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The role of the inwardly rectifying K^+ current in resting potential and thyrotropin-releasing-hormone-induced changes in cell excitability of GH_3 rat anterior pituitary cells

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Abstract. Exposure of GH_3 rat anterior pituitary cells to cholera toxin for 2–4 h significantly increased the thyrotropin-releasing-hormone(TRH)-induced inhibition of the inwardly rectifying K^+ current studied in patch-perforated voltage-clamped cells. On the other hand, the current reduction became almost totally irreversible after washout of the neuropeptide. Comparison of the effects elicited by the toxin with those of 8-(4-chlorophenylthio)-cAMP or forskolin plus isobutylmethylxanthine indicated that, although the irreversibility may be due, at least in part, to elevations of cAMP levels, the enhancement of the TRH-induced inhibition of the current is not mediated by the cyclic nucleotide. Only reductions on the inwardly rectifying K^+ current, but not those elicited by TRH on voltage-dependent Ca^{2+} currents, were increased by the treatment with cholera toxin. In current-clamped cells showing similar rates of firing, the second phase of enhanced action-potential frequency induced by TRH was also significantly potentiated by cholera toxin. Measurements of $[Ca^{2+}]_i$ oscillations associated with electrical activity, using video imaging with fura-2-loaded cells, demonstrated that cholera toxin treatment causes a clear reduction of spontaneous $[Ca^{2+}]_i$ oscillations. However, this did not prevent the stimulatory effect of TRH on oscillations due to the action potentials. In cholera-toxin-treated cells, the steady-state, voltage dependence of inactivation of the inward rectifier was shifted by nearly 20 mV to more negative values. These data suggest that the inwardly rectifying K^+ current plays an important role in maintenance of the resting K^+ conductance in GH_3 cells. Furthermore, the TRH-induced reductions on this current may be an important factor contributing to the increased cell excitability promoted by the neuropeptide.

Key words: Thyrotropin-releasing hormone – Inwardly rectifying K^+ current – GH_3 cells – Cholera toxin – Cell excitability – Anterior pituitary

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Introduction

Stimulation of prolactin release by thyrotropin-releasing hormone (TRH) in clonal rat anterior pituitary GH_3 cells is biphasic and linked to a similarly biphasic increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (for reviews see [4, 7, 13, 26]). TRH induces an acute enhancement of prolactin secretion followed by a plateau phase of sustained enhancement, which are primarily dependent on release of Ca^{2+} from the intracellular stores and on influx of extracellular Ca^{2+} respectively [13, 23, 33]. The initial phase of intracellular Ca^{2+} and secretion increase is accompanied by a transient hyperpolarization of the cell membrane due to activation of Ca^{2+}-dependent K^+ channels [9, 20, 23, 30]. It has also been established that this phase is due to production of intracellular mediators, which act by liberating Ca^{2+} from both inositol 1,4,5-triphosphate[Ins(1,4,5)P_3]-sensitive and Ins(1,4,5)P_3-insensitive intracellular compartments [23] (see also [11, 17]). This transient phase of TRH effects is followed by an enhanced generation of action potentials for an extended period, causing a sustained plateau of elevated $[Ca^{2+}]_i$ and secretion [1, 6, 13, 26, 33]. It has been shown that the TRH-induced increase in action potential production is at least partly caused by a decrease in the membrane K^+ conductance [26]. It has also been suggested that activation of protein kinase C is involved in the sustained effects of TRH [4, 7, 13, 26] including the modulation of the K^+ channel activity, which determines the firing rate [4, 7, 10, 12, 25, 26]. However, the exact conductance pathway(s) responsible for the resting potential and conductance changes underlying the increased frequency of action potentials and hence the mechanism for the TRH-induced facilitation still remain controversial. We have recently shown that an inwardly rectifying K^+ current [3], present at the resting potential and reduced by TRH, could play a major role in determining the firing rate of GH_3 cells and its enhancement by TRH [2, 6]. Our results also suggested that a phosphorylation/dephosphorylation mechanism is involved in regulation of this current and of action po-

tential frequency by the neuropeptide [2, 6]. An important role of inward-rectifier K^+ channels in the maintenance of cell resting membrane potential and in controlling cell excitability has been previously recognized [15, 19, 22]. However, the lack of specific inhibitors makes difficult the implication of the inwardly rectifying K^+ currents in a given cellular response. On the other hand, few examples of modulation of this current by physiological effectors are yet available. In this report, the effect of treatment of GH_3 cells with cholera toxin on the response of the inwardly rectifying K^+ current to TRH is explored. The parallel enhancement of TRH effects on current reduction and cell excitability emphasizes the important role of the inwardly rectifying K^+ current in resting potential and firing rate changes induced by TRH. Furthermore, the possibility that cAMP-independent enhanced activation of a protein kinase is the reason of the increased TRH effects following treatment of the cells with cholera toxin is discussed.

