Ionic currents underlying HTRP3 mediated agonist-dependent Ca^{2+} influx in stably transfected HEK293 cells

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Abstract h*Trp3* **is a human homologue of the** *Drosophila* **gene responsible for a transient receptor potential (*trp*) mutation. When stably expressed in HEK293 cells, hTrp3 formed ion channels that were active under resting conditions but could be further stimulated by carbachol or ATP via endogenous muscarinic or purinergic receptors, respectively. Agonist evoked currents reversed polarity near 0 mV in physiological ionic conditions and were associated with a significant increase in the current variance. These results suggest the involvement of a non-selective cation channel with relatively large unitary amplitude. Consistent with this, resolved unitary events had a conductance of approximately 60 pS in the negative voltage range and an extrapolated reversal potential near 0 mV.**

Key words: Trp; hTrp3; Channel; Current; Patch-clamp; Carbachol

1. Introduction

Unlike phototransduction in the vertebrate eye, the photoreceptors in *Drosophila*, like most invertebrates, depolarize in response to light (for review, see [1]). In the *Drosophila* photoreceptor, light mediated membrane depolarization involves the opening of two homologous cation channels, *D*Trp (*Drosophila* transient receptor potential) and *D*Trpl (Trp-like). These channels open in response to the G-protein mediated activation of phospholipase C (PLC) and subsequent breakdown of phosphatidyl inositol 4,5-bisphosphate (PIP_2) (for reviews, see [1–4]). In addition to their excitatory role, these channels play a crucial role in the refilling of Ca^{2+} stores by allowing Ca^{2+} to flow into the cell from the extracellular environment, an important process for light adaptation. Indeed, the *Drosophila trp* mutant becomes blinded by intense light as it is not able to mount a Ca^{2+}-dependent light adaptation response [5,6].

The cDNAs encoding *Drosophila* Trp and Trpl have been cloned and functionally expressed in heterologous cell systems [7–14]. *D*Trp is a Ca^{2+} selective channel that is activated by an unknown mechanism associated with the release of Ca^{2+} from endoplasmic reticulum stores [11,12]. *D*Trpl is a non-selective cation channel that is activated by G-protein coupled signaling pathways but opens independently of the filling state of Ca^{2+} stores [9–11,13–16]. Both IP_3 [9] and G proteins of the $G_{q/11}$ subfamily [16] have been implicated as important signaling molecules for the activation of *D*Trpl.

Several mammalian homologues of *DTtrp* and *DTrpl* have recently been cloned and functionally expressed [17–21]. One such gene, h*Trp3*, when co-transfected with the muscarinic M5 receptor into COS cells, increases Ca^{2+} influx in response to the muscarinic agonist carbachol (CCh) [18]. The aim of the present work was to characterize the ionic currents underlying the hTrp3 mediated agonist evoked Ca^{2+} influx. The experiments described here were performed by measuring ionic currents from HEK293 cells stably expressing hTrp3 using the perforated patch-clamp technique.

2. Materials and methods

2.1. Nomenclature

*D*Trp is the protein encoded by the *Drosophila trp* gene; *D*Trpl is the protein encoded by the *Drosophila trp-like* gene; hTrp3 is the protein encoded by the h*Trp3* gene; *D* = *Drosophila*, h = human; Trp3 cells are HEK293 cells stably expressing hTrp3.

