Thyrotropin-releasing Hormone Activates K_{Ca} Channels in Gastric Smooth Muscle Cells Via Intracellular Ca^{2+} Release

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Abstract. Thyrotropin-releasing hormone (TRH) is released in high concentrations into gastric juice, but its direct effect on gastric smooth muscles has not been studied yet We undertook studies on TRH effect on gastric smooth muscle using contraction and patch clamp methods TRH was found to inhibit both acetylcholine- and $BaCl_2$-induced contractions of gastric strips TRH, applied to single cells, inhibited the voltage-dependent Ca^{2+} currents and activated the whole-cell K^+ currents The TRH-induced changes in K^+ currents and membrane potential were effectively abolished by inhibitors of either intracellular Ca^{2+} release channels or phospholipase C Neither activators, nor blockers of protein kinase C could affect the action of TRH on K^+ currents

In conclusion, TRH activates K^+ channels *via* inositol-1,4,5-trisphosphate-induced release of Ca^{2+} in the direction to the plasma membrane, which in turn leads to stimulation of the Ca^{2+}-sensitive K^+ conductance, membrane hyperpolarization and relaxation The data imply that TRH may act physiologically as a local modulator of gastric smooth muscle tone

Key words: Thyrotropin-releasing hormone — Smooth muscle — Stomach — K^+ channels — Calcium

Introduction

Thyrotropin-releasing hormone (TRH) is an amidated tripeptide secreted by the hypothalamus, and it stimulates the release of thyrotropin and other tropic hormones from the anterior pituitary (for review, see O'Leary and O'Connor 1995) The central nervous system (CNS) effects of TRH resulting from its direct application to specific brain regions or from intracerebroventricular injection include cardiovascular and respiratory effects (Paakkari 1990, Jarvinen and Paakkari 1991), arousal (Tanaka et al 1997) and analeptic effects, antiepileptic and anticonvulsant

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effects (Knoblach and Kubek 1997; Przewlocka et al. 1998; Wan et al. 1998) as well as gastrointestinal effects. Some of the extensively described TRH effects on the CNS include: increase of the intragastric pressure and pyloric motility in rats (Krowicki and Hornby 1993), stimulation of gastric motility in rats (Heymann-Monnikes et al. 1991; Bond et al. 1992; Tachibana et al. 1995), inhibition of contractions of duodenal smooth muscle in rats (Jarvinen and Paakkari 1991), control over both gastric acid secretion and motility in cats (Feng et al. 1990), stimulation of colonic activity in rabbits (Smith et al. 1977). Intravenous administration of TRH also induces various gastrointestinal effects that have been studied extensively: effect on the skate and cod stomach myoelectric activity (Shparkovsky and Kozak 1989), stimulation of gastric action potentials in dogs (Morley et al. 1979), inhibition of pentagastrin-stimulated gastric secretion in dogs (Scarpignato et al. 1981), etc. However there are just a few reports on the direct effect of TRH on gastrointestinal smooth muscle (Tonoue et al. 1979; Harada et al. 1996). TRH-like immunoreactivity is detected not only in the CNS but also in body fluids of mammals and humans (Prasad 1987; Klootwijk et al. 1997) as well as in various smooth muscle tissues like uterus and the gastrointestinal tract (Dolva et al. 1983; Grasso et al. 1992; Kaneko et al. 1992; Parkman et al. 1993). There is also evidence that TRH is secreted by the endocrine pancreas (Duntas and Malfertheiner 1993) and by some endocrine cells of the gastric antrum (Hökfelt et al. 1989). It is well known that TRH is not degraded by general peptidases (O'Leary and O'Connor 1995), and concentrations of immunoreactive TRH in the gastric juice may therefore reach sub-milimolar levels (Nagai et al. 1995; Konagaya et al. 1998a,b). A question arises about the possible direct effect of TRH on gastrointestinal smooth muscle and its mechanism of action. This question is even more important on the background of the increasing interest towards the use of TRH and newly synthesized TRH analogs for diagnosis and therapy of various pathologies (for review, see Horita 1998). The problem concerns the possible side effects of such drugs in tissues that express TRH receptors with unknown selectivity and intracellular targets.