Materials and methods

Nystatin, TRH, cholera toxin, forskolin, 8-(4-chlorophenylthio)-cAMP (ClSPh-cAMP), isobutylmethylxanthine and caffeine were purchased from Sigma. Fura-2/AM and pluronic F-127 were purchased from Molecular Probes (Oregon, USA). For electrophysiological experiments, GH_3 cells (ATCC-CCL 82.1) were plated in 35-mm tissue-culture plastic dishes containing Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DME/F12 1:1 mixture, Sigma) supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Maintenance of the cells and the conditions for perforated-patch recordings have been previously described in detail [1, 2, 6]. Unless otherwise stated, perforation of the patches with nystatin was achieved under voltage-clamp mode, setting the pipette voltage at a value of -50 to -70 mV in the patch-clamp amplifier. Access resistance values analogous to those previously obtained under current clamp (i. e. 20–50 MΩ, see [2]) were reached after 5–30 min of perforation. Treatments with cholera toxin were performed by addition of 1 μg/ml toxin directly to tissue-culture dishes from an aqueous stock containing 1 mg/ml. Incubation was continued for the indicated times at 37°C in the humidified atmosphere of 95% air and 5% CO_2 used to grow the cells. Electrical activity and ionic current recordings were made at room temperature (20–25°C) in a recording chamber of 0.2–0.3 ml. Seals and subsequent perforation of the patches were performed in a standard extracellular-like solution containing, (in mM) 140 NaCl, 1 KCl, 1 $MgCl_2$, 10 glucose, 10 $CaCl_2$ and 10 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) titrated to pH 7.4 with NaOH. This solution was used in experiments in which the electrical activity of the cells was monitored under current clamp. The rate of firing of the individual cells was set to a similar basal value by injection of a constant current, which was subsequently maintained all along the course of the experiment. The standard extracellular-like solution was also used to monitor voltage-dependent Ca^{2+} currents under voltage clamp. Recordings of inwardly rectifying K^+ currents were performed in voltage-clamped cells after changing the extracellular medium to high-K^+ Ca^{2+}-free solution once permeabilization of the patches had been completed. This solution contained (in mM) 140 KCl, 4 $MgCl_2$, 10 [ethylenebis(oxonitrilo)]tetraacetic acid (EGTA) and 10 HEPES titrated to pH 7.4 with KOH. The tip of the pipette was initially filled with nystatin-free solution containing (in mM) 65 KCl, 30 K_2SO_4, 10 NaCl, 1 $MgCl_2$, 50 sucrose and 20 HEPES titrated to pH 7.4 with KOH. The remainder of the pipette was back-filled with the same solution also containing 0.25 mg/ml nystatin. In experiments designed to study Ca^{2+} currents, Cs^+ replaced K^+ in the pipettes. The plotted current-clamp records were obtained at a sampling rate of 10 ms/point. Such a low rate imposed a considerable filtering effect on recordings, but was very adequate to monitor changes of action potential frequency over extended periods of time. The action potential frequency was determined as previously described [1, 6]. Records of Ca^{2+} currents, filtered at 1 kHz, were sampled every 0.2 ms for plotting. Leak and capacitive currents were subtracted on-line using a P/4 procedure. Inwardly rectifying K^+ current records were sampled every 0.5 ms and digitally filtered at 500 Hz. Data are shown without correction for leakage. Uncompensated capacitive transients were subtracted as described [2]. Test solutions were applied by continuous perfusion of the chamber at 1 ml/min. Drugs added to standard Na^+- and Ca^{2+}-containing medium for long-term treatments were also maintained in high-K^+ Ca^{2+}-free solutions until entry of TRH. Experiments performed with identical stimulation rates and along the same time courses are averaged in the graphs.

For single-cell fluorescence measurements, GH_3 cells were allowed to attach to poly-L-lysine-coated glass coverslips and grown for 24 h. Then the coverslips were transferred to wells containing 1 ml medium of the following composition (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES titrated to pH 7.4 with NaOH. Fura-2/AM (5 μM), horse serum (0.1%) and pluronic F-127 (0.01%) were added from concentrated stocks and the coverslips were incubated for about 60 min at 30°C. The coverslips were then mounted under the microscope (Nikon, Diaphot) in a chamber thermostatically controlled at 36°C and epilluminated alternately at 340 nm and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, UK) and analysed using a Joyce-Loebl Magical image processor (Newcastle, UK) with 32 Mbyte video RAM. Four video frames of each wavelength were averaged by computer, with an overall time resolution of 3 s for each pair of images at alternate wavelengths. Consecutive frames obtained at 340 nm and 380 nm excitation were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated from F_{340}/F_{380} by comparison with fura-2 standards [14, 32]. Data are expressed as means ± SEM together with the number of cells (n).

Results

The inwardly rectifying K^+ currents present in GH_3 cells can be measured in isolation using nearly symmetrical K^+ solutions free of Na^+ and Ca^{2+} [2, 3]. Figure 1 compares the effect of TRH on inwardly rectifying K^+ currents in patch-perforated voltage-clamped GH_3 cells which had or had not been previously incubated with cholera toxin. As shown in the lower part of the figure, hyperpolarization of the membrane to -100 mV elicited inward currents showing an initial maximum, followed by a period in which they inactivated to reach a steady state. The magnitude of the specific inwardly rectifying K^+ current was estimated from the difference between peak and steady-state currents once complete inactivation had been achieved. The hyperpolarization pulse was preceded by a 100-ms voltage ramp from 0 to -50 mV, which yielded an estimation of the membrane conductance within this voltage range (see also [2]). Furthermore, a transient inward tail current was obtained after return to the holding potential, because of activation of the transient K^+ current present in these cells, which could be used as an additional control for specificity of the effects observed on the inward rectifier. In the absence of cholera toxin treatment, the perfusion of the cells for about 1 min with a solution containing 100

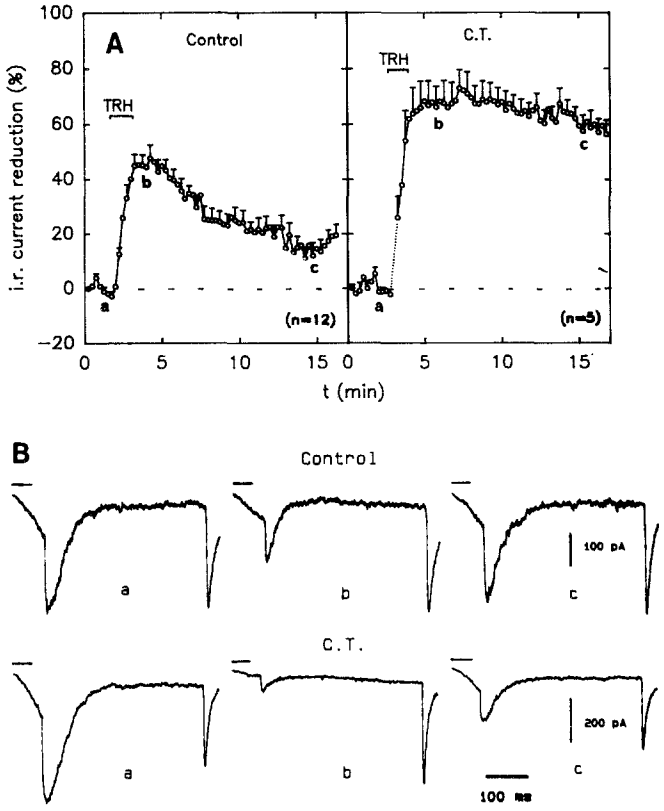

Fig. 1A, B. Effect of treatment with cholera toxin (*C.T.*) in modulation of inwardly rectifying K^+ current by thyrotropin-releasing hormone (*TRH*). **A** Time course of the TRH effects on GH_3 control cells (*left*) and on cells treated with 1 μg/ml cholera toxin for 2–4 h (*right*). The magnitude of the currents was obtained from the difference between peak and steady-state currents during maintained hyperpolarizations at −100 mV. Holding potential −20 mV. TRH was used at a concentration of 100 nM. The 500-ms hyperpolarization pulses to −100 mV were preceded by a 100-ms voltage ramp from 0 to −50 mV. Perfusion of the cells with TRH is indicated by *horizontal lines*. The number of averaged experiments is shown in parentheses. Only alternate error bars are represented for clarity. The *dotted line* between data points corresponds to times at which membrane conductance increases during phase I of TRH action [2] precluded an accurate estimation of inwardly rectifying K^+ currents. **B** Representative current traces obtained at the times marked *a–c* in **A** for both control untreated (*top*) and toxin-treated cells (*bottom*). Zero current level is indicated by a *horizontal line* on top of the traces

