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Stimulation of single L-type calcium channels in rat pituitary GH_3 cells by thyrotropin-releasing hormone

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Hormonal stimulation of voltage-dependent Ca^{2+} channels in pituitary cells is thought to contribute to the sustained phase of Ca^{2+} entry and secretion induced by secretion stimulating hormones and has been suggested as a mechanism for refilling the Ca^{2+} stores. Using the cell-attached patch-clamp technique, we studied the stimulation of single Ca^{2+} channels by thyrotropin-releasing hormone (TRH) in rat GH_3 cells. We show that TRH applied from the bath switched the activity of single L-type Ca^{2+} channels from a gating mode with very low open probability (p_o) to a gating mode with slightly smaller conductance but 10 times higher p_o. Interconversions between these two gating modes were also observed under basal conditions, where the equilibrium was shifted towards the low p_o mode. TRH applied from the pipette had no effect, indicating the involvement of a cytosolic compound in the stimulatory pathway. We show that TRH does not potentiate all the L-type Ca^{2+} channels in a given membrane patch and report evidence for co-expression of two functionally different L-type Ca^{2+} channels. Our results uncover the biophysical mechanism of hormonal stimulation of voltage-dependent Ca^{2+} channels in GH_3 cells and are consistent with differential modulation of different subtypes of dihydropyridine-sensitive Ca^{2+} channels.

Key words: Ca^{2+} channel/G protein/gating mode/patch-clamp/signal transduction

Introduction

In pituitary cells, thyrotropin-releasing hormone (TRH) and other secretion stimulating hormones trigger a biphasic elevation of cytosolic Ca^{2+} concentration and a consequent biphasic release of hormones (see Ozawa and Sand, 1986, for a review). The initial transient increase in Ca^{2+} is mainly due to Ca^{2+} release from intracellular stores in response to inositol 1,4,5-trisphosphate (IP_3) produced by TRH-induced activation of phospholipase C, while the subsequent sustained elevation of intracellular Ca^{2+} is due to a persistent stimulation of Ca^{2+} influx from the extracellular space, a large part of which is mediated by voltage-dependent Ca^{2+} channels sensitive to dihydropyridine drugs (Schlegel *et al.*, 1987; Benham, 1989; Stoilkovic *et al.*, 1992).

In pituitary GH_3 cells, TRH exerts opposite modulatory effects on L-type Ca^{2+} channels through different mechanisms. TRH induces a Ca^{2+}-dependent inhibition of Ca^{2+} current secondary to IP_3-mediated Ca^{2+} mobilization when the intracellular stores are loaded (Gollasch *et al.*, 1991; Kramer *et al.*, 1991) and on the other hand it induces stimulation of the Ca^{2+} current when the intracellular stores are emptied (Gollasch *et al.*, 1991). Hormonal stimulation of voltage-dependent Ca^{2+} channels in GH_3 cells and other endocrine cells (Cohen *et al.*, 1988; Hescheler *et al.*, 1988; Mollard *et al.*, 1988; Thorn and Petersen, 1991; Doupnik and Pun, 1992) probably contributes to the sustained phase of Ca^{2+} entry and secretion induced by secretion stimulating hormones and has been suggested as a mechanism for refilling the Ca^{2+} stores (Gollasch *et al.*, 1991).

In contrast to the well-studied stimulation of cardiac L-type Ca^{2+} channels by β-adrenergic agonists (see Tsien *et al.*, 1986, for a review), hormonal stimulation of L-type channels in pituitary cells is not mediated by cAMP-dependent phosphorylation and is independent of intracellular cAMP levels (Mollard *et al.*, 1988; Rosenthal *et al.*, 1988; Gollasch *et al.*, 1993). The TRH-induced stimulation of L-type Ca^{2+} channels involves pertussis toxin (PT)-sensitive G proteins of the G_i family (mainly G_{i2}) and requires concurrent protein kinase C (PKC) activation (Gollasch *et al.*, 1993). The involvement of PT-sensitive G proteins is a feature that also distinguishes hormonal stimulation of L-type Ca^{2+} channels in pituitary cells from both the direct stimulation of cardiac L-type channels by G_s (Yatani *et al.*, 1987) and the stimulation of smooth muscle L-type channels by contractile agonists, that involves PT-insensitive G proteins and is probably mediated by PKC (Nelson *et al.*, 1988; Loirand *et al.*, 1990; Baron *et al.*, 1993).

Complex modifications of gating properties of single L-type Ca^{2+} channels, including an increase in channel availability and a shift to a mode of gating with longer open times (mode 2) (Hess *et al.*, 1984; Pietrobon and Hess, 1990), account for the protein kinase A (PKA)-mediated stimulation of Ca^{2+} current (Tsien *et al.*, 1986; Artalejo *et al.*, 1990; Ochi and Kawashima, 1990; Yue *et al.*, 1990; Herzig *et al.*, 1993; Ono and Fozzard, 1993). Contractile agonists seem to similarly affect the gating properties of single smooth muscle L-type channels (Bonev and Isenberg, 1992; Kamishima *et al.*, 1992; Tomasic *et al.*, 1992). The TRH-induced stimulation of L-type Ca^{2+} channels in pituitary cells has not been studied at the single channel level and therefore the biophysical mechanism of Ca^{2+} channel modulation remains unknown.

Molecular biology has revealed a great molecular diversity of L-type Ca^{2+} channels, arising from multiple

genes for α_1 and β subunits, multiple splice variants for each subunit and multiple possible subunit combinations (Hui *et al.*, 1991; Snutch *et al.*, 1991; Tsien *et al.*, 1991; Chin *et al.*, 1992; Hullin *et al.*, 1992; Perez-Reyes *et al.* 1992; Williams *et al.*, 1992). Recently, evidence for co-expression of functionally different L-type Ca^{2+} channels has been obtained in several cell types (Forti and Pietrobon, 1993; Neveu *et al.*, 1993), including rat pituitary melanotropic cells (Keja and Kits, 1994). Both class C and D α_1 subunits of L-type channels are expressed in the pituitary gland (Snutch *et al.*, 1991; Chin *et al.*, 1992). It remains unknown if functionally different L-type Ca^{2+} channels are expressed in GH_3 cells and if TRH differentially modulates the different possible subtypes.

To address these questions we obtained single Ca^{2+} channel recordings from GH_3 cells. We show that TRH applied from the cell bath stimulates single L-type Ca^{2+} channels in cell-attached patches by switching their activity from a low p_o gating mode to a high p_o gating mode different from mode 2. We report some evidence for co-expression of two functionally different L-type Ca^{2+} channels in GH_3 cells. Our results are consistent with selective potentiation of only one subtype by TRH.