The aim of the present work was to study the effect of TRH on muscle contraction and ionic currents of single smooth muscle cells isolated from the guinea pig stomach in relation to the intracellular signaling cascade involved in TRH-induced contractile and electrical phenomena.

Materials and Methods

Smooth muscle preparation for isometric recording of tension

Male guinea pigs weighing 350–400 g were killed by decapitation. Smooth muscle cells were isolated from the circular layers of the gastric fundus, and strips for isometric recording of the contractile force were excised to study the contractile effects of the hormone.

Contraction experiments

The preparations for contraction studies were according to a previously described procedure (Petkov and Boev 1996). After excision the fundus strips were mounted vertically in 10 ml organ baths and stretched under tension of 10 mN. The organ baths were filled with Ca-free Krebs solution (see *Solutions and drugs*) and strips were left to relax for 20 min. The bath solution was then changed to 2.5 mmol/l Ca^{2+}-containing Krebs to initiate contractions, and the strips were left to equilibrate for 60–90 min until a stable spontaneous contractile activity developed. The bath solutions were thermostatically controlled (37 °C) and continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. During the equilibrium period the bath solution was changed every 15 min. The spontaneous isometric contractions of preparations were recorded in the presence of 1 μmol/l tetrodotoxin and 10 μmol/l atropine to block the release of neurotransmitters, especially acetylcholine. Grass Instruments force-displacement transducers with a capacitance $\pm$10 g were used. Contraction traces were recorded on a paper line recorder type MTA 175 (Kutesz, Hungary). The amplitude of contractions was expressed as mean $\pm$ S.E.M. for n, number of preparations.

Whole-cell patch clamp experiments

Whole-cell voltage clamp experiments were performed on single smooth muscle cells freshly isolated from the circular layer of the guinea-pig gastric fundus. Muscle strips were cut into small pieces and placed in a Ca^{2+}-free physiological salt solution (PSS – see *Solution and drugs*), prewarmed to 37 °C and containing 1 g/l collagenase (type 1A, SIGMA, St. Louis, USA), 1 g/l soybean trypsin inhibitor and 1 g/l bovine serum albumin. After 35–55 min incubation at 37 °C, the enzyme was carefully washed out from the pieces with 20 ml prewarmed Ca^{2+}-free PSS. Single muscle cells were then obtained by gentle agitation of the pieces in 1 ml Ca^{2+}-free solution for cell isolation (see *Solution and drugs*) until the solution became cloudy. Cells were stored up to 10 h in this solution at 6 °C. The bath chamber where cells were left to adhere to the glass bottom was continuously perfused with Ca^{2+}-containing PSS in which the drugs were dissolved. The cells were first washed from the isolation solution with Ca^{2+}-containing PSS for 8 min, and then nicardipine (2 μmol/l) was added to stop subsequent Ca^{2+} entry, except when Ca^{2+} currents were studied.

The values of current densities expressed as i = μA/cm^2 of membrane surface assuming the specific membrane capacitance of 1 μF/cm^2 were plotted *versus* the potential applied in order to obtain comparable data for statistical analysis. The whole-cell mode of the patch-clamp technique was employed. The patch electrodes from borosillicate filamented glass (World Precision Instruments, Sarasota, U.S.A.), when filled with intracellular solutions, had a resistance of approximately 2.5–3 MΩ. Membrane currents were registered *via* an EPC-7 (List Electronics, Darmstadt, Germany) amplifier. Current signals were recorded and further analyzed on an AT 286 PC through a TL-1 DMA (AXOPATCH) interface, using Square Wave Cell Tester software (Shkodrov 1995). In all experiments with potassium currents the

holding potential (V_h) was −50 mV and in all experiments with calcium currents V_h = −80 mV. The temperature was 25 °C.

Statistics

The data were assessed for statistical significance using Student's *t*-test at $p < 0.05$, or by Tukey-Kramer multiple comparison test where appropriate. Significance is labeled with asterisks or open circles as follows: * for $p < 0.05$; ** for $p < 0.01$, *** for $p < 0.001$.