nM TRH produced a reduction of the inwardly rectifying K^+ current (Fig. 1, control; see also [2]). The magnitude of this reduction reached a maximum of 49.0 ± 2.6% (n = 13) at the −20-mV holding potential used in these experiments. This effect of TRH was gradually reversed upon washout of the hormone (12/13 cells). It is important to emphasize that the TRH-induced inhibition was only reversed after withdrawal of the hormone from the chamber. Thus, no recovery of current inhibition was detectable during 5-min applications of TRH, even in cells in which the inhibition was reversed more than 60% after 10 min in hormone-free medium (3 cells). This indicates that, although the inhibition lasted longer than the duration of the TRH application, the continuous presence of the neuropeptide was required for full maintenance of the inhibition over prolonged periods of time.

Figure 1 B shows that treatment of GH_3 cells with 1 μg/ml cholera toxin for 2–4 h prior to TRH addition significantly increased the inhibition of the inwardly rectifying K^+ current up to 62.8 ± 4.8% (n = 11; $P < 0.015$, Student's t-test). In 9 additional cells no clear effect of TRH was apparent. On the other hand, the reduction became almost totally irreversible after washout of the neuropeptide. Thus, in 10 of the 11 cells showing response to TRH, less than 20% of the inhibition was reversed after 10 min of washout. As shown in the lower part of the figure, the time course of inactivation in toxin-treated cells was well fitted by a single exponential with a time constant of 118 ± 13 ms (n = 13). Although in some individual cells the time constant was about half this value, the averaged value was not significantly different from that previously obtained in control non-treated cells [2]. It is also interesting to note that the magnitude of the inwardly rectifying K^+ current before TRH was added was not affected by the treatment with cholera toxin [290 ± 27 pA (n = 22) in control versus 359 ± 35 pA (n = 20) in toxin-treated cells; P = 0.12, Student's t-test].

The ability of cholera toxin to ADP-ribosylate the α subunit of G_s (the GTP-binding protein that stimulates adenylyl cyclase) and to increase intracellular cAMP levels is well known [24]. To examine the possibility that elevation of intracellular cAMP levels is the cause of the enhanced TRH-induced inhibition, the cells were exposed to either Cl-SPh-cAMP (a cAMP analogue with 100 times the potency of cAMP to stimulate protein kinase A), or forskolin plus isobutylmethylxanthine (a potent activator of adenylyl cyclase and an inhibitor of cAMP phosphodiesterase respectively). None of these treatments had any effect on inwardly rectifying K^+ currents when the drugs were added to the extracellular medium for 10–15 min. Treatment of the cells with 0.3 mM ClSPh-cAMP for 5 min did not modify the inhibition caused by 100 nM TRH (43.0 ± 9.0%, n = 3; see also [2]). In this case, the currents recovered from the TRH-induced reduction upon washout of the neuropeptide. The percentage inhibition caused by TRH also remained largely unaltered after treatment of the cells for 15–30 min with 2 μM forskolin plus 5 μM isobutylmethylxanthine (46 ± 7%, n = 7). However, this treatment made the inhibition almost totally irreversible upon TRH washout in 6 of the 7 cells studied (Fig. 2 A). As shown in Fig. 2 B, similar results were obtained when the cells were exposed to 0.3 mM ClSph-cAMP for 30–60 min. In this case, the reduction elicited by TRH averaged 36 ± 4% (n = 5) and it was not reversed after washout of the neuropeptide for 10 min. It is important to note that these longer treatments were performed by adding the drugs for the indicated times to the standard Na^+- and Ca^{2+}-containing medium.

Maintenance of the cells for such long times in the high-K^+ EGTA-containing solution prevented the TRH-induced inhibitory response. Thus, only 2 out of 15 cells showed a detectable response when the neuropeptide was added 10–15 min after introduction of the high-K^+ Ca^{2+}-free medium. This contrasts with nearly 70% of the cells showing a response to TRH when the neuropep-

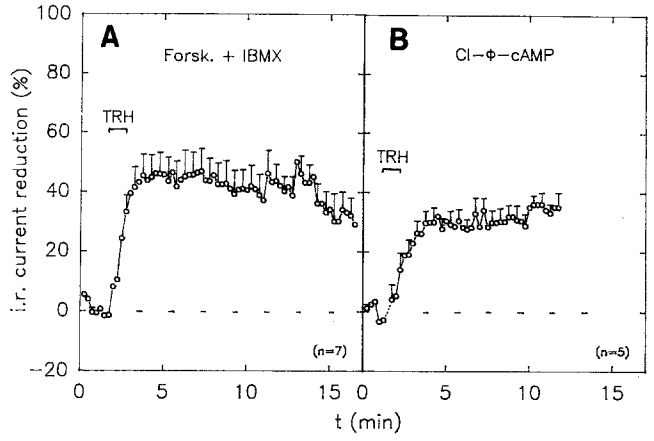

Fig. 2A, B. Effect of treatments that elevate intracellular cAMP levels in modulation of inwardly rectifying K^+ currents by TRH. **A** Time course of the TRH effect on GH_3, cells treated 15−30 min with 2 μM forskolin plus 5 μM isobutylmethylxanthine. **B** Time course of the TRH effect on cells treated 30−60 min with 0.3 mM ClSPh-cAMP. Holding potential −20 mV. Measurements of inwardly rectifying K^+ currents in patch-perforated cells were performed as indicated in the legend of Fig. 1. Addition of 100 nM TRH is indicated by *horizontal lines*