Results

Figure 1A shows representative traces of the single L-type Ca^{2+} channel activity most frequently observed in cell-attached patches of GH_3 cells recorded before and after perfusion with the dihydropyridine (DHP) agonist (+)-(S)-202-791. As shown by the insignificant open channel peak in the amplitude histogram and the very small average current in Figure 1B, the control activity was characterized by very low open probability (p_o = 0.020 ± 0.004 in three single channel patches and p_o = 0.039 ± 0.04 in six two channel patches at +10 mV; the value of p_o for each patch was obtained by averaging the open probabilities measured in any active sweep). The large majority of openings were very brief and unresolved. The few well-resolved and the rare spontaneous long openings (mode 2, Figure 1D, inset) revealed the existence of two prevailing single channel current levels, with unitary current amplitudes of 1.05 ± 0.002 and 1.23 ± 0.008 pA at +10 mV (averages of 80 and 94 openings respectively from 22 patches) and similar conductances (g = 19.8 ± 0.1 and 21.5 ± 0.4 pS, obtained by averaging the unitary conductances of six patches; Figure 1D). Strikingly, the same patches after addition of the DHP agonist showed mainly a higher single channel current (i = 1.44 ± 0.001 pA, average of 200 openings) and conductance (g = 24.9 ± 0.5 pS, n = 6; Figure 1D) and only brief sojourns in the lower conductance states prevailing under control conditions. Thus, contrary to what has been concluded for cardiac L-type Ca^{2+} channels (Hess *et al.*, 1984), in GH_3 cells the long opening gating pattern induced by the DHP agonist is clearly different from the rare spontaneous long opening mode observed in the absence of drugs. To avoid confusion, from here on we will use the term mode 2 to refer only to the spontaneous long opening mode.

Less frequently, in some patches we observed a rather different DHP-sensitive single channel activity characterized by a more than 10 times higher open probability at each voltage (p_o = 0.298 ± 0.036 at +10 mV, n = 5) in the absence of the DHP agonist (Figure 2A and B). Short, well-resolved openings separated by brief closings were typically clustered in long bursts. Both the average single channel conductance (g = 19.1 ± 0.38 pS, n = 7 patches) and current (i = 1.01 ± 0.01 pA, average of 44 openings at +10 mV from seven patches) of this high p_o gating pattern were similar to those of the smaller of the two main conductance states of the low p_o gating pattern in Figure 1 (Figure 2D). Transitions to a higher conductance state (similar to the larger conductance of the low p_o gating) were occasionally observed (Figure 2D, inset). The single channel current (i = 1.43 ± 0.001, average of 66 openings at +10 mV from five patches) and conductance (g = 25.3 ± 0.6 pS, n = 5) of the prolonged openings observed upon addition of the DHP agonist were again larger than those obtained under control conditions (Figure 2D). Interestingly, in the presence of the DHP agonist the low p_o and high p_o gating patterns became hardly distinguishable, not only in terms of elementary current and conductance, but also in terms of open probability and open and closed time distributions (cf. Figures 1A and C and 2A and C, see legends).

In principle the low p_o and high p_o DHP-sensitive gating patterns shown in Figures 1 and 2 can be interpreted either as two different modes of activity of the same L-type Ca^{2+} channel or as single channel activities of two structurally different L-type Ca^{2+} channels. Cell-attached patches containing only one channel allow a discrimination between these two possibilities. If the different gating patterns are different modes of activity of the same L-type channel, we would expect to observe interconversions between the two gating patterns during long recordings of single channel patches. On the other hand, if interconversion between the two gating patterns is not observed, one can reasonably conclude that the two gating patterns represent the single channel activities of two structurally different L-type Ca^{2+} channels. In GH_3 cells, single channel patches were rare (only 15 out of a total of 370 patches) and could be identified with certainty only after addition of an agonist, given the extremely low p_o of the gating pattern in Figure 1. However, in one out of the 15 patches containing only one channel we did observe during the experiment conversion from the high p_o gating pattern present at the beginning to the low p_o gating pattern: after almost 6 min in the high p_o mode the channel switched to the low p_o mode and remained in this mode for 14 min, until the end of the recording (with the exception of a single trace in the high p_o mode 1 min after the switch). Considering also recordings from patches with two or three channels (lasting from at least 5 to 50 min), out of a total of 102 patches, in 41 we exclusively observed the low p_o gating pattern, in 33 we observed rare interconversions between the largely prevailing low p_o gating and short periods of high p_o gating (ranging from a few hundred milliseconds to 1–2 min) and in the remaining 28 we observed mixed activity with the high p_o gating present from the beginning (lasting up to 14 min). We can therefore conclude that GH_3 cells express L-type Ca^{2+} channels that can freely interconvert between two different modes of activity (shown in Figures 1 and 2). Under basal conditions the equilibrium between the two modes is largely shifted towards the low p_o mode.