Solutions and drugs

The Krebs solution used in contraction experiments consisted of (in mmol/l): 110 NaCl; 6 KCl; 1.2 $MgCl_2$; 1.2 NaH_2PO_4; 11 glucose; 25 $NaHCO_3$; 2.5 $CaCl_2$, and was bubbled with 95 % O_2 and 5 % CO_2 to achieve a pH of 7.2 at 37 °C. The physiological salt solution (PSS) for single cell voltage and current clamp experiments contained (in mmol/l): 126 NaCl; 6 KCl; 20 HEPES; 10 taurine; 20 glucose; 1.2 $MgCl_2$; 1.8 $CaCl_2$; 5 Na-pyruvate; pH adjusted to 7.4 with NaOH. The solution for cell isolation consisted of (in mmol/l): 85 NaCl; 30 $NaNO_3$; 5 $MgCl_2$; 20 taurine; 5 Mg-ATP; 5 Na-pyruvate; 5 creatine; 5 oxalacetic acid; 1 g/l bovine serum albumin; pH adjusted to 7.25 with NaOH. The solutions in the recording pipette contained (in mmol/l): 125 KCl; 10 Hepes; 1 $MgCl_2$; 5 phosphocreatine; 5 oxalacetic acid; 5 succinic acid; 3 EGTA and 1.71 $CaCl_2$ (giving free Ca^{2+} of 100 nmol/l); pH adjusted to 7.4 with KOH. The intracellular Ca^{2+} concentration was estimated according to the calculation guide of Schubert (Schubert 1996). In experiments where Ca^{2+} currents were studied KCl in the pipette solution was replaced by equimollar CsCl and pH was adjusted with CsOH.

All substances used to prepare solutions as well as $BaCl_2$, acetylcholine, atropine sulphate and 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC) were obtained from Sigma Chemical Co. GF 109203X (2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl), was a product of Tocris Cookson Ltd (Bristol, UK). The other drugs used were purchased from RBI (Natick, U.S.A.).

Results

Contraction studies

Contraction experiments were performed to test if TRH acted on spontaneous, $BaCl_2$- and acethylcholine-evoked contractions of fundus strips. TRH cumulatively added to the bath at concentrations between 1 nmol/l and 100 μmol/l had no effect on spontaneous fundus contractions characterized as typically tonic (Petkov and Boev 1996). Strips responded to 5 mmol/l $BaCl_2$ and 0.1 μmol/l acetylcholine, with a slowly developing tonic tension, reaching up to 358 ± 9.7 % (n = 6, $p < 0.001$) of the control (in the case of $BaCl_2$) and 262 ± 8.2 % (n=5, $p < 0.001$) of the control (in the case of acetylcholine). Bath application of TRH (1 μmol/l) led to a decrease of $BaCl_2$-induced tonic contraction by 23 ± 6.9 % (n = 7, $p < 0.001$)