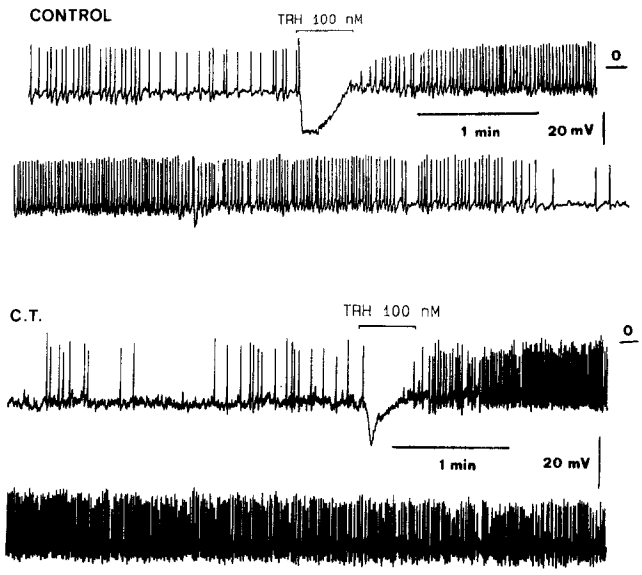

Fig. 3. Effect of treatment with cholera toxin (*C.T.*) on TRH-evoked increases in action potential frequency. 100 nM TRH was applied as shown to two representative patch-perforated current-clamped GH_3 cells either untreated (*top*) or treated with 1 μg/ml toxin for 2 h (*bottom*). The two lanes of the traces represent a continuous recording obtained at a sampling rate of 10 ms/point. 0 mV is represented by *horizontal lines* on the *right*

tide was introduced 2−5 min after start of the perfusion with the EGTA-containing medium. Perhaps a prolonged incubation in this solution causes a severe depletion of intracellular Ca^{2+}, which prevents the TRH effect. The failure of cAMP elevations to reproduce the whole effect of cholera toxin is not due to differences in the incubation conditions. Thus, the TRH-evoked inhibition averaged 42.5 ± 5.4% ($n = 6$) when ClSPh-cAMP was directly added to tissue-culture dishes and incubation was continued for 3−6 h at 37°C in the humidified atmosphere of 95% air and 5% CO_2 used to grow the cells. Furthermore, the TRH effect was not reversed upon washout of the hormone for 10 min (not shown). It is important to indicate that in these experiments the permeable cAMP analogue was also included in the extracellular saline solutions before addition of TRH to the recording chamber. This excludes the possibility that any difference between cholera-toxin-treated and ClSPh-cAMP-loaded cells is due to leakage of the cAMP analogue out of the cells during the perforation period. These data suggest that the irreversibility observed in toxin-treated cells may be due, at least in part, to elevations of intracellular cAMP levels. However, they also indicate that in toxin-treated cells the enhancement of the TRH-induced inhibition of the inwardly rectifying K^+ current is not mediated by the cyclic nucleotide.

It has been previously suggested that the inwardly rectifying K^+ current could play a major role in determining the firing rate of GH_3 cells and its enhancement by TRH [2, 3, 6]. On the other hand, we have recently shown the ability of the perforated-patch configuration of the patch-clamp method to preserve fully the electrical response of GH_3 cells to neuropeptides under current clamp [1, 6]. Subsequently experiments were performed in order to check whether the cholera-toxin-induced increased inhibition of the inwardly rectifying K^+ current was paralleled by an enhanced effect of TRH on action potential production along the second phase of increased firing. Figure 3A shows the typical biphasic response to TRH, consisting of a transient hyperpolarization after a delay of several seconds, followed by a second phase of sustained increase in action potential frequency for several minutes. As shown in Fig. 3B, the enhancement of electrical activity during the second phase of TRH action was considerably potentiated by pretreatment with cholera toxin for 2 h. Under these conditions, the increase in action potential frequency averaged 346 ± 62% ($n = 5$) at 100 nM TRH in cells showing a basal rate of firing of 0.25 ± 0.04 Hz before addition of the neuropeptide. This value is significantly higher ($P < 0.001$; Student's t-test) than that obtained in control cells not treated with the toxin (163 ± 11%, $n = 28$; see also [1, 6] showing similar basal rates of firing (0.22 ± 0.02 Hz).

Besides reductions on the inwardly rectifying K^+ current, TRH-induced inhibitions in Ca^{2+}-dependent K^+ currents have been implicated in enhanced excitability during the second phase of TRH action [18, 31]. In a previous study, we proposed that inhibition of the Ca^{2+}-dependent K^+ currents by TRH was independent of alterations in voltage-dependent Ca^{2+} currents, since the Ca^{2+} currents remained unaffected after addition of TRH [1]. Subsequent work from different laboratories indicated that, under apparently similar conditions, the L-type Ca^{2+} current of GH_3 cells was reduced by TRH, leading to reduced activation of Ca^{2+}-dependent K^+ channels [18, 31]. We have found that, when Cs^+-containing electrodes and perforated patches are used to monitor the effect of TRH on Ca^{2+} currents, the detection of the TRH-induced reductions strongly depends on the perforation conditions. In our initial studies [1] we systematically performed the perforations in current-

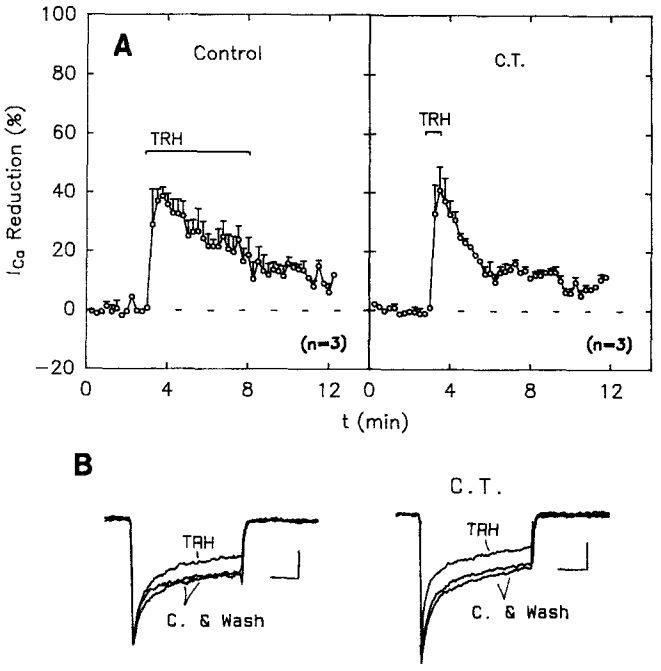

Fig. 4A, B. Effect of treatment with cholera toxin (*C.T.*) in modulation of voltage-dependent Ca^{2+} currents by TRH. **A** Time course of the TRH effect on the L-type Ca^{2+} current component of untreated cells (*left*) and cells treated with 1 μg/ml cholera toxin for 3 h (*right*). The magnitude of the currents was measured in patch-perforated cells at the end of 100-ms depolarization pulses to 0 mV from the holding potential of −50 mV. TRH was used at a concentration of 100 nM. Perfusion of the cells with the neuropeptide is indicated by *horizontal lines*. **B** Representative current traces obtained from two individual cells before (*C.*), 1 min after start of TRH application (*TRH*) and after washout of the neuropeptide (*Wash*). The traces on the *left* correspond to a control untreated cell. Current traces from a cell treated with 1 μg/ml cholera toxin for 2 h are represented on the *right*. *Calibration bars: vertical,* 100 pA; *horizontal,* 25 ms