2.2. Stable expression of hTrp3 in HEK293 cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Stable cell lines expressing hTrp3 were developed by transfection of cDNA encoding hTrp3 containing a hemagglutinin (HA) epitope at the C terminus in pcDNA3 vector by the calcium phosphate/glycerol shock method [22]. After 24 h, cells were harvested, diluted in medium supplemented with 400 μg/ml G418, and transferred to wells of 96-well plates at different dilutions. G418-resistant transformants were expanded into 12-well plates. Cell lines expressing the HA epitope were identified by immunocytochemical staining in 96-well plates as described by Vannier et al. [23] using a monoclonal HA antibody, 12CA5 (Babco Inc., Berkeley, CA, USA), as the primary antibody and anti-mouse IgG conjugated with peroxidase (Amersham) as the secondary antibody. Expression of HA-tagged hTrp3 in stain-positive cell lines was confirmed by immunoprecipitation with 12CA5 as described [24]. Two cell lines were used for electrophysiological analysis. Two G418-resistant control cell lines were selected from HEK293 cells stably transfected with cDNA encoding rat V1a vasopressin receptor in pcDNA3 developed similarly as the Trp3 cell lines. The stable cell lines were diluted twice weekly and maintained in medium supplemented with 400 μg/ml G418. For electrophysiological recordings, cells were seeded on circular coverslips (5 mm in diameter) pre-coated with poly-D-lysine (100 μg/ml). Detailed results on immunoprecipitation of hTrp3 from the Trp3 cells and the effect of hTrp3 expression on Ca^{2+} influx of HEK293 cells in response to receptor activation and store depletion are described elsewhere [25].

2.3. Electrophysiology

Stably transfected HEK293 cells were voltage-clamped using the perforated patch-clamp technique [26]. Patch pipettes (2–5 MΩ) were filled by first adding a pipette solution free of nystatin. The remainder of the pipette was back filled with the same solution containing nystatin (50 μg/ml). The pipette solution was composed of the

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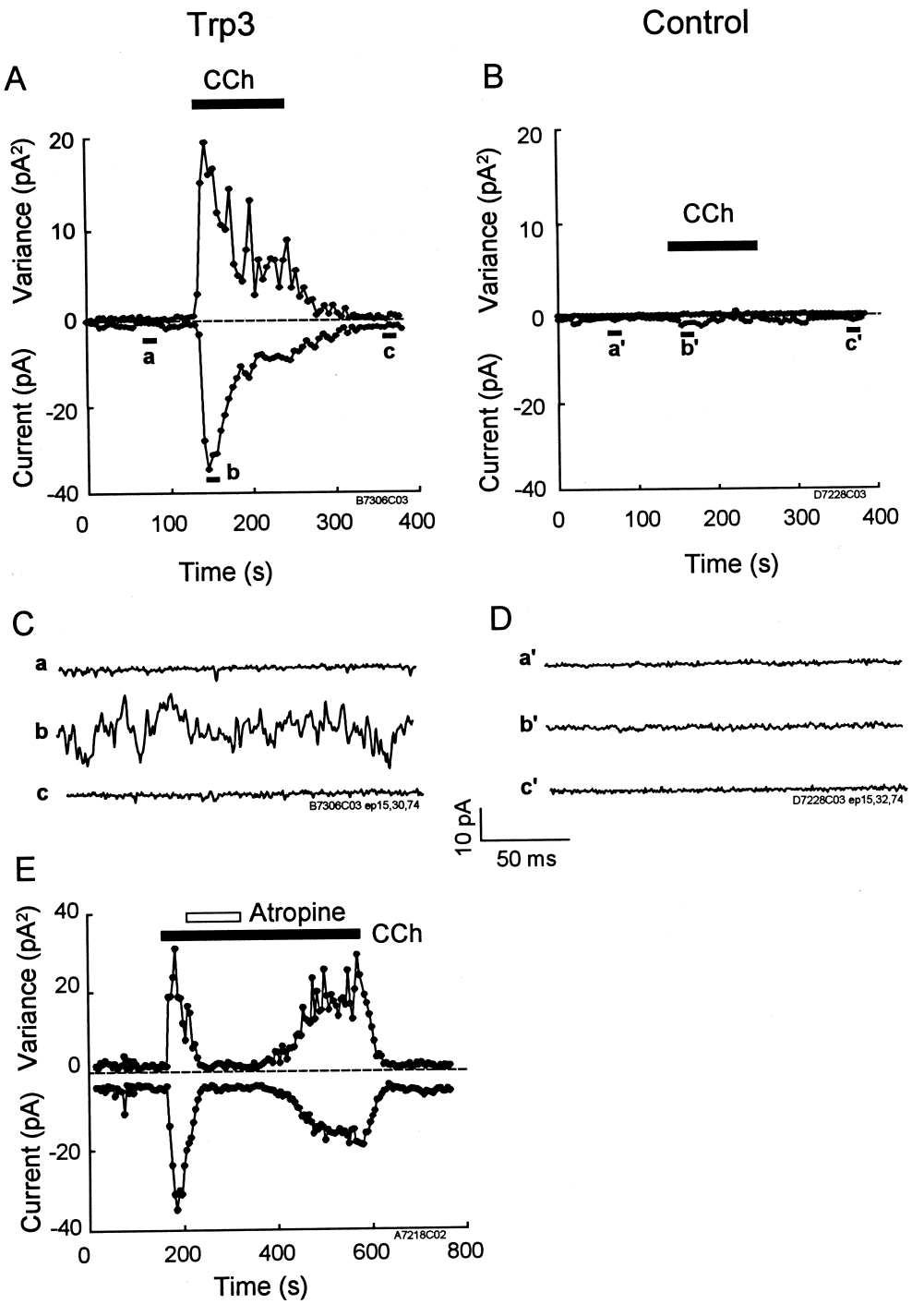