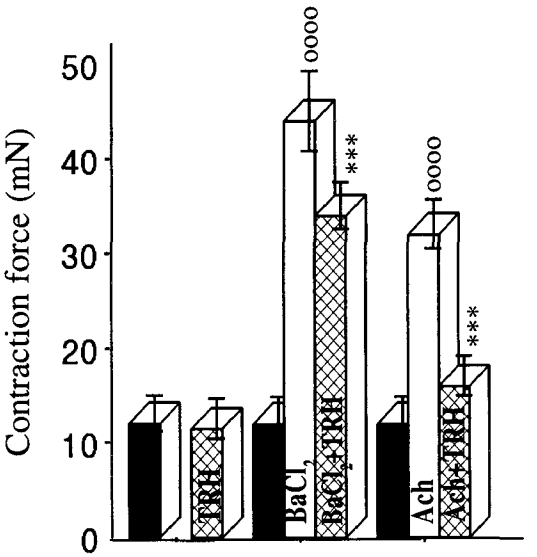

Figure 1. TRH (1 μmol/l)-induced decrease of 5 mmol/l $BaCl_2$- and 0 1 μmol/l acetylcholine-activated contractions of the strips, isolated from the guinea pig gastric fundus First, a contraction agonist was applied (either $BaCl_2$ or acetylcholine) that elicited tonic contractions which stabilized usually within 15 min Then, TRH was added to the bath and its relaxing effect was evaluated usually in 20 min when the tone stabilized at the new level The amplitudes of contractions were plotted in absolute values (mN) and were compared to the control spontaneous amplitudes (black bars) as measured before application of contracting substances Data are means ± S E M of 7 experiments (in the case of $BaCl_2$) and means ± S E M of 6 experiments (in the case of acetylcholine) Abbreviations Ach – acetylcholine, TRH – thyrotropin-releasing hormone Asterisks $p < 0\ 001$, controls *vs* the effect of $BaCl_2$ or Ach Significant differences between the control values of contraction and those observed in the presence of contraction-inducing agents ($BaCl_2$ or Ach) are marked with circles (in both cases, $p < 0\ 001$)

and of acetylcholine-induced tone by 48 5 ± 13.2% ($n = 6$, $p < 0.001$) (Fig. 1) These data suggest that TRH inhibits the tonic contractions of the fundus by a mechanism that involves changes in both plasma membrane ion conductivity and intracellular calcium homeostasis

Whole-cell patch clamp experiments

Control outward currents expressed by fundus smooth muscle cells in PSS and in the presence of nicardipine are shown in Fig. 2 After reaching their maximal amplitude at the given potential (times for reaching the peak amplitude were between 10 ms and 25 ms), K^+ currents stabilized at that level during pulses lasting 500 ms (longer pulses were not applied). Figure 2A shows the outward current waveforms elicited by increasing voltage depolarizations from −60 mV to +40 mV in 20 mV