clamp mode, only occasionally switching to voltage-clamp mode to follow the progress of capacitive transients. In this case, the perforations were made in voltage-clamp mode at −50 to −70 mV (see Materials and methods). As shown in Fig. 4, once the perforation has been completed under these conditions, the magnitude of the Ca^{2+} currents measured at the end of a 100-ms depolarization from −50 to 0 mV is clearly reduced by 100 nM TRH. Maintenance of the cells at a holding potential of −50 mV and quantification of the currents after 100 ms of depolarization guarantees that only the L component of the Ca^{+} current is measured. The magnitude of the reduction reached a maximum of 36 ± 2% (n = 9). On the other hand, the inhibition was gradually reversed even although the neuropeptide was maintained in the medium for periods of up to 5 min. Addition of 10 mM caffeine after recovery of the TRH-induced inhibition caused a similar reduction (mean reduction 29 ± 3%, n = 5). However, the caffeine-induced inhibition lasted for the duration of caffeine application (up to 5 min) and was rapidly and fully reversible upon washout (not shown). Among previous results [18], this indicates that reductions of voltage-dependent Ca^{2+} currents are indirectly caused by elevation of intracellular Ca^{2+} levels. This also opened the possibility that the failure to detect a consistent response to TRH after perforation under current-clamp mode [1] was caused by accumulation of Ca^{2+} along the perforation period, resulting from the depolarization originated by replacement of intracellular K^{+} with Cs^{+}. Our interpretations are further supported by the following observations. (a) When cells in which patches have been perforated at −70 mV under voltage clamp were maintained for 1−3 min at −10 or 0 mV, the magnitude of the L-type Ca^{2+} currents elicited by depolarization pulses from −50 to 0 mV averaged 68 ± 10.8 pA (n = 9). This value was similar to that obtained in cells of similar size with patches permeabilized under current-clamp mode (122 ± 24.8 pA, n = 20). On the other hand, it was significantly lower than the 287 ± 39.1 pA (n = 19; $P < 0.001$, Student's t-test) obtained by measuring the current immediately after the perforation had been completed at −70 mV under voltage clamp. (b) Addition of 100 nM TRH 2−3 min after the cells had been kept depolarized, as above, did not cause any clear inhibition of the Ca^{2+} current. However, the inhibitory response returned when the cells had again been held for a long time at −70 mV. (c) Reduction of Ca^{2+}-dependent K^{+} currents subsequent to inhibition of L-type Ca^{2+} channels could normally be observed if perforations were achieved in current-clamp mode with K^{+}-containing electrodes [1]. These data indicate that, after perforation under current-clamp mode with Cs^{+}-containing electrodes, the entry of external Ca^{2+} during the perforation period would limit or abolish for quite a long time the possibility of detecting any consistent inhibition of the Ca^{2+} current by TRH.

As shown in Fig. 4B, the TRH-induced reduction of the Ca^{2+} current was not altered by treatment of the cells with cholera toxin. Thus, in cells treated for 2−3 h with the toxin, addition of 100 nM TRH caused maximal reductions of 40 ± 8% (n = 3). This value was 28 ± 3% (n = 3) when 10 mM caffeine was used. This demonstrates that, regardless of the mechanism(s) leading to inhibition of the Ca^{2+} current, the potentiation of the TRH-induced inhibition after the cells had been treated with cholera toxin is specifically exerted on inwardly rectifying K^{+} currents.

The aforementioned results clearly disagree with a recent report in which treatment of GH_3/B6 cells with cholera toxin prevented the inhibition of the inwardly rectifying K^{+} current by TRH and the concomitant production of the second phase of increased electrical activity [3a]. In order to validate further the results obtained in electrophysiological experiments, the oscillatory behaviour of intracellular Ca^{2+} levels was explored by video imaging in fura-2-loaded GH_3 cells [5, 23, 32, 33]. Figure 5A, B shows the behaviour of 2 individual cells both before and after the addition of TRH. Before the neuropeptide was added, most of the cells showed spontaneous oscillatory activity of $[Ca^{2+}]_i$, although the frequency and extent of the oscillations were rather variable among individual cells. In the presence of 100 nM TRH, two major effects were observed. (a) There was a fast and transient increase of $[Ca^{2+}]_i$, which peaked

