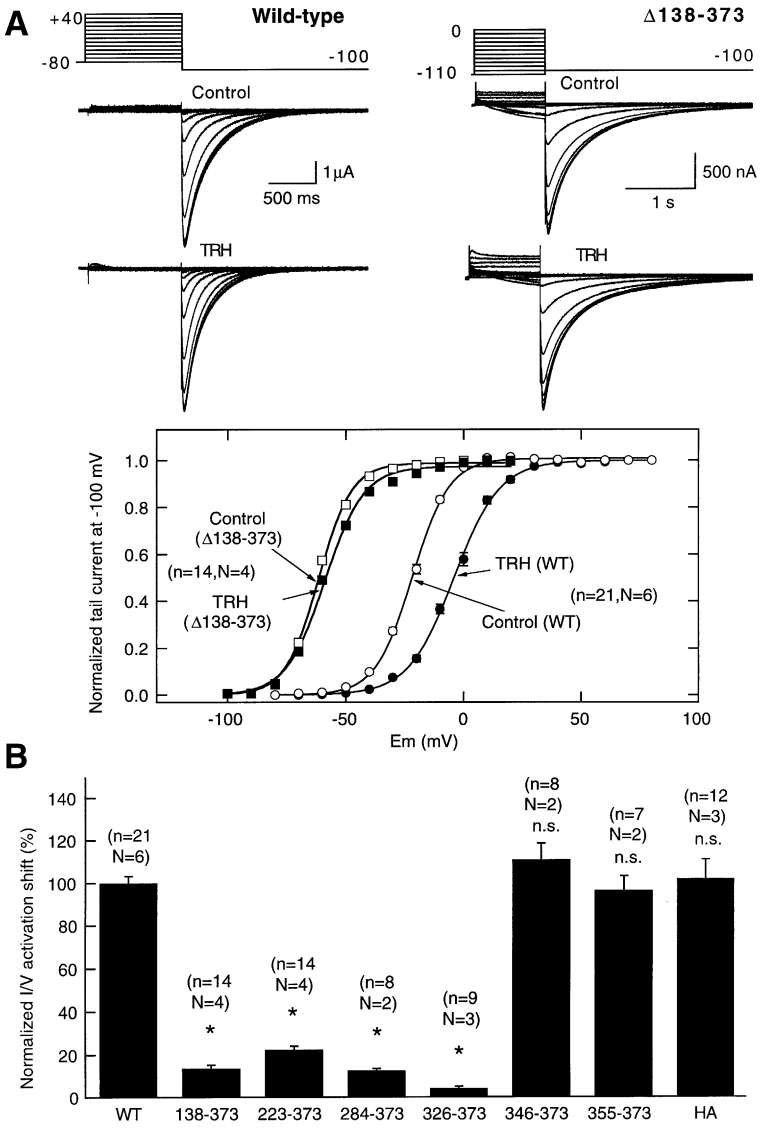

Fig. 3. Effect of amino-terminal modifications on TRH-induced shifts in HERG activation voltage dependence. A: Voltage dependence of activation was studied in the absence (control) or the presence of 1 μM TRH by varying the magnitude of a depolarising prepulse according to the voltage protocols shown at the top. The continuous lines in the lower panel correspond to Boltzmann curves $h(V) = I_{max}(1/(1+\exp(V-V_{1/2})/k))$, which best fitted the data with $V_{1/2}$ of −22 and −4 mV for control and TRH-treated wild-type channels. $V_{1/2}$ values of Δ138–373 channels corresponded to −61 and −59 mV in the absence or the presence of TRH, respectively. B: Comparison of TRH-induced shifts in activation voltage dependence on channels carrying different alterations in the amino-terminus. Shifts in $V_{1/2}$ value were normalised to that observed in wild-type channels. Control $V_{1/2}$ values for the different channel variants were −59 (Δ223–373), −68 (Δ284–373), −60 (Δ326–373), −44 (Δ346–373), −42 (Δ355–373) and −26 (HA-HERG) mV.

channels from steady-state conditions. Application of 3- and 10-s depolarisations to wild-type channels yielded $V_{1/2}$ values of −24.1 ± 1.2 and −28.6 ± 0.6 mV, respectively. However, the *I*/*V* curves were still shifted by TRH to −3.0 ± 1.7 and −16.7 ± 0.7 mV under these conditions ($n = 6$, $N = 2$). These results show again that the segment between amino acids 325 and 346 is required for the TRH-induced effects on activation gating. They also suggest that a functionally intact eag/PAS domain is not a requisite for the hormonal effects.