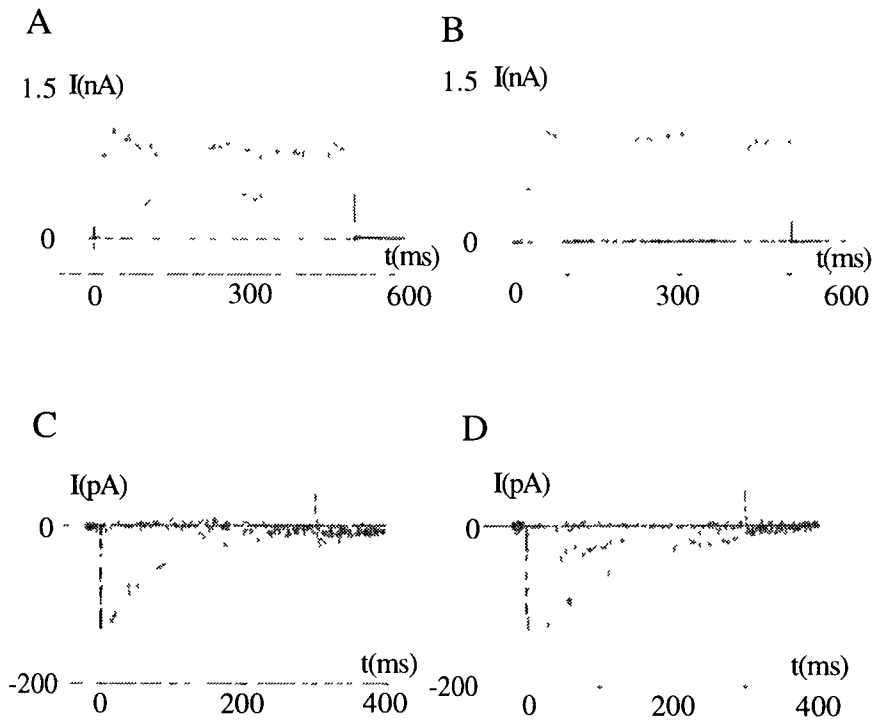

Figure 2. Representative outward (A,B) and inward (C,D) currents expressed in the fundus smooth muscle cells at the 5th minute after the start of dialysis (A and C) and 1 hour later (B and D) Fig 2A,B shows the current waveforms elicited by depolarizing voltage steps from −60 mV to +40 mV applied in 20 mV increments from V_h = −50 mV The cells were bathed in 2 mmol/l Ca^{2+}-containing solution for 8 min Then, nicardipine (2 μmol/l) was added to this solution and was left in the bath till the end of the study of potassium currents Standard intracellular solutions were used (K^+-rich in A,B and Cs^+-rich in C,D) (see Materials and Methods) Ca^{2+} currents were elicited by voltage pulses from −60 mV to +40 mV applied in 20 mV increments from V_h = −80 mV A and B a cell with a capacitance of 44 pF and an input impedance of 1 1 GΩ C and D a cell with a capacitance of 49 pF and input impedance of 1 2 GΩ

steps from holding potential (V_h) = −50 mV at the 5th minute after the start of dialysis; Fig. 2B shows the current waveforms obtained under the same conditions 60 minutes later. From the control measurements made it is clear that there was no run-down of the outward currents during the time of the experiment (see the legend to the Figure). Figure 2C presents control inward currents elicited by increasing voltage depolarizations from −60 mV to +40 mV in 20 mV steps from V_h = −80 mV expressed in fundus cells 5 minutes after the start of the dialysis, and Figure 2D shows the control currents registered 1 hour after the beginning of the experiment It is obvious that there was no run-down of the inward currents

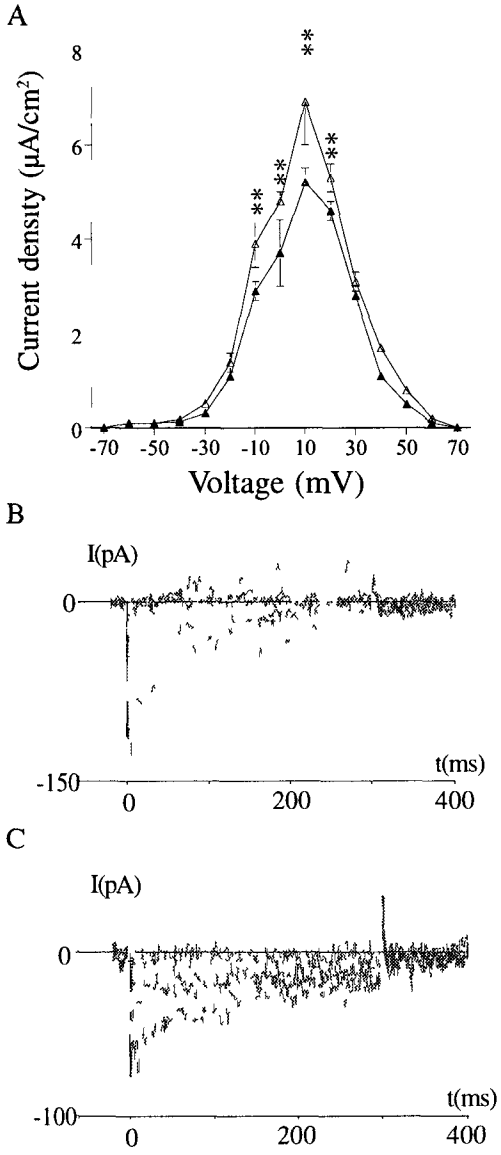

Figure 3. (A) Voltage dependence of the current densities of I_{Ca}, measured in control conditions (open triangles) and 30 min after addition of 1 μmol/l TRH to the perfusing solution (closed triangles) Voltage pulses of 500 ms were consecutively applied from V_h = −80 mV to potentials between −70 mV and +70 mV in 10 mV increments Data are means ± S E M of 7 cells The asterisks represent statistically significant differences in current densities (at $p < 0\ 01$) (B) and (C) representative traces of I_{Ca}, elicited by depolarization pulses starting from −60 to +40 mV at 20 mV steps in control conditions (5 min after the start of dialysis) (B), and 20 min after bath application of 1 μmol/l TRH (C) V_h = −80 mV Maximal inward calcium current was measured at 0 mV test stimulus (B) and at 0 mV and +20 mV (C) Cell with a capacitance of 48 pF and an input impedance of 1 1 GΩ

during the experiment in control conditions Therefore any changes in the currents, outward or inward, observed during this time could be considered as being due to the effects of the drugs applied