Fig. 1. hTrp3 mediated CCh evoked current. Response of Trp3 cells (left panels; A,C,E) and control cell (right panels; B,D) to stimulation by CCh. A and B: Mean current (negative values) and current variance (positive values) recorded during repeated 200 ms sweeps at a holding potential of −60 mV. The horizontal bars indicate the presence of CCh (100 μM). C and D: Individual sweeps of 200 ms at −60 mV before (a and a′) and during (b and b′) the application of CCh and after its wash-out (c and c′). Sweeps were taken from the regions in A and B indicated with lower case letters. The capacity of the Trp3 and control cell was 21.9 pF and 21.0 pF, respectively. E: Mean current and current variance evoked from a different Trp3 cell challenged with CCh (100 μM, solid bar) ± the co-application of atropine (1 μM, open bar); the capacity of this cell was 16.7 pF.

following (in mM): potassium methanesulfonate 130, KCl 10, $CaCl_2$ 0.5, $MgCl_2$ 2.0, EGTA 5.0, Na-HEPES 5.0, pH 7.2 (~280 mosM). Nystatin was diluted into the pipette solution about every 3 h from a 50 mg/ml stock in dimethylsulfoxide (DMSO). All patch pipettes were pulled from borosilicate glass capillary tubing (Warner Instrument Corp., Hamden, CT). The bath was composed of the following (in mM): NaCl 140, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 10, HEPES 15, pH 7.4 (~305 mosM). Carbachol, atropine, ATP and $LaCl_3$ were diluted into the bath solution from stock solutions dissolved in water.

In experiments measuring current at a constant potential, the current was digitized at 400 μs/point after being filtered at 400 Hz. In single channel experiments, the current was digitized at 200 μs/point