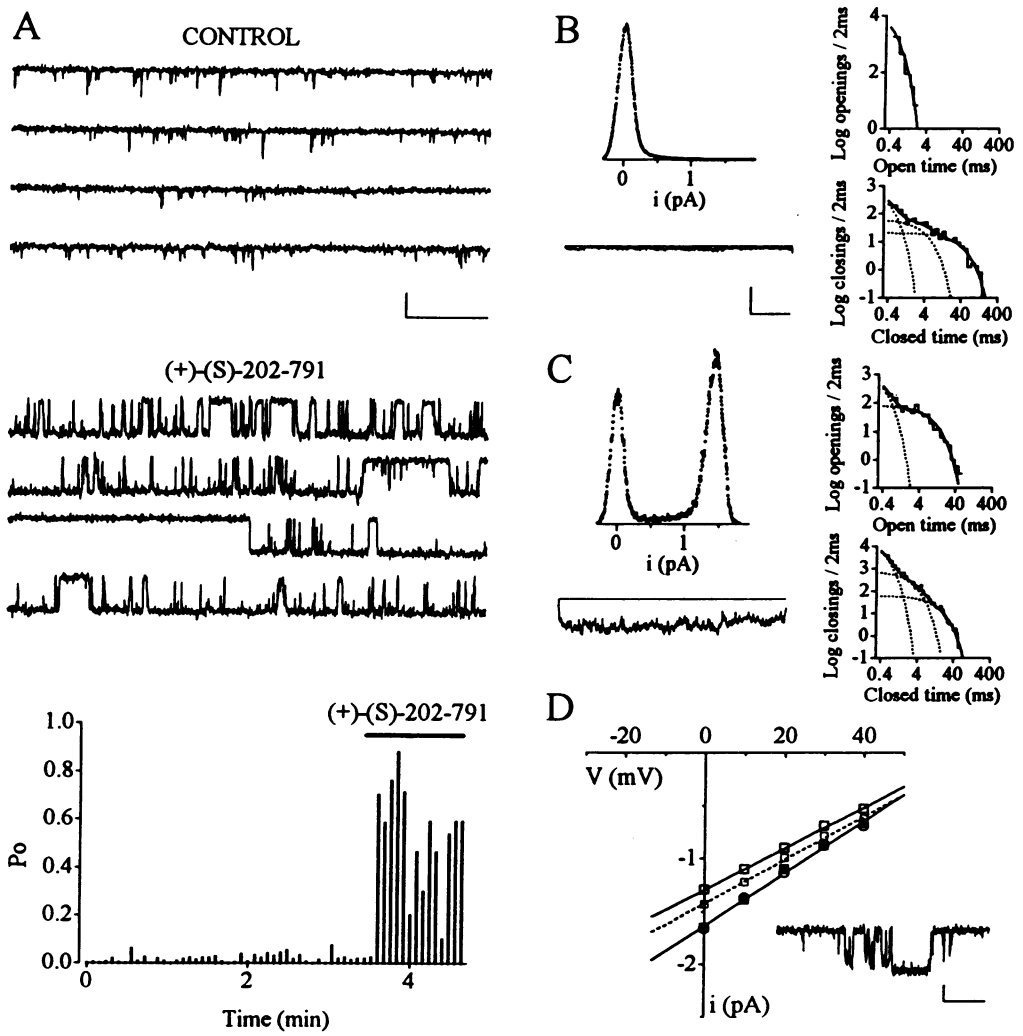

Fig. 1. Elementary properties of L-type Ca^{2+} channels with low p_o gating pattern in GH_3 cells. (**A**) Cell-attached recordings with 90 mM Ba^{2+} as charge carrier from a patch containing a single L-type Ca^{2+} channel (as judged by the lack of overlapping events after addition of a DHP agonist) and corresponding plot of open probability p_o versus time. Four representative current traces at +10 mV are shown before (control) and after perfusion with 1 μM (+)-(S)-202-791 in the bath solution. Calibration bars, 1 pA, 80 ms. Bars in the p_o versus time plot represent the probability of the channel being open at +10 mV in successive depolarizations. Cell G55A. (**B**) (Left) Normalized current amplitude histogram from all control traces with activity (cell G55A) and average single channel current (from three single channel patches, cells G55A, G68B and G37E, n = 84; calibration, 0.4 pA, 80 ms). V = +10 mV. (Right) Log–log plots of the open and closed time distributions before addition of the DHP agonist. V = +10 mV. The dark solid line in each plot is the best fit sum of exponential components. Each exponential component is shown as a dotted line. Open times were poorly resolved (time constant of exponential fit 0.25 ms). The closed time distribution was best fitted by the sum of three exponential components with time constants of 0.27, 3.55 and 37.9 ms and relative areas of 23, 17 and 60%. Cell G55A. (**C**) As (B), but in the presence of (+)-(S)-202-791. Cell G55A. Ensemble average current from three single channel patches, cells G55A, G96A and S02D. Open and closed time distributions were best fitted by the sum of two and three exponential components respectively, with time constants of 0.25 and 7.99 ms for the open times and 0.29, 2.26 and 12.2 ms for the closed times and relative areas of 39 and 61% for open times and 72, 20 and 8% for closed times. (**D**) Unitary current–voltage relations for the prevailing conductance levels under control conditions (open squares; continous line, slope conductance g = 19.4 pS; dashed line g = 20.5 pS) and in the presence of (+)-(S)-202-791 (closed squares; g = 24.7 pS; open circles: L-type channels with an inactivating gating pattern as in Figure 5). Unitary current values i are averages from three different patches (SEMs are smaller than the symbol size). For each patch, values of i at a given voltage are averages of many measurements on well-resolved openings. The trace in the inset shows the two prevailing current levels in the absence of (+)-(S)-202-791, during a rare period of mode 2 activity. Calibration bars, 0.5 pA, 40 ms.

Figure 3 shows that TRH stimulates the Ca^{2+} current of GH_3 cells by switching the activity of L-type channels from the low p_o mode prevailing under control conditions to the high p_o mode shown in Figure 2. In fact, the high p_o mode induced in cell-attached patches by bath addition of TRH had elementary current (i = 0.85 ± 0.001 pA at +20 mV from 45 well-resolved openings in four patches) and open probability (p_o = 0.394 ± 0.003 at +20 mV, n = 4 patches) not significantly different from those of the high p_o mode sometimes observed under control conditions (i = 0.83 ± 0.05 from 27 openings in nine patches; p_o = 0.366 ± 0.002, n = 7 patches). Amplitude and open and closed time histograms (cf. Figures 3B and 2B), as well as single channel conductance (Figure 2D), of the TRH-induced high p_o mode were all very similar to those of the sponteneous high p_o mode. Figure 3C shows that the activation curves for the control low p_o mode and for the high p_o mode after TRH can be fitted to Boltzmann distributions with similar $V_{1/2}$ but widely different $p_{o\,max}$. As a result of the TRH-induced switch to the high p_o mode, the average single channel current increased >600% (616 ± 26, n = 4 patches containing