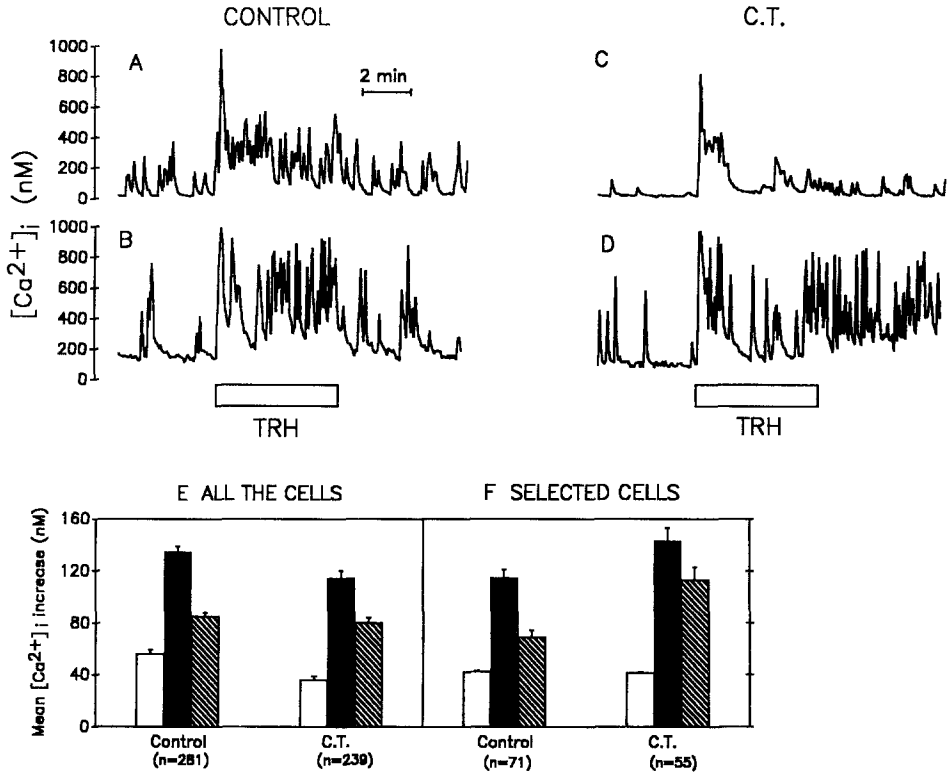

Fig. 5A–F. Effect of pretreatment with cholera toxin (*C.T.*) on the TRH-induced $[Ca^{2+}]_i$ oscillations. Cells were treated with 1 μg/ml toxin for 2–4 h and then $[Ca^{2+}]_i$ was measured as described in Materials and methods. **A–D** Representative traces of individual cells, either control (**A, B**) or toxin-treated (**C, D**). Cells were perfused with 100 nM TRH during the period shown by the *bar*. **E, F** Average values of the $[Ca^{2+}]_i$ oscillations in control and toxin-treated cells. The mean $[Ca^{2+}]_i$ increases (see text for explanation) observed within the 2-min periods before TRH, 3 min after TRH addition and 3 min after TRH removal (*Recovery*) are shown. **E** All the studied cells were included in the analysis. **F** Only the cells with a given level of spontaneous oscillations were included (mean $[Ca^{2+}]_i$ increase ranging between 30 nM and 60 nM; see text for details). The activity during the *TRH* and *Recovery* periods was significantly bigger in toxin-treated cells ($P < 0.02$ and $P < 0.001$ respectively; two-tailed Student *t*-test); before TRH (□), TRH (■), recovery (▨)

within 5–10 s. This increase is also observed in Ca^{2+}-free medium and is due to Ca^{2+} release from intracellular stores, corresponding to phase I of the TRH action. (b) There was also a period of increased frequency and amplitude of $[Ca^{2+}]_i$ oscillations, which persisted for several minutes (phase II). Both the spontaneous oscillations before TRH and the oscillations during phase II were dependent on extracellular Ca^{2+} and largely prevented in the presence of 100 nM nimodipine. Thus, the Ca^{2+} oscillations must reflect Ca^{2+} entry through voltage-dependent Ca^{2+} channels (mainly L-type channels) followed by Ca^{2+} removal from the cytoplasm. It is clear that the extent of the Ca^{2+} oscillations and electrical activity must be correlated, although it is not possible to assess the firing frequency of the cells from these data since a single Ca^{2+} peak could reflect a burst of action potentials rather than a single one. However, it is also clear that such a burst or an increase in action potential frequency and/or duration is likely to produce larger and/or longer Ca^{2+} transients. Although the control of channel functions has been studied only indirectly in this case, the use of less invasive techniques makes these data especially relevant physiologically [5].

In order to quantify the behaviour of the cells in terms of Ca^{2+} oscillations, we calculated the mean $[Ca^{2+}]_i$ increase over the resting level. This parameter was computed as the average of all the $[Ca^{2+}]_i$ values minus the "basal" value, arbitrarily chosen as the lowest $[Ca^{2+}]_i$ value of the whole trace. It then represented the mean area under the $[Ca^{2+}]_i$ peaks. Integration periods of 2 min either just before or 3 min after TRH addition were compared. Addition of 100 nM TRH to control cells untreated by cholera toxin increased the mean $[Ca^{2+}]_i$ rise in most of the cells studied. Thus whereas, the value of this parameter prior to TRH addition was 56 ± 3 nM (n = 281), it increased to 135 ± 4 nM in the presence of the neuropeptide (Fig. 5 E). It is interesting to note that a positive correlation was found between the values observed before and after TRH, i. e. the values obtained after TRH addition were larger in the cells showing higher spontaneous activity. This suggests that TRH modulates a pre-existent activity rather than inducing a new one. On the other hand, the relative increase was bigger in cells showing smaller oscillations at rest.

Treatment of the cells with 1 μg/ml cholera toxin for 3 h had little or no effect on the TRH-induced Ca^{2+} release from the intracellular stores (phase I) according to the magnitude and duration of the initial Ca^{2+} transient (Fig. 5 C, D). This suggests that toxin treatment does not interfere with either inositol phosphate production or Ca^{2+} pumping/releasing mechanisms from the stores. In contrast, the spontaneous oscillatory activity was usually reduced by the toxin, a large fraction of the cell population showing little or no spontaneous activity (see Fig. 5 C, representative of the behaviour of most of the cells). The mean $[Ca^{2+}]_i$ increase before TRH was reduced to 36 ± 3 nM (n = 239, Fig. 5 E). In spite of this clear decrease in spontaneous activity, the increase in Ca^{2+} oscillations induced by TRH was not prevented by cholera toxin (Fig. 5 C). Thus, the mean $[Ca^{2+}]_i$ increase was raised to 115 ± 5 nM (Fig. 5 E). It is interesting to indicate that the TRH-evoked increases in oscillatory activity were not restricted to individual cells showing little or no basal activity (Fig. 5 C), but also appeared in

a minor fraction of cells in which the initial level of oscillation was relatively high (Fig. 5 D).

Direct comparison of the relative increase of oscillatory activity induced by TRH in control and toxin-treated cells is complicated by the decrease of the spontaneous activity induced by the toxin. Cells showing no spontaneous activity would show apparently near infinite relative activity increases. In order to overcome this problem, we selected for comparison subgroups of cells showing the same spontaneous activity (namely, mean $[Ca^{2+}]_i$ increases ranging between 30 nM and 60 nM) within the control and the toxin-treated groups. As shown in Fig. 5 F, the oscillations of subgroups with the same basal activity were significantly higher in the toxin-treated group after addition of TRH (143 $\pm$ 10 nM, $n = 55$ versus 115 $\pm$ 6 nM, $n = 71$). This indicates that pretreatment with the toxin potentiates the effect of TRH.