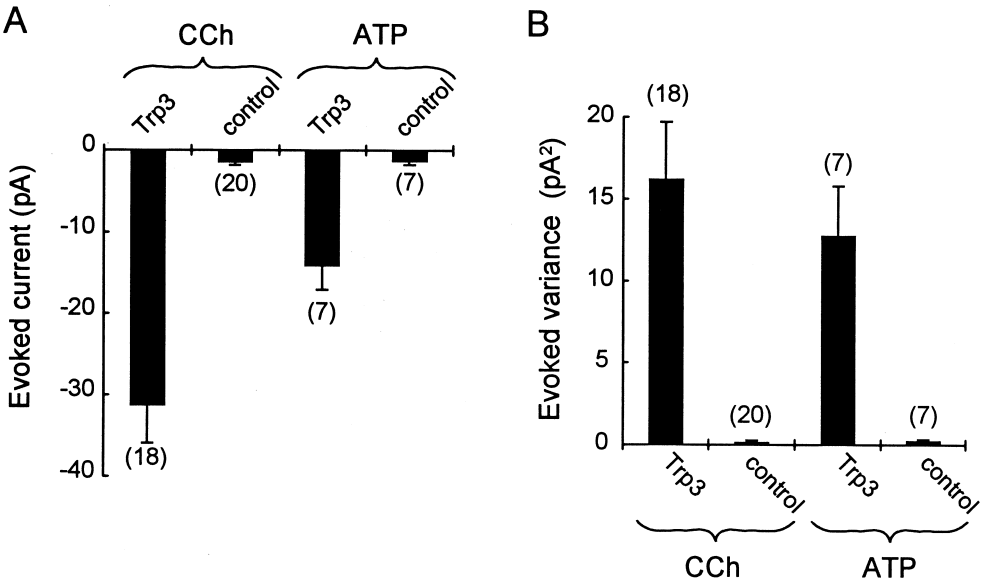

Fig. 2. CCh and ATP evoked current and current variance. Bar plots of the mean evoked current (A) and current variance (B) from Trp3 and control cells elicited by either CCh (100 or 200 μM) or ATP (100 μM). Individual values were obtained by subtracting the baseline current (i.e. current prior to agonist exposure) from the peak inward current during the agonist application. There was no difference in the evoked current amplitude by 100 μM ($n=5$) and 200 μM ($n=13$) CCh ($P>0.2$). The data were normalized to 20 pF which is approximately the average cell capacity of 20.7 ± 0.9 pF ($n=78$). Numbers in parentheses are the number of cells tested and error bars show the S.E.M.

and filtered at 1 kHz. Voltage ramps were delivered from −80 mV to 20 mV over 200 ms, data were acquired at 500 μs/point and filtered at 400 Hz. All experiments were performed at room temperature.

2.4. Data analysis

Evoked current in response to drug application was quantified by subtracting the average current preceding drug application (control value) from the peak inward current measured during drug application. Similarly, the evoked variance was quantified by subtracting the control variance preceding drug application from the maximum variance measured during drug application. Averaged data were normalized to 20 pF of membrane capacity which was approximately the average cell capacity of 20.7 ± 0.9 pF (± S.E., $n=78$). The ratio of current variance to mean current was derived from the linear regression fit to the variance as a function of the mean current in individual cells. Reversal potentials were estimated from macroscopic current recordings by fitting a linear regression to the data between −80 and −20 mV and extrapolating the fit to the zero current potential. All data are represented as the mean ± S.E. and n = number of cells. Statistical analyses were performed with the two-tailed Student's t distribution.

3. Results and discussion

3.1. Agonist evoked hTrp3 currents

When challenged with CCh, Trp3 cells rapidly developed an inward current at −60 mV that decayed to a small plateau upon continued exposure to the agonist for up to 100 s (Fig. 1A). The current further decayed back to its resting value when CCh was washed out. Similar results were observed in two different HEK293 cell lines stably transfected with h*Trp3*. CCh evoked currents were always associated with an increased noise or current variance (Fig. 1A,C) that most likely reflects a relatively small number of non-synchronous channel openings and closings upon CCh stimulation [27,28]. Both the CCh evoked current and increased current noise were reversibly blocked by atropine, a specific antagonist of the muscarinic receptor (Fig. 1E). In contrast to the Trp3 cells, CCh did not evoke either a measurable current or current variance in two different control cell lines that were stably transfected with the rat V1a vasopressin receptor (Fig. 1B,D, see also Fig. 2).

The purinergic receptor agonist ATP, which also stimulates a $G_{q/11}$ protein, evoked a similar inward current when bath applied to Trp3 cells voltage clamped at −60 mV (Fig. 2A). ATP evoked current, like that of CCh, was associated with an increased current variance (Fig. 2B). Both of these effects were reversed upon wash-out of ATP. There was no measurable increase in either the current amplitude or current variance in control cells treated with the same concentration of ATP.

Since CCh and ATP act through a common signaling pathway, the currents evoked from Trp3 cells by either agonist should be mediated by the same underlying channels. Consistent with this idea, the ratio of current variance to current amplitude, which gives a lower estimate of the single channel amplitude [27–29] was not different for currents evoked by these two agonist ($P>0.5$). The mean ratio of variance to mean current at −60 mV was −0.6 ± 0.1 pA^2/pA ($n=15$) for currents evoked by CCh and −0.7 ± 0.1 pA^2/pA ($n=7$) for currents evoked by ATP. The similar ratio of current variance to mean current suggests that the channels underlying CCh and ATP stimulated current had a similar single channel amplitude at this potential.