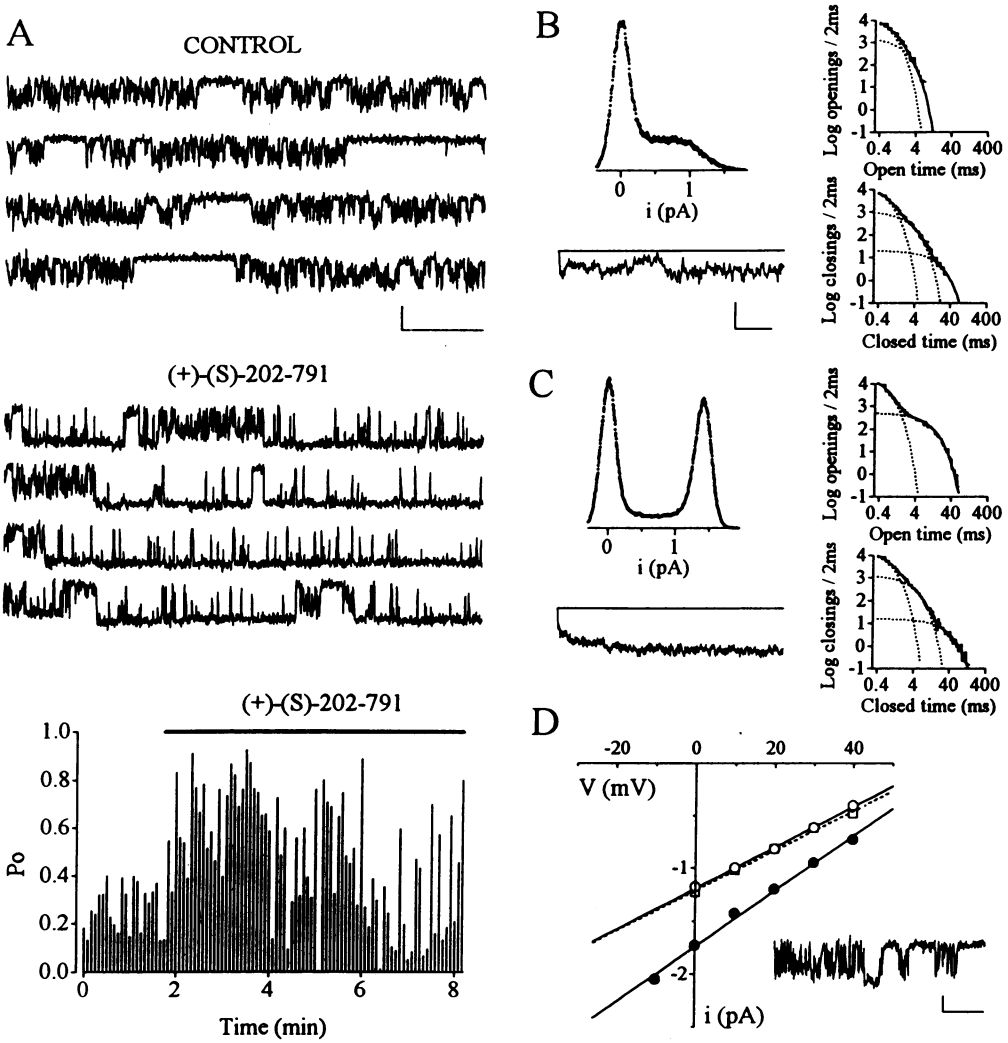

Fig. 2. Elementary properties of L-type Ca^{2+} channels with a high p_o gating pattern in GH_3 cells. Voltage protocol and conditions as in Figure 1, but here the L-type Ca^{2+} channel displayed a high p_o gating pattern before the addition of (+)-(S)-202-791. (**A**) Representative traces from cell-attached recordings and corresponding plot of p_o versus time. $V = +10$ mV. Holding potential -80 mV. Calibration bars, 1 pA, 80 ms. Cell G96E. (**B**) (Left) Normalized current amplitude histogram from all control traces with activity and average single channel current (cell G96E, $n = 16$; calibration, 0.4 pA, 80 ms). $V = +10$ mV. (Right) Log–log plots of the open and closed time distributions before addition of the DHP agonist. $V = +10$ mV. Open and closed time distributions were best fitted by the sum of two and three exponential components respectively, with time constants of 0.51 and 1.33 ms for the open times and 0.47, 2.48 and 14.4 ms for the closed times and relative areas of 26 and 74% for open times and 72, 25 and 3% for closed times. Cell G96E. (**C**) As (B), but in the presence of (+)-(S)-202-791. Cell G96E. Average current from 70 single channel traces. Open and closed time distributions were best fitted by the sum of two and three exponential components respectively, with time constants of 0.47 and 9.43 ms for the open times and 0.5, 2.48 and 15.0 ms for the closed times and relative areas of 72 and 28% for open times and 67, 28 and 5% for closed times. (**D**) Unitary current–voltage relations in the absence (open circles; $g = 19.1$ pS) and in the presence of (+)-(S)-202-791 (closed circles; $g = 26$ pS). Unitary current values are averages from six and three different patches in the absence and presence of (+)-(S)-202-791 respectively (SEMs are smaller than the symbol size). Open squares and dashed line, unitary current–voltage relation of the high p_o mode induced by TRH (cell G36B). The control trace in the inset shows a rare transition to a higher conductance state, similar to that in Figure 1D. Calibration bars, 0.5 pA, 40 ms.

1–3 channels). The effect of TRH was reversed upon washing, although not completely (cf. residual brief bursts of high p_o mode in traces in Figure 3) and not always. We observed a channel switching to the high p_o mode after addition of TRH to the bath in only five out of 22 cell-attached patches. On the other hand, we did not observe switching from low p_o to high p_o mode in 11 experiments in which TRH was present in the pipette but not added to the bath. A diffusible second messenger appears then to be involved in the modulation. In evaluating these statistics on the effectiveness of TRH in modulating single L-type Ca^{2+} channels in cell-attached patches, one should take into account the fact that even in whole cell experiments, stimulation of the Ca^{2+} current by TRH was seen in only 60% of cells on average. Moreover, the effect of TRH appeared to be labile and critically dependent on some unknown factors, since in many cell batches none of the cells were responsive to TRH. Cell-attached patches from these batches were not included in the statistics.

The overlapping openings in the representative TRH traces in Figure 3A (cf. the second and fourth traces) show that only one of the two Ca^{2+} channels in the cell-attached patch shifted to the high p_o mode upon bath addition of TRH. The selective modulation of only one of the channels in the patch was also observed in the