Effect of TRH on Ca^{2+} currents

It is well documented that voltage-sensitive Ca^{2+} current (I_{Ca}) densities recorded

Table 1. Concentration-dependence of the TRH action on I_K in single smooth muscle cells of gastric fundus

	Concentration of the hormone (nmol/l)				
Membrane potential	Control ($n = 7$)	TRH 1 ($n = 6$)	TRH 10 ($n = 7$)	TRH 100 ($n = 6$)	TRH 1000 ($n = 6$)
−40	0 3 ± 0 02	1 0 ± 0 02	1 2 ± 0 3	1 3 ± 0 1	1 43 ± 0 03
−20	3 5 ± 0 1	4 4 ± 0 22	6 4 ± 0 7	9 8 ± 0 9	11 42 ± 1 10
0	7 2 ± 0 2	11 3 ± 0 28	12 7 ± 0 7	14 2 ± 0 5	15 82 ± 0 30
+20	12 8 ± 2 1	18 6 ± 1 32	24 3 ± 1 1	28 1 ± 1 1	32 82 ± 1 83
+40	26 3 ± 2 1	32 5 ± 2 13	41 6 ± 2 2	49 9 ± 1 2	58 45 ± 2 51

TRH was added in increasing concentrations to the perfusion solution, current amplitudes were registered after stabilizing, during three consecutive measurements Ca^{2+} concentration in the pipette solution was 100 nmol/l The hormone effect was tested on numbers of cells (n) given in the parentheses

from guinea-pig fundus cells are relatively low (Mitra and Morad 1985), and the only type of voltage-sensitive Ca^{2+} channel present is the L-type. Figure 3 shows that TRH (1 μmol/l) inhibits I_{Ca} densities by about 25% in cells dialyzed with 100 nmol/l free Ca^{2+}-containing pipette solution Ca^{2+} currents were elicited by voltage pulses with increasing amplitudes from −70 mV to +70 mV in two separate voltage protocols with odd and even potentials for a better resolution. To answer the question whether the TRH-induced inhibition of I_{Ca} was due to an increased Ca^{2+} concentration near the plasma membrane or to direct inhibition of the channels, we studied the effect of TRH on Ca^{2+} sensitive K^+ currents.

Effect of TRH on K^+ currents

TRH added in increasing concentrations to the perfusion chamber led to a marked increase of the outward current amplitudes. The concentration-dependent activation of outward currents induced by TRH, which had an ED_{50} of about 100 nmol/l (for the current densities measured at 0 mV) is shown in Table 1. The increase in outward current amplitudes was manifested 15 to 30 minutes after the application of the hormone (Fig. 4A). The double pulse protocol used in the presence of TRH revealed that the TRH-activated currents had a reversal potential close to that of the K^+ at equilibrium (Fig. 4B). Potassium currents (I_K) expressed in smooth muscle cells of the guinea-pig gastric fundus have been extensively investigated during the last 3 years (Duridanova et al. 1995, 1996). About 90% of the whole cell I_K in these cells was shown to be carried through a population of charybdotoxin-blockable Ca^{2+}-sensitive K^+ channels. In the present study it was found that following TRH application I_K amplitudes increased and reached a steady state level within 10 min. The effect of the hormone was stable for the next 40 minutes following its bath application and was not reversible upon washout. TRH (1 μmol/l) caused a pro-