The data of Fig. 5 F allow further comparisons. We have noticed that, in cholera-toxin-treated cells showing spontaneous activity, the TRH-induced stimulation of Ca^{2+} activity tended to persist more than in controls once TRH was washed out (compare traces A and B with trace D in Fig. 5). When the activity remaining 3 min after TRH was washed out was compared in subgroups of cells with similar spontaneous activity ("Recovery" in Fig. 5 E), it became clear that it was significantly larger in toxin-treated cells (113 $\pm$ 10 nM versus 69 $\pm$ 5 nM). Thus, these results suggest that pretreatment with cholera toxin reinforces the effects of TRH both in amplitude and duration.

As shown above, the extent of the TRH-induced enhancement on electrical activity shows a positive correlation with the size of the hormone-evoked inhibition of inwardly rectifying K^+ currents. Such a comparison can be easily performed when the basal rate of firing is set to similar initial values under current clamp. However, neither ionic gradients nor membrane potential values can be accurately controlled using less invasive techniques as those employed in the video-imaging experiments. Since the reduction of the oscillatory activity in toxin-treated cells very probably reflects a concomitant decrease in the spontaneous electrical activity of the cells, the assumption that the inwardly rectifying K^+ channels could play an important role in maintenance of resting membrane K^+ conductance, and hence in electrical behaviour, prompted us to check the possible effect of treatment with cholera toxin on kinetic parameters of the inwardly rectifying K^+ current. Figure 6 A shows the current/voltage relationship for the current in a cell treated with 1 μg/ml cholera toxin for 3 h. Comparison of the current magnitude at both the peak (I_p) and the end of 500-ms depolarization pulses (I_{ss}) indicates that the time-dependent inactivation of the current occurs at potentials more negative than −60 mV (4 cells). This contrasts with the −40 mV value previously found in control toxin-untreated cells [2]. In order to investigate more precisely the hypothesis that treatment with cholera toxin is altering the inactivation properties of the current, the steady-state voltage dependence of inactivation was studied with 2-s prepulses to different potentials followed by test pulses to −100 mV or −120 mV [2]. As shown in Fig. 6 B, the prepulse voltage at which the current is half-maximal is shifted nearly 20 mV to more negative values in cholera-toxin-treated cells. Given the strong inhibitory effect promoted by TRH in cells incubated with the toxin, we did not perform a similar kinetic analysis on the residual currents remaining after addition of TRH. However, these data indicate that the reduction of spontaneous oscillatory activity observed in toxin-treated cells can be due, at least in part, to the shift in the steady-state voltage dependence of inactivation of the inwardly rectifying K^+ current.

Discussion

Exposure of GH_3, cells to cholera toxin for 2−4 h significantly increased the TRH-induced inhibition of the inwardly rectifying K^+ current. The exact contribution of this current to the whole membrane K^+ conductance in physiological K^+ solutions is not known. However, several facts point to an important role of the inwardly rectifying K^+ current in the maintenance of resting membrane conductance and regulation of the electrical activity of GH_3 cells by TRH. (a) The TRH-promoted reductions of the current extend for a period well into the time required to enhance the frequency of spikes. (b) Enhancement of the neuropeptide-induced inhibition of inwardly rectifying K^+ currents in cholera-toxin-treated cells is paralleled by a potentiation of the second phase of enhanced action potential frequency in cells showing similar basal rates of firing. This is also consistent with the potentiation of the TRH-induced $[Ca^{2+}]_i$ oscillations in toxin-treated cells. (c) Only reductions on the inwardly rectifying K^+ currents, but not those elicited by TRH on vollage-dependent Ca^{2+} currents, were increased by treatment of the cells with cholera toxin. (d) A similar positive correlation between the size of the TRH-evoked inhibition of inwardly rectifying K^+ currents and the extent of the TRH-induced enhancement of electrical activity has also been demonstrated after treatment of the cells with phosphatase inhibitors [2, 6]. Again, neither the reductions caused by the neuropeptide of Ca^{2+}-dependent K^+ currents [2] nor those exerted on voltage-dependent Ca^{2+} currents (Barros F., unpublished) were altered by the phosphatase inhibitors. (e) Video imaging with fura-2-loaded cells demonstrated that cholera toxin treatment causes a clear reduction of spontaneous Ca^{2+} oscillations associated with electrical activity. The fact that treatment with the toxin also causes a prominent shift in the voltage dependence of inactivation of the inwardly rectifying K^+ current towards more negative values would also be consistent with the proposed role of this current in controlling resting membrane conductance and electrical behaviour.

It has previously been shown that, in addition to its inhibitory effect on inwardly rectifying K^+ currents, TRH shifts to more positive values the steady-state voltage dependence of inactivation of the currents [2, 3]. Rigorous quantification of kinetic parameters of the current in the presence of TRH in toxin-treated cells is