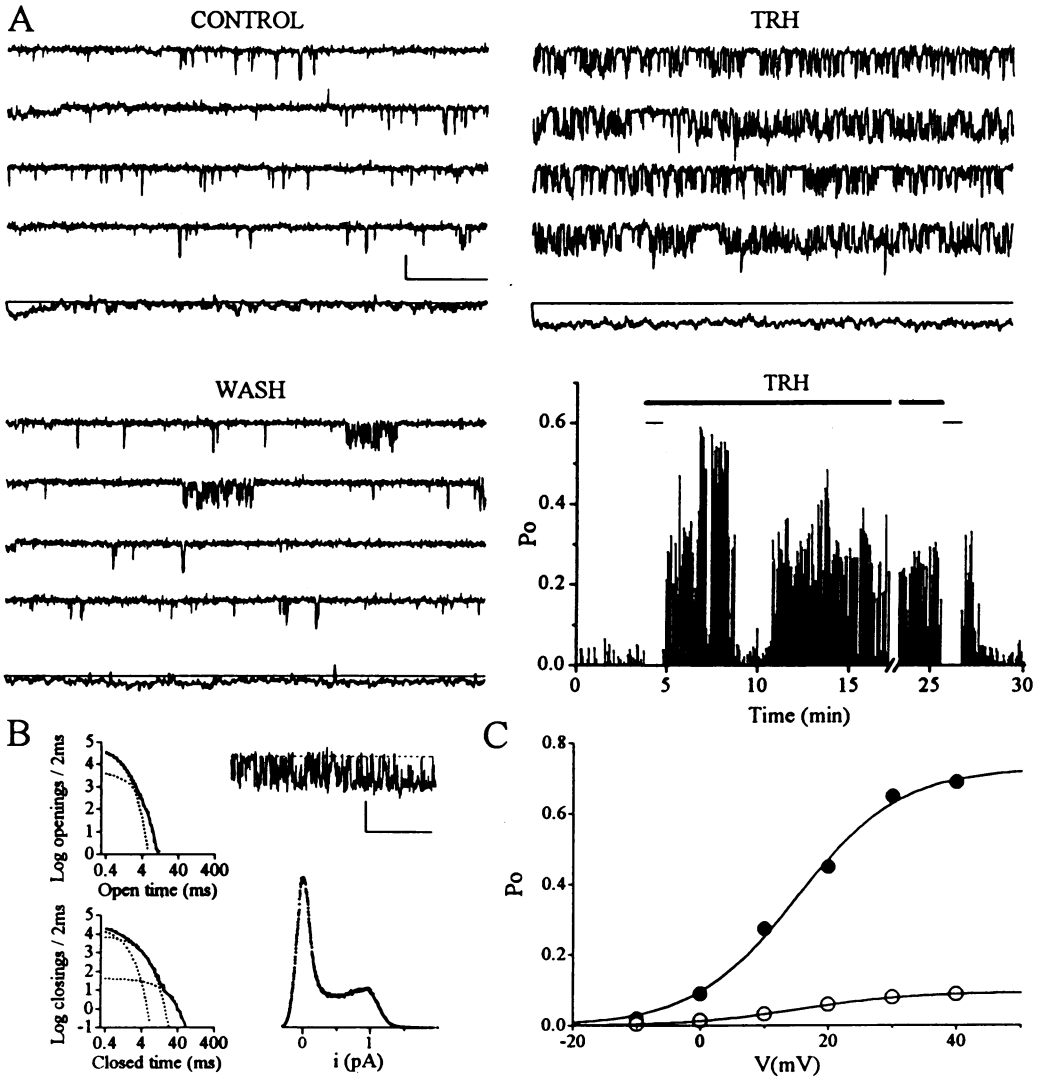

Fig. 3. Modulation by TRH of single L-type Ca^{2+} channels in GH_3 cells. (**A**) Cell-attached recordings from a patch containing two L-type channels (as judged from the overlapping events in the presence of TRH) and corresponding plot of p_o versus time. $V = +10$ mV. Holding potential -50 mV. Four representative current traces (calibration, 1 pA, 80 ms) and the corresponding ensemble average currents (calibration, 0.25 pA, 80 ms) before (control, $n = 31$) and after perfusion with 1 μM TRH in the bath solution (TRH, $n = 171$) are shown together with traces and average current after reperfusion with control solution (wash, $n = 38$). The average current after TRH was 7.2 times higher than the control average current. The patch also contained a T-type Ca^{2+} channel (cf. small amplitude openings at the beginning of the second trace) which accounts for the inactivating component in the control average current. Bars in the p_o versus time plot represent p_o at +10 mV in successive depolarizations. During the period not shown, the test potential was changed in the continuous presence of TRH to obtain current–voltage relation and activation curves. The thin horizontal lines mark the period of perfusion of the recording chamber, during which the additional noise hampered a meaningful calculation of p_o. Cell G36B. (**B**) (Left) Log–log plots of open and closed time distributions of the high p_o single channel activity induced by TRH. Open and closed time distributions were best fitted by the sum of two and three exponential components respectively, with time constants of 0.57 and 1.35 ms for the open times and 0.62, 2.09 and 11.7 ms for the closed times and relative areas of 85 and 15% for open times and 48, 51 and 1% for closed times. (Right) Normalized amplitude histogram at +10 mV of all traces with activity in the presence of TRH. Cell G36B. The trace shows a transition from the high p_o mode to a less frequent mode of gating with even higher p_o and longer open times. In the p_o versus time plot in (A), the cluster of depolarizations with average $p_o = 0.52 \pm 0.003$, clearly above the average $p_o = 0.281 \pm 0.004$ of the remaining depolarizations in the presence of TRH, denote a period of 'very high p_o' mode lasting more than 1 min. This very high p_o mode was also observed in some control patches. (**C**) Voltage dependence of the open probability of the high p_o mode induced by TRH (closed symbols, cell G36B) and of the control low p_o mode (open symbols, average of four different patches, SEMs smaller than the symbol size). For each patch, p_o values at a given voltage were obtained by averaging the open probabilities measured in each active sweep. The data points are fitted by Boltzmann distributions of the form $p_o = p_{o\,max} \times \{1 + \exp[-(V - V_{1/2}) \times zF/RT]\}^{-1}$, with $z = 3.19$, $V_{1/2} = 15.1$ mV, $p_{o\,max} = 0.73$ (closed symbols) and $z = 2.96$, $V_{1/2} = 15.5$ mV, $p_{o\,max} = 0.094$ (open symbols).

other patches with two or three channels where TRH was effective. Figure 4 shows additional representative traces displaying superimposed openings of a potentiated L-type Ca^{2+} channel in the high p_o mode and a non-potentiated Ca^{2+} channel remaining in the low p_o mode after addition of TRH. The non-potentiated Ca^{2+} channels were L-type Ca^{2+} channels, because on rare occasions they displayed the typical spontaneous long opening mode (mode 2) superimposed on the high p_o mode of the potentiated L-type channel (cf. the first and fourth traces in Figure 4). The conclusion that the Ca^{2+} channels differentially modulated by TRH in cell-attached patches are both L-type Ca^{2+} channels is also supported by the fact that, in agreement with previous whole cell experiments (Gollasch