As a further indication that the proximal domain is necessary for the regulatory influence of TRH on HERG, we checked the effect of modifying the amino-terminus on the acceleration of deactivation induced by the hormone [13]. Again, the TRH-induced acceleration of the tail current decay was almost abolished in channels lacking the whole proximal domain (Δ138–373; Fig. 4). Surprisingly, TRH-induced accelerations lying between those recorded in full-length and Δ138–373 channels were observed with the Δ223–373 mutant. Furthermore, TRH induced modifications similar to those obtained with wild-type HERG in oocytes expressing Δ284–373 and Δ326–373 constructs (Fig. 4B,C). Studies of hormonal effects on deactivation properties of HA-HERG channels were hampered by the strong modification in closing rates induced by introduction of the HA epitope itself [12]. Once more, these results demonstrate that the presence of the proximal domain is necessary for the hormonal effects. However, since amino acids 326–345 are required for the TRH effects on activation, but the effects on deactivation are partially recovered in Δ223–373 and they are totally normal after deletion of residues 284–373, they also suggest that different protein seg-

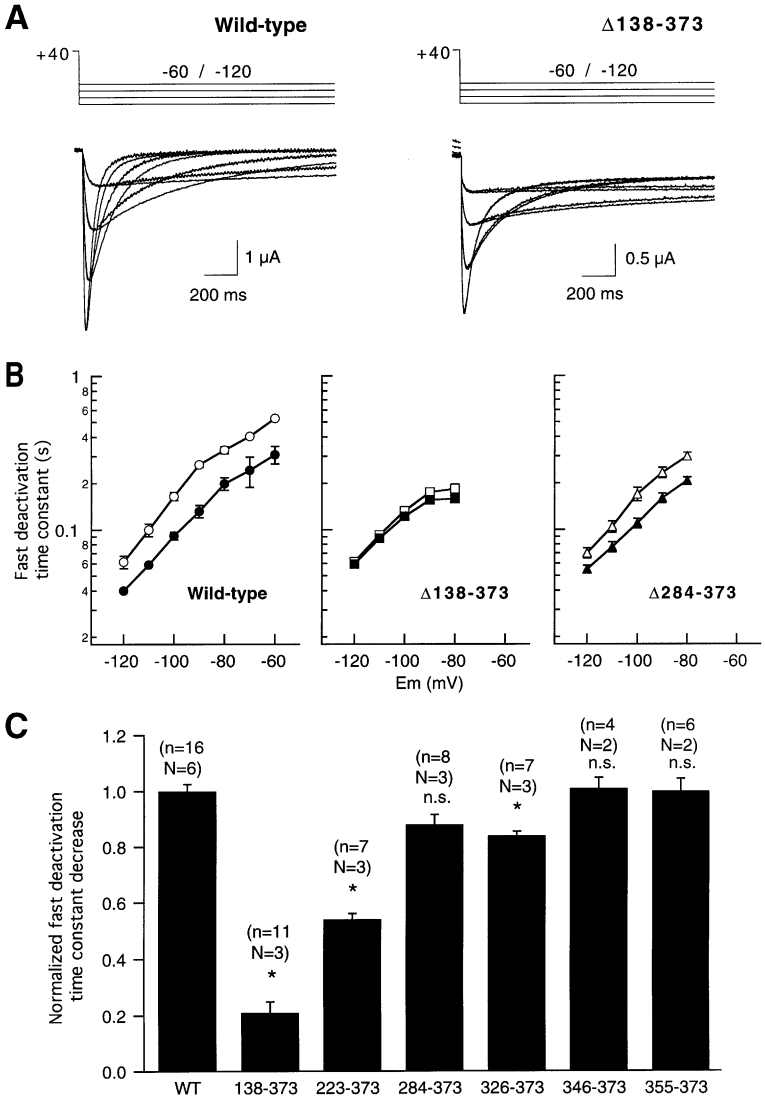

Fig. 4. Effect of amino-terminal modifications on TRH-induced acceleration of HERG deactivation. A: Current traces in the absence (control) or the presence of 1 μM TRH are shown superimposed and normalised to peak for better comparison of decay rates. In all cases faster decay corresponds to currents recorded following TRH treatment. Currents were obtained using the protocols shown at the top of the traces. Membrane currents are shown starting at the end of the 1-s depolarisation pulses used to activate the channels. B: Variation of fast deactivation time constant with repolarisation voltage. Deactivation time constants were quantified by fitting a double exponential to the decaying portion of the currents during repolarisation pulses as in A. Only values corresponding to the fast component of deactivation that predominates at negative potentials are plotted, for simplicity. Time constants before (open symbols) and after (closed symbols) TRH treatment are shown. Significant differences ($P < 0.05$) were observed in TRH-treated cells for wild-type and Δ284–373 channels at all voltages. Closing rates at −60 to −80 mV were not assessed for Δ138–373 and Δ284–373 constructs to prevent influence of channel opening on deactivation rates along this voltage range. C: Comparison of TRH effects on deactivation kinetics in channels carrying different alterations in the amino-terminus. Bars in the histogram represent TRH-induced reductions in the magnitude of fast deactivation time constant at −100 mV as compared to that observed in wild-type channels.

ments and/or structural rearrangements are involved in hormonal regulation of activation and deactivation gating.

Evidence for participation of protein kinase A (PKA) in HERG modulation has been obtained from site-directed mutagenesis experiments in which elimination of the four consensus sites for PKA phosphorylation (Ser283, Ser890, Thr895 and Ser1137) abolished the cAMP-dependent regulation of the channel both in oocytes and in CHO cells [17,18]. Interestingly, most of the PKA-dependent phosphorylation and more than half of the modification on activation parameters induced by cAMP analogues were impaired after the single mutation of Ser283 [18]. A major participation of Ser283 could be the reason for the lack of TRH effects in