3.2. Current-voltage properties

Voltage ramps from −80 mV to 20 mV were applied to Trp3 cells before and after addition of CCh to determine the reversal potential of the evoked current in physiological ionic conditions using the nystatin perforated patch-clamp technique [26]. CCh evoked currents were approximately linear in the voltage range between −80 mV and −20 mV and reversed at 1 ± 2 mV ($n=5$) suggesting a non-selective cation current with little voltage dependence over this voltage range (Fig. 3A). A relatively large endogenous voltage-dependent current present in both the Trp3 cells and control cells often

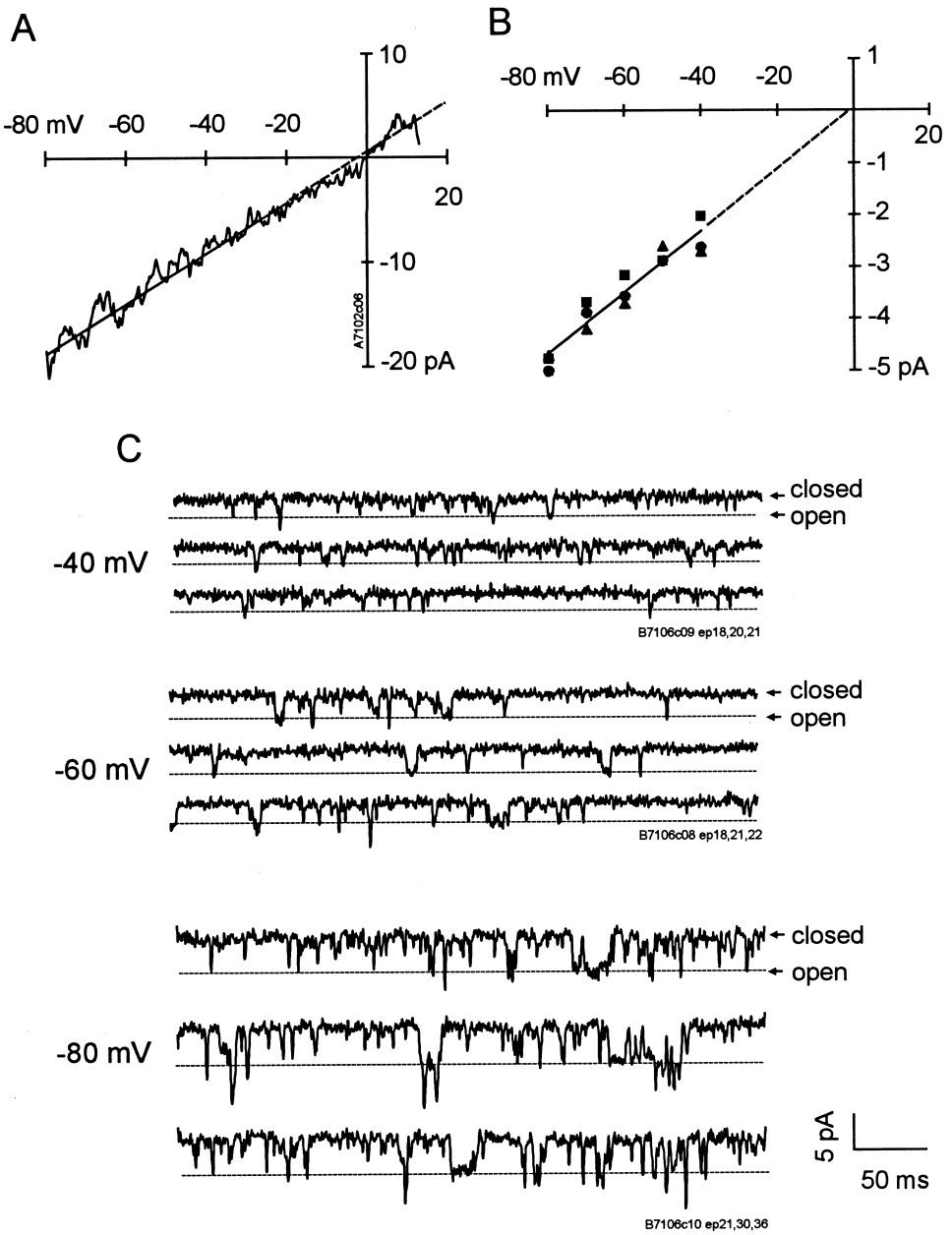

Fig. 3. Macroscopic and single channel current-voltage relationship. A: CCh evoked current-voltage relationship was obtained by subtracting current recorded prior to CCh stimulation from the current measured during bath application of CCh (200 μM). Voltage ramps were given from −80 mV to +20 mV over 200 ms. The average of 11 ramps prior to CCh stimulation and 6 ramps during CCh exposure were used for this analysis. The continuous straight line shows the linear regression fitted to the data between −80 and −20 mV. The dashed line is the extrapolation of the fit through the reversal potential. B: Current voltage relationship obtained by manually measuring the amplitude of single channel events from three different Trp3 cells (circles, squares and triangles). Five measurements were averaged per cell at every potential. The continuous line is a linear regression fitted to the mean values. The dashed line is the extrapolation to the zero current potential. C: Individual current sweeps obtained at −40 mV, −60 mV and −80 mV showing downward channel openings. The dashed horizontal lines indicate the unitary channel amplitude.

obscured the smaller CCh evoked currents at potentials positive to about −20 mV, therefore no conclusion can be drawn from these data regarding the linearity of the current-voltage relationship at more positive potentials.

During the course of this work, we recorded from three different Trp3 cells that had a combination of a small evoked whole-cell current and high input resistance (15.4, 18.5 and 6.2 GΩ). This combination allowed the resolution of apparent single channel events in the presence of CCh. While many of the events were fast and probably truncated by the filter (1 kHz), some events appeared longer with discernible amplitudes (Fig. 3C). By manually measuring the amplitudes of these resolved events, a single channel current-voltage relationship (i-V) was constructed for each of the three cells (Fig. 3B). The mean conductance determined by this analysis was 59 ± 2 pS ($n = 3$). Supporting the validity of this measurement, the extrapolated reversal potential for the three i-V curves was 0 ± 4 mV ($n = 3$), consistent with the results obtained from the macroscopic current-voltage relationship.