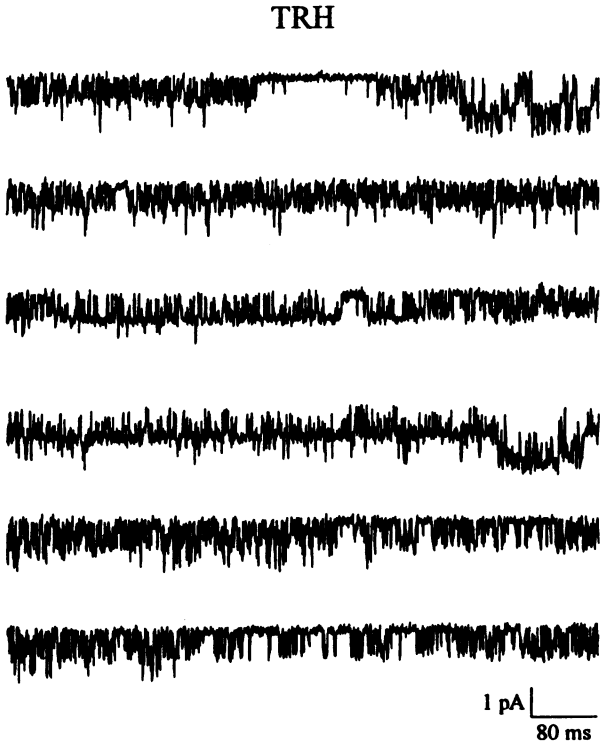

Fig. 4. Selective modulation of single L-type Ca^{2+} channels by TRH. Representative traces of cell-attached recordings from two different patches containing 2–3 L-type Ca^{2+} channels after perfusion with 1 μM TRH in the bath solution. $V = +20$ mV. Holding potential −50 mV. First three traces, cell G36B. Last three traces, cell G25B. As shown by the overlapping openings, in both patches only one of the L-type channels shifted to the high p_o mode after addition of TRH. Note the very high p_o gating mode in the third and fourth traces and the mode 2 openings of the non-potentiated L-type channel superimposed on the high p_o (first trace) and very high p_o (fourth trace) openings of the potentiated L-type channel.

et al., 1991), in our single channel recordings we did not find evidence for DHP-insensitive Ca^{2+} channels in GH_3 cells, except for the tiny, low threshold T-type Ca^{2+} channels (cf. Figure 3 legend and the second trace).

As a possible explanation for the observed selective modulation by TRH of single L-type Ca^{2+} channels, one can suggest that GH_3 cells co-express two different L-type Ca^{2+} channels, characterized by similar low p_o gating patterns under control conditions, only one of which can be potentiated by TRH. Some support for this explanation is provided by Figure 5. In many patches, the activity of L-type channels with control low p_o gating similar to that shown in Figure 1 changed, upon addition of the DHP agonist, into a quite different gating pattern, characterized by long closures between openings and an inactivating average current. The inactivating behaviour, together with the long closures between openings (cf. closed time histograms) account for the low open probability of the L-type channel in Figure 5 compared with that in Figure 1 (cf. amplitude histograms and p_o versus time plots). The two different gating patterns in Figures 1 and 5 have very similar unitary currents and conductances (Figure 1D, average conductance for L-type channels with inactivating behaviour $g = 24.8 \pm 0.5$ pS, $n = 7$). They are probably due to different L-type Ca^{2+} channel isoforms, because in nine recordings from single channel patches (lasting from at least 5 up to 35 min) we never observed interconversions between them.

The existence of two different subtypes of DHP-sensitive Ca^{2+} channels, only one of which can be modulated by TRH, would also help explain (i) the fact that the average single channel current was increased >600% by TRH, while the whole cell current was stimulated only ~40% (Gollasch *et al.*, 1991) and (ii) the limited number of experiments in which TRH was effective. Due to the lability of the TRH effect and the rarity of single channel patches, we were unable to establish with certainty which of the two L channel subtypes (which have very similar low p_o gating under control conditions) were potentiated by TRH. However the non-inactivating L-type Ca^{2+} channels shown in Figure 1 appear the most likely effectors of TRH stimulation, given the similarity of their activity in the presence of a DHP agonist with that of the high p_o mode after addition of a DHP agonist (cf. Figure 2).

Discussion

In this report we have shown the following. (i) GH_3 cells express L-type Ca^{2+} channels that can freely interconvert between two main modes of activity, one characterized by very low open probability and one characterized by a 10 times higher open probability and a slightly smaller conductance. Under basal conditions the equilibrium between the two gating modes is shifted towards the low p_o mode. TRH stimulates the Ca^{2+} current of GH_3 cells by switching the activity of single L-type Ca^{2+} channels from the low p_o gating mode to the high p_o gating mode. (ii) A diffusible second messenger is involved in the hormonal stimulation, because TRH potentiates single L-type Ca^{2+} channels in cell-attached patches when added to the bath, but not when present only in the pipette. (iii) TRH does not potentiate all the L-type Ca^{2+} channels contained in a given membrane patch. (iv) GH_3 cells co-express two functionally different L-type Ca^{2+} channels, which most probably correspond to structurally different channel isoforms.

The biophysical mechanism of hormonal stimulation of Ca^{2+} channels in pituitary cells uncovered by our data adds to the growing list of examples of neuromodulation of channel function through alteration of the relative frequency of occurrence of different gating modes (Yue *et al.*, 1990; Bonev and Isenberg, 1992; Tomasic *et al.*, 1992; Herzig *et al.*, 1993; Delcour and Tsien, 1993; Marrion, 1993; Ono and Fozzard, 1993; Wilson and Kaczmarek, 1993) and supports the view of gating modes as modulatory states of the channels (superimposed on the set of conformational states occupied during normal gating) due to basal activity of intracellular regulatory processes that can be stimulated by neurotransmitters or hormones (Bean, 1989; Delcour *et al.*, 1993; Rittenhouse and Hess, 1994). Restricting the discussion to voltage-dependent Ca^{2+} channels, the PKA-mediated enhancement of calcium influx following β-adrenergic stimulation in the heart has been shown to be mainly due to a redistribution of the relative proportions of modes of L-type channels, with selective reduction of modes 0 and 0a (in which the channels are either unavailable to open or open with very low p_o) and potentiation of two highly active modes (mode 1, bursts of brief openings, and mode 2, long lasting