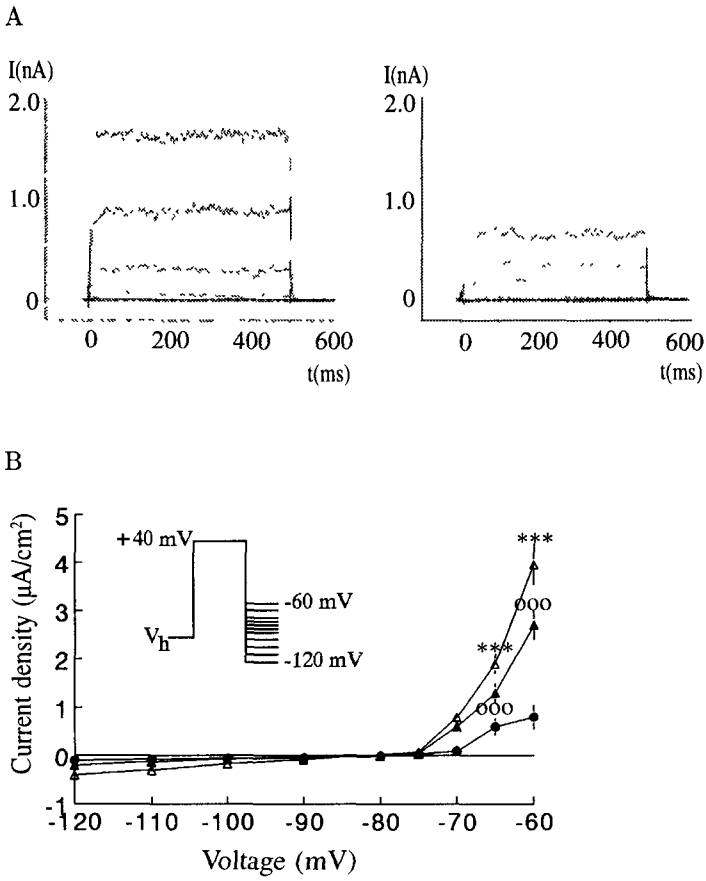

Figure 4. (A) TRH (1 μmol/l)-induced activation of I_K 20 min after its application (*left panel*), compared to control current values (*right panel*), as expressed in a cell with a capacitance of 54 pF and an input impedance of 1.3 GΩ. (B) Voltage dependence of tail current densities, monitored during a double pulse protocol (given in the inset) to reveal the selectivity of outward currents for K^+. Closed circles control current ($n = 5$). Triangles: the current measured 20 min after bath application of 0.1 μmol/l TRH (closed symbols, $n = 6$) or 1 μmol/l TRH (open symbols, $n = 5$). Data are means $\pm$ S.E M. The holding potential was -50 mV. Significance between groups according to the Tukey-Kramer multiple comparison test: control current *vs.* current after 0.1 μmol/l TRH $p < 0.001$; control current *vs.* current after 1 μmol/l TRH $p < 0.001$. Significantly different values as compared to control ones are marked with the asterisks and circles.

nounced hyperpolarization of the cell membranes by 18 ± 5 mV ($n = 7$, $p < 0.001$) as revealed under current clamp mode (not shown). I_K amplitudes exceeded the control ones about 3 times – a result which was constantly reproduced in each of the cells studied.