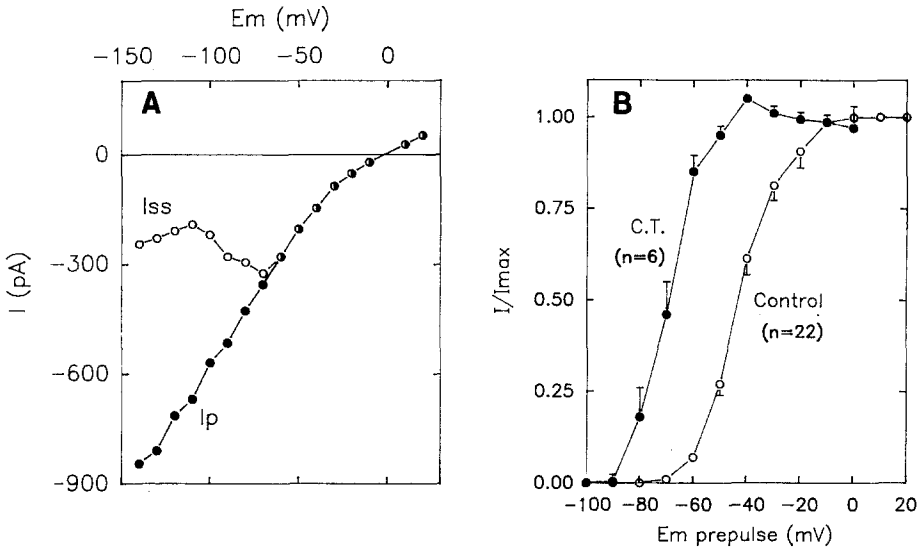

Fig. 6A, B. Effect of treatment of GH_3 cells with cholera toxin (*C.T.*) on kinetic parameters of inwardly rectifying K^+ currents in patch-perforated GH_3 cells. **A** Current/voltage relationship for membrane currents elicited by 500-ms voltage pulses to the potentials indicated on the *abscissa*. Pulses were delivered every 15 s to a voltage-clamped cell treated with cholera toxin for 3 h. Holding potential 0 mV. The amplitude of the maximal currents (*Ip*) and the currents at the end of the voltage step (*Iss*) are plotted versus the test pulse potential. Note that both current magnitudes start to differ at potentials more negative than −60 mV, which contrasts with the −40 mV value previously obtained in control untreated cells studied with an identical protocol (Fig. 2 in [2]). **B** Effect of treatment with cholera toxin on steady-state voltage dependence of inactivation of inwardly rectifying K^+ currents. Test pulses to −100 or −120 mV were preceded by 2-s prepulses to the potentials indicated on the *abscissa*. Holding potential 0 mV. The ratio of peak inward current during the test pulses to maximum inward current (*I/Imax*) is plotted as a function of prepulse potential. I_{max} represents the magnitude of the current on test pulses following prepulses to potentials that do not cause inactivation of the inwardly rectifying currents. Ratio values at every prepulse potential are averaged on the graph for the number of cells indicated in parentheses. Curves generated using control untreated cells (*Control*, ○) and toxin-treated cells for 2−4 h (*C.T.*, ●) are shown. See [2] for details on the protocols used. Inwardly rectifying current magnitudes at −100 mV in the subpopulation of toxin-treated cells used to generate the graph (324 ± 56 pA, $n = 6$) were not significantly different from those recorded in control cells (290 ± 27 pA, $n = 22$; $P = 0.57$, Student's *t*-test)

largely hampered by the strong reduction of the current magnitude elicited by the neuropeptide. However, both the reductions of K^+ conductance and the shift of the inactivation values in the positive direction would be factors leading to enhanced electrical activity. The kinetic properties of the inwardly rectifying K^+ current make it a good candidate to act as a "potential clamp" around the resting potential of the cell. Thus, the inward rectification and the inactivation properties of the current would maintain it maximally active near rest. This is further supported by the striking increase in excitability when the current is suppressed by TRH. It could be argued that the rectification properties of the current would limit its participation in regulation of the cell membrane potential when more physiological low-K^+ medium is used. An important role of inwardly rectifying K^+ channels in determining the resting membrane potential has been largely recognized in many systems [15, 19, 22]. Since the input resistance of GH_3 cells is large (of the order of 1−5 GΩ [8]) very small currents would also be able to exert a large effect on resting membrane potential.

Apart from the inwardly rectifying K^+ current, it has been suggested that inhibition by TRH of various outward currents can be involved in resting potential and conductance changes underlying the neuropeptide-induced enhancement of electrical activity. These include several voltage- and/or Ca^{2+}-activated K^+ currents [1, 9, 18, 20, 21, 31]. The participation of the voltage-dependent transient outward K^+ current in these effects [9] has been recently challenged [1, 31]. A direct inhibitory effect of TRH on a voltage- and/or Ca^{2+}-activated K^+ conductance has not been demonstrated. However, the inhibition of L-type Ca^{2+} currents in response to TRH (Fig. 5; see also [18, 31]) makes it possible that reduced Ca^{2+} influx along each individual action potential results in reduced activation of Ca^{2+}-activated K^+ channels during the after-hyperpolarizations and the interspike intervals [18, 31]. The presence in GH_3 cells of Ca^{2+}-activated K^+ channels sensitive to elevations in cytosolic Ca^{2+} at membrane potentials near rest [20, 21] also supports this mechanism. Measurements of the kinetic behaviour of intracellular Ca^{2+} variations in individual cells and a better understanding of the spatiotemporal regulation of Ca^{2+} movements immediately beneath the plasma membrane would help to resolve the relative contribution of different K^+ conductances to regulation of excitability by TRH.

The reason for the enhanced inhibitory effect of TRH on inwardly rectifying K^+ currents in cholera-toxin-treated cells is not known. Our results using procedures that raise intracellular levels of cAMP suggest that a chronic elevation of internal cAMP could cause the irreversibility of the TRH-evoked reductions on inwardly rectifying K^+ currents. It is possible that protein kinase A inhibition of a protein phosphatase is involved in this

effect. However, they also indicate that the enhancement of the TRH-induced inhibition is not mediated by the cyclic nucleotide. Among previous data obtained using protein phosphatase inhibitors [2], we have recently obtained a more direct demonstration of the participation of a protein kinase in the inhibition of the inward rectifier by TRH: the TRH-evoked inhibition was almost abolished by introduction into the cell of a non-hydrolysable ATP analogue through the patch pipette in whole-cell mode. Furthermore, although the TRH-induced current inhibition becomes irreversible under those conditions, it is readily reversed by addition of purified specific protein phosphatases to the pipette buffer (Barros, Mieskes, del Camino and de la Peña, manuscript in preparation). The magnitude of the inwardly rectifying K^+ current is not modified by cholera toxin *per se* or by raising intracellular cAMP concentrations (this report and [2]). Since the toxin-induced enhancement of the inhibition caused by TRH is not mimicked by cAMP, it seems reasonable to conclude that cAMP-independent enhanced activation of a protein kinase is the reason for the increased TRH effects following treatment of the cells with cholera toxin. It is known that modulation of the inwardly rectifying K^+ current by TRH is mediated by a G protein [3]. The regulation of phospholipase C by TRH in GH_3 cells is not sensitive to cholera or pertussis toxins [16]. This would be consistent with our results showing that phase I of TRH action is not modified in cholera-toxin-treated cells. Our preliminary results and those of Bauer et al. [3] seem to indicate that a cholera-toxin-sensitive pathway may be involved in some of the effects of TRH. Coupling of TRH receptors to a cholera-toxin-sensitive GTP-binding protein has been reported [34]. Furthermore, although TRH acts as a poor activator of adenylate cyclase in GH_3 cells, a direct interaction of the GH_3 cell TRH receptor with G_s or a G_s-like protein has also been recently demonstrated [27]. Thus, it is tempting to speculate that, in addition to the well-known toxin-insensitive transduction pathway linked to phospholipase C activation, a cholera-toxin-sensitive pathway could link the GH_3 cell TRH receptor to regulation of inwardly rectifying K^+ currents. The validity of this hypothesis and the possible relationship of this dual pathway with the recent demonstration of two different isoforms of the TRH receptor in GH_3 cells [28, 29] is currently under investigation.

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