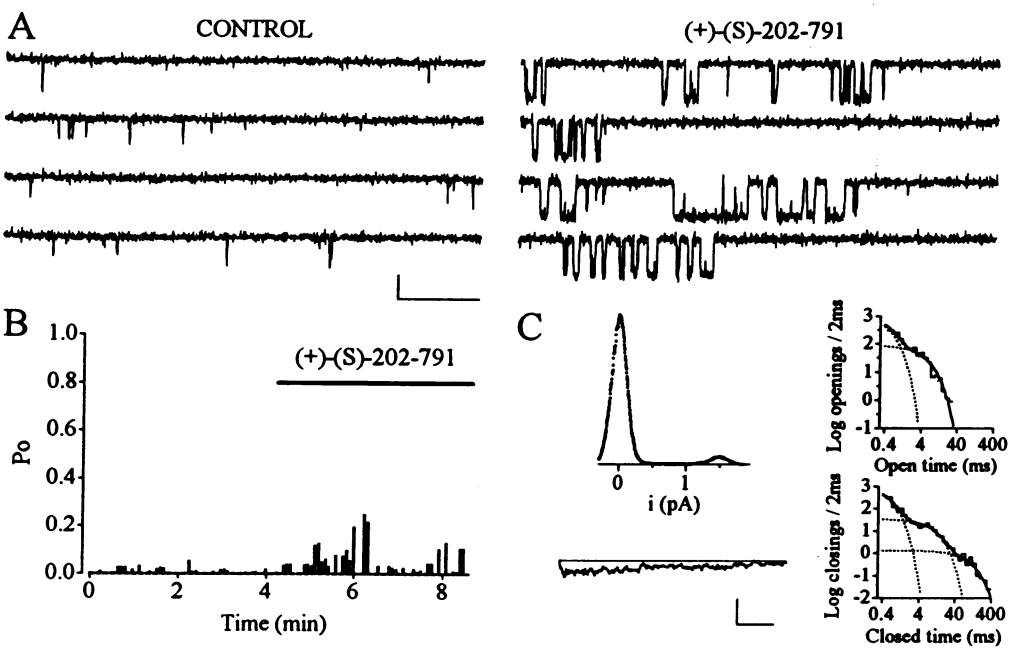

Fig. 5. Elementary properties of L-type Ca^{2+} channels with an inactivating gating pattern in GH_3 cells. Voltage protocol and conditions as in Figure 1. (**A**) Representative traces from cell-attached recordings. The patch contained a single L-type channel. $V = +10$ mV. Holding potential -80 mV. Calibration bars, 1 pA, 80 ms. Cell G94A. (**B**) p_o versus time plot for the same experiment (cell G94A). (**C**) Histograms and average current of L-type channels with gating similar to that in (A) in the presence of (+)-(S)-202-791 (1 μM). (Left) Normalized current amplitude histogram from all traces with activity (cell G94A) and average single channel current from three patches (cells G94A, G92B and G55C, $n = 93$; calibration, 0.4 pA, 80 ms). $V = +10$ mV. (Right) Log–log plots of open and closed time distributions. $V = +10$ mV. Open and closed time distributions were best fitted by the sum of two and three exponential components respectively, with time constants of 0.41 and 5.14 ms for the open times and 0.42, 8.51 and 85.9 ms for the closed times and relative areas of 52 and 48% for open times and 53, 34 and 13% for closed times. Cell G94A.

openings) (Ochi and Kawashima, 1990; Yue *et al.*, 1990; Herzig *et al.*, 1993; Ono and Fozzard, 1993). On the other hand, an alteration in the weighting of modes of N-type channels, with a selective reduction of a high p_o mode and enhancement of both low p_o and silent modes of activity, has been shown to underly the G protein-mediated inhibition of calcium influx by noradrenaline in sympathetic neurons (Delcour and Tsien, 1993).

Both analogies and differences can be found between the gating modes here described for L-type channels subject to modulation by TRH in GH_3 cells and the different gating modes previously described for L-type channels in cardiac and neuronal cells (Hess *et al.*, 1984; Pietrobon and Hess, 1990; Yue *et al.*, 1990; Forti and Pietrobon, 1993) and for N-type channels in neurons (Delcour and Tsien, 1993; Delcour *et al.*, 1993; Rittenhouse and Hess, 1994). The prevailing mode of activity of L-type channels in GH_3 cells, characterized by a very low p_o and very short openings, resembles mode 0a of cardiac L-type channels (Yue *et al.*, 1990), and also the low p_o mode prevailing in the presence of noradrenaline of N-type channels in frog sympathetic neurons (Delcour and Tsien, 1993). The high p_o gating mode stabilized by TRH has some similarities with mode 1 of cardiac L-type channels, but differs from it in having a conductance smaller than that of both the long opening mode induced by DHP agonists and the rare spontaneous mode 2. The recognition of different conductance states differentially stabilized by different modulatory agents is new for L-type Ca^{2+} channels. On the other hand, the observation that the low p_o mode of L-type channels in GH_3 cells displays a slightly higher unitary current than the high p_o modes stabilized by TRH is similar to what has been observed for the gating modes of N-type channels (Delcour *et al.*, 1993; Rittenhouse and Hess, 1994). In terms of alterations of modal gating, hormonal stimulation of L-type channels in pituitary cells approaches a mirror image of neurotransmitter inhibition of N-type channels in neuronal cells (Delcour and Tsien, 1993), which is known to be mediated by G proteins via a membrane delimited pathway (see Hille, 1992; Clapham, 1994, for reviews). Since the inhibitory modulation of N-type channels could not be obtained in cell-attached patches by bath addition of agonist, a direct interaction between G proteins and N-type Ca^{2+} channels has been suggested as the biochemical mechanism of the modulation (Forscher *et al.*, 1986; Lipscombe *et al.*, 1989; Bernheim *et al.*, 1991).

By using specific antisense oligonucleotides, the involvement of both G_{i2} (and to a minor extent G_{i3}) and $G_{q/11}$ in the stimulatory modulation of L-type channels in GH_3 cells has been clearly established (Gollasch *et al.*, 1993). It was proposed that PKC-mediated phosphorylation of either the channel or the G protein was necessary to allow the $G_{i2}-Ca^{2+}$ channel interaction and consequent channel stimulation (Gollasch *et al.*, 1993). Even though in this study we did not specifically address the biochemical mechanism of hormonal stimulation, our observation of modulation of L-type channels in cell-attached patches after bath addition of TRH (without TRH in the pipette) makes a direct interaction between G_{i2} and L-type Ca^{2+} channels an unlikely mechanism for channel stimulation. If one excludes the possibility of translocation of G_i proteins from the membrane to the cytosol after stimulation (however, cf. Spiegel, 1992), a direct interaction of G_{i2} with L-type channels would be compatible with our data only if one suppose that there is some tonic activation of

G_{i2} (or G_{i3}) in the absence of a receptor agonist and that switching of single L-type channels to high p_o gating modes, as a consequence of PKC-mediated phosphorylation induced by bath addition of TRH, can be observed only in cell-attached patches containing some tonically active G_{i2} (or G_{i3}). In this case, as suggested for G_s in the β-adrenergic modulation of cardiac L-type channels (Cavalie' *et al.*, 1991), G_{i2} might act by priming Ca^{2+} channels for phosphorylation. This interpretation would help explain the limited number of patches in which the modulation was observed. On the other hand, the hypothesis that would also be compatible with our data, of a G protein-mediated tonic inhibition of Ca^{2+} channels that is relieved as a consequence of PKC-mediated phosphorylation induced by bath addition of TRH, appears incompatible with the lack of increase in basal Ca^{2+} current density after selective inhibition of G_{i2} expression with antisense oligonucleotides and after PT pretreatment (Gollasch *et al.*, 1993 and unpublished data).