Δ138–373 and Δ223–373 channels lacking Ser283. However, it totally disagrees with the similar absence of TRH effects in Δ284–373 and Δ326–373 channels still carrying the Ser283 PKA phosphorylation site. To exclude that abolition of the hormonal effect is due to a disruption of the structural environment around residue 283 as a result of the deletions, the TRH effect was also studied with a mutant channel in which Ser283 was replaced by alanine (S283A). In this case, the $V_{1/2}$ of the HERG availability curves was shifted by TRH from -36 ± 0.3 to -9 ± 1.0 mV ($n = 5$). This indicates that maintenance of the consensus site for PKA phosphorylation in the amino-terminus is not necessary for the TRH-induced modifications of HERG activation voltage dependence.

The shifts in HERG activation kinetics induced by TRH in oocytes have been shown to be minimised by the protein kinase C (PKC) inhibitor GF109203X [13]. However, previous results in rat adenohypophyseal cells endogenously expressing ERG channels and TRH-Rs indicated that phosphorylation by PKC and/or PKA was not involved in channel regulation by TRH [6,19–21]. Some differences between different cell systems are also observed on the current parameters modified by the hormone [6,13,19–21]. To exclude that the abolition of the TRH effects in proximal domain-deleted channels is related to use of the oocyte as the expression system, the hormone-induced alterations on activation kinetics were studied in a HEK293 cell line permanently expressing HERG and the TRH-R. As in oocytes, addition of TRH to cells expressing full-length channels induced a clear shift of the HERG *I/V* curves in the depolarising direction. In this case, the $V_{1/2}$ of the curves was shifted by TRH from -6.8 ± 0.8 to 14.3 ± 2.4 mV ($n = 5$). However, no such modification in the activation voltage dependence was induced by the hormone in cells expressing the Δ138–373 construct. Thus, $V_{1/2}$ values of -24.5 ± 4.9 and -25.0 ± 2.6 mV ($n = 5$) were obtained with and without TRH when these channels lacking the proximal domain were studied.

The amino-terminal sequences located between the initial distal domain and the channel core of voltage-dependent K^+ channels were initially considered as a simple anchor for the inactivating ball in channels showing N-type inactivation or as a determinant of tetramerisation and assembly. However, recent evidence indicates that they act as important regulators of channel gating and modulation by cytoplasmic factors [22–25]. We recently proposed that some conformational changes in the proximal domain of HERG constitute a constraining factor to efficiently progress through the activation pathway, acting as an important determinant of the inwardly rectifying properties and hence of HERG's physiological role in cardiac cells and other cell types [11,12]. The proximal domain of HERG also seems to regulate the interaction of the eag/PAS domain with the gating machinery [11]. The results presented here demonstrate a crucial role of the proximal domain for hormonal regulation of HERG. Interestingly, cytoplasmic N-terminal domains of voltage-dependent channels are also known as points for protein–protein interactions with regulatory factors such as small GTPases, protein kinases and phosphatases [22,24]. It would be interesting to know if direct modification (e.g. phosphorylation) of the proximal domain itself or some alteration in a regulatory component associated with it is involved in the hormonal regulation of HERG properties.

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