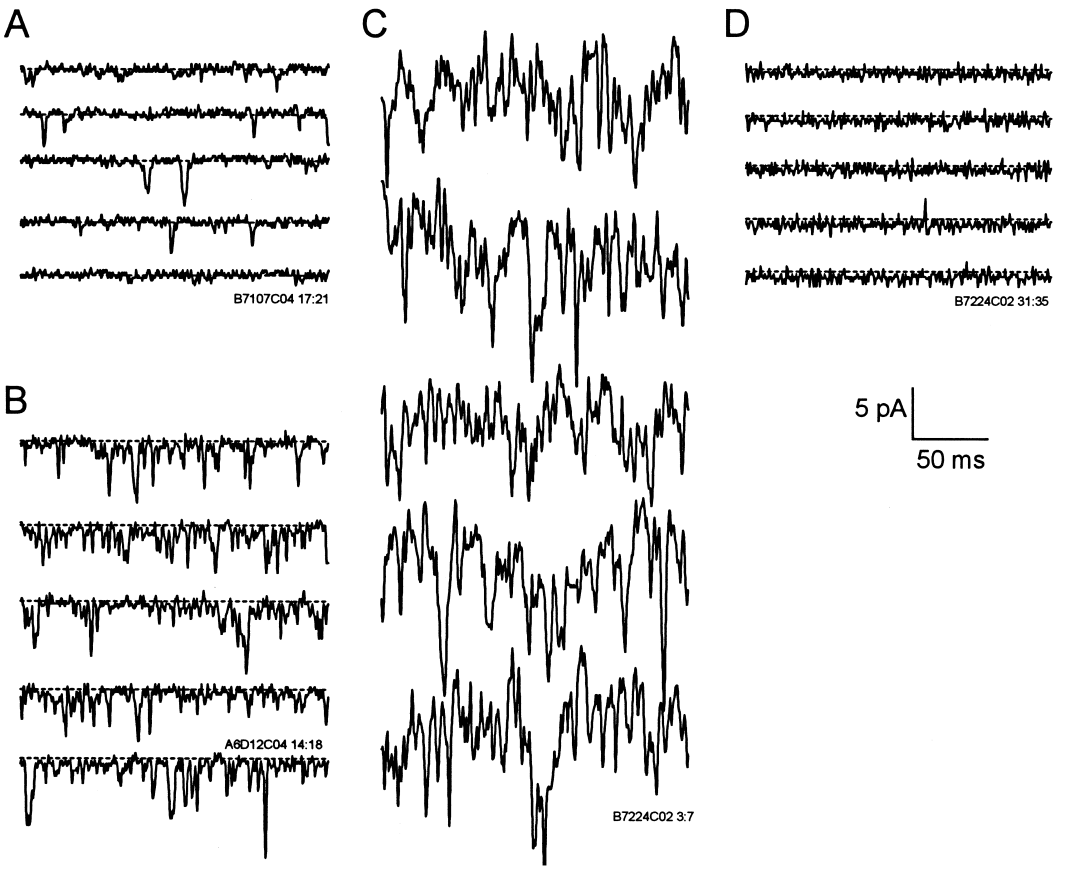

Fig. 4. Trp3 cells display variable amounts of basal channel activity. A–C: Consecutive current sweeps from three different Trp3 cells prior to agonist exposure. D: Consecutive current sweeps from the same cell as C but in the presence of $LaCl_3$ (200 μM). Dashed horizontal lines in A, B and D indicate the zero current level. The average current amplitude in C was approximately −20 pA.

3.3. Constitutive channel activity

Most Trp3 cells tested displayed some level of channel activity before the cell was ever exposed to agonist. This basal activity ranged from individual channel openings to several picoamps of mean current in which single events could not be resolved (Fig. 4A–C). Most cells tested had a basal activity somewhere within the range of that shown in Fig. 4A,B. The basal channel activity was not measurably reduced by atropine (1 or 3 μM, $n = 7/7$) but was completely blocked by the non-specific Ca^{2+} channel blocker La^{3+} at 200 μM ($n = 7/7$; Fig. 4D).

Two lines of evidence suggest that the channels underlying the basal activity resulted from the expression of hTrp3 and were not endogenous to the HEK293 cells. First, no such activity was every observed in two different control HEK293 cell lines expressing rat V1a vasopressin receptors and were otherwise maintained under identical conditions ($n = 31$). Second, the basal activity for hTrp3 had a similar ratio of variance to mean current as the agonist evoked response. The ratio of variance to mean current for the basal activity was -0.6 ± 0.1 pA^2/pA ($n = 7$) compared to -0.6 pA^2/pA and -0.7 pA^2/pA for CCh and ATP evoked current, respectively (see above). Only those cells that had a basal inward current ≥ -9 pA that could be completely blocked by La^{3+} (200 μM) were considered for this analysis ($n = 7$; the mean basal current of those cells was -15.4 ± 2.0 pA). Though the mechanism underlying this basal channel activity is not known, it should be noted that this constitutive activity is reminiscent of that seen with *Drosophila* Trpl expressed in *Sf*9 cells [10,11,15,30].

4. Conclusions

The main findings described in this work are the following. (1) Stable expression of hTrp3 in HEK293 cells resulted in constitutive channel activity that could be increased by agonist of $G_{q/11}$ protein coupled receptors; neither component was observed in control cells. (2) The evoked currents were voltage independent in the range of −80 mV to −20 mV and reversed polarity near 0 mV under physiological ionic conditions. (3) Single channel events had a slope conductance of about 60 pS in the negative voltage range and had an extrapolated reversal potential near 0 mV in physiological ionic conditions.

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