As an alternative to mechanisms based on direct G_i–Ca^{2+} channel interaction, our data are consistent with and appear to favour mechanisms based on G_{i2}-mediated production of some unknown second messenger, which may well be membrane-associated, given the numerous examples of modulation of ion channels by G_i proteins via membrane-delimited pathways (Brown and Birnbaumer, 1990; Clapham, 1994). In this respect, the ongoing debate on whether cardiac K^+ channels regulated by the muscarinic receptor are activated directly by $G_{\beta\gamma}$ or indirectly via $G_{\beta\gamma}$-mediated activation of phospholipase A_2 appears relevant (Clapham and Neer, 1993).

Another finding of this report is the evidence for co-expression in GH_3 cells of two functionally different L-type Ca^{2+} channels. The two L-type Ca^{2+} channels can be clearly distinguished in the presence of a DHP agonist on the basis of their different inactivation and gating properties, but they show very similar low p_o gating patterns under control conditions. The alternative interpretation of different modulatory states of the same L-type channel, giving rise to inactivating and non-inactivating behaviours, is unlikely (although not disproven) given the lack of interconversion between the inactivating and non-inactivating gating patterns in single channel experiments. Co-expression of two types of DHP-sensitive Ca^{2+} channels having similar conductances but different gating properties (especially of inactivation) has also recently been reported in rat cerebellar granule neurons (Forti and Pietrobon, 1993) and in rat pituitary melanotropic cells (Keja and Kits, 1994). Given the widespread co-expression of both class C and D α_1 subunits in many neurons and also in the pituitary gland (Snutch *et al.*, 1991; Chin *et al.*, 1992), the non-inactivating and inactivating L-type Ca^{2+} channels may correspond to the two different class C and D α_1 subunit genes or, alternatively, given the widely different inactivation rates obtained by co-expressing different β subunits with the same α_1 subunit (Hullin *et al.*, 1992; Ellinor *et al.*, 1993), they may be formed by different β subunits.

The unexpected and rather striking finding that in membrane patches containing two or three L-type Ca^{2+} channels with a low p_o gating pattern, usually only one of the L-type Ca^{2+} channels switched to the high p_o mode upon addition of TRH can be explained if TRH selectively potentiates only one of the two different subtypes of DHP-sensitive Ca^{2+} channels co-expressed in GH_3 cells. An alternative interpretation, that we cannot completely refute, is that for some reason not all the channels in the patch are accessible to whatever the modulating factor is. However, selective potentiation of only one subtype of the two functionally different L-type Ca^{2+} channels of GH_3 cells would also help explain the large quantitative discrepancy between the increase in average single channel current (>600%) and the stimulation of whole cell Ca^{2+} current (~40%) induced by TRH. It is also consistent with and may explain the low probability of observing stimulatory modulation of single L-type channels in cell-attached patches.

The existence of functionally different L-type Ca^{2+} channels, which may be differentially modulated, offers the interesting possibility that inhibition of the whole cell Ca^{2+} current due to TRH-induced Ca^{2+} release from stores (Gollasch *et al.*, 1991; Kramer *et al.*, 1991) may actually be due to Ca^{2+}-dependent inhibition of L-type channels different from those stimulated by TRH. Further studies are necessary to verify this hypothesis, as well as the other interesting hypothesis that the Ca^{2+} channel subtype stimulated by TRH may be preferentially localized at defined areas within the cell membrane, possibly close to the sites of vesicle release (Thomas *et al.*, 1990).

Materials and methods

Rat pituitary GH_3 cells were obtained from the American Type Culture Collection and cultured as described (Rosenthal *et al.*, 1988).

Single channel patch-clamp recordings followed standard techniques (Hamill *et al.*, 1981). All recordings were obtained in the cell-attached configuration. Single channel currents were recorded with a DAGAN 3900 patch-clamp amplifier, low-pass filtered at 1 kHz (−3 dB, 8-pole Bessel filter), sampled at 5 kHz and stored for later analysis on a PDP-11/73 computer. Depolarizations were 720 ms long and were delivered every 4 s. Linear leak and capacitative currents were digitally subtracted from all records used for analysis. A channel opening or closure was detected when more than one sampling point crossed a discriminator line at 50% of the elementary current. Histograms of open and closed times were fitted to sums of decaying exponentials. The best fit was determined by maximum likelihood maximization (Colquhoun and Sigworth, 1983) and the best minimum number of exponential components was determined by the maximum likelihood ratio test (Rao, 1973; Horn and Lange, 1983). Log binning and fitting of the binned distributions was done as described by McManus *et al.* (1987) and Sigworth and Sine (1987). In log–log plots of fitted open or closed time distributions, the presence of an exponential component is signalled by a bump in the distribution of the data. Open probability was computed by measuring the average current $<I>$ in a given single channel current record and dividing it by the unitary single channel current i. Current amplitude histograms were obtained from the data directly, with bin width equal to our maximal resolution (323.6 points/pA). For display, each histogram was normalized to the value of the zero current peak. Open channel current amplitudes for i–V were measured by manually fitting cursors to well-resolved channel openings. Values at each voltage are averages of many measurements.

The pipette solution contained 90 mM $BaCl_2$, 10 mM tetraethylammonium (TEA) chloride, 15 mM CsCl and 10 mM HEPES (adjusted to pH 7.4 with TEA hydroxide). The bath solution contained 140 mM potassium gluconate, 5 mM EGTA, 35 mM L-glucose and 10 mM HEPES (pH adjusted with KOH). The high potassium bath solution was used to zero the membrane potential outside the patch. The DHP agonist (+)-(S)-202-791 (a gift from Dr Hof, Sandoz Co., Basle, Switzerland) was added during experiments by gravity perfusion or added to the bath before recording. TRH was applied by bath perfusion or included in the pipette solution. Experiments were performed at room temperature (21–25°C).

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