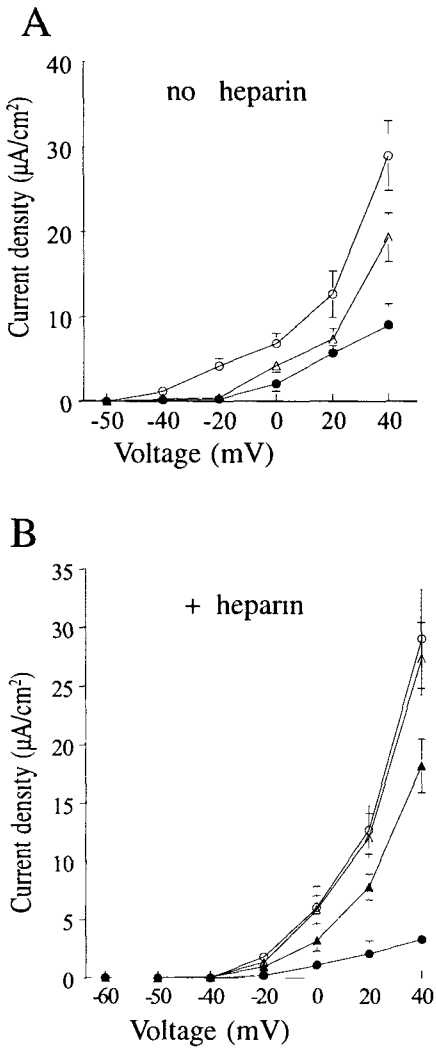

Figure 5. Voltage dependence of current densities of I_K, measured in the absence (A) or in the presence (B) of 0 1 μmol/l heparin in the dialyzing solution (A) Control conditions (open circles), 30 min after bath application of 10 μmol/l ryanodine (triangles), and 15 min after addition of 1 μmol/l TRH in the ryanodine-containing bath (closed circles) A multiple comparison test showed a significant difference between the values marked with the open circles and those marked with both the closed circles and triangles at potentials above -20 mV ($p < 0$ 05), and significant differences between the three curves at potentials between 0 mV and +40 mV at $p <$ 0 01 (B) Control conditions (open circles), 15 min (open triangles), 20 min (closed triangles) or 40 min (closed circles) following the bath application of 1 μmol/l TRH Each point represents mean $\pm$ S E M of 5 measurements Voltage pulses of 500 ms were applied from $V_h = -50$ mV to voltages between -60 mV and +40 mV in 20 mV increments The multiple comparison test showed no significant difference between the values marked with the open circles and open triangles ($p > 0$ 05) Significant differences were observed between the values marked with the closed triangles and those marked with the closed circles ($p < 0$ 001) as well as compared to control values ($p < 0$ 01)

Dependence of TRH-induced activation of I_K by calcium released from the sarcoplasmic reticulum (SR)

Bath application of 10 μmol/l ryanodine resulted in a transient activation of I_K. It lasted for about 10 min and was followed by a decrease in I_K densities (Fig. 5A) during the next 7 $\pm$ 2 min ($n = 8$). A steady-state level was reached around the 20th min after the drug application. Subsequent addition of 1 μmol/l TRH to the bath solution led to a further significant decrease of the I_K densities in 15 minutes (Fig. 5A). When cells were dialyzed with a heparin-containing pipette solution

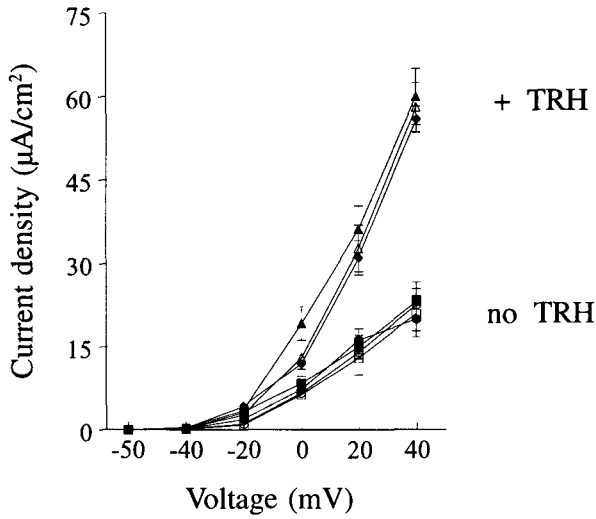

Figure 6. Effect of blockers of nucleotide-dependent protein kinases on voltage dependencies of I_K densities Control, open circles Rp-cAMPS (10 μmol/l) for 10 min, closed circles Rp-cGMPS (10 μmol/l) for 10 min, open squares H-8 (20 μmol/l) for 15 min, closed squares TRH (1 μmol/l) still activated I_K on the background of Rp-cAMPS (25 min after TRH application), open triangles, on the background of Rp-cGMPS (25 min after TRH application), closed triangles, on the background of H-8 (25 min after TRH application, closed diamonds) Each point represents mean ± S E M of 5 measurements Voltage pulses were applied from $V_h = -50$ mV to potentials between −60 mV and +40 mV in 20 mV increments for 500 ms A multiple comparison test showed significant differences between the currents expressed in the presence of TRH, and the ones measured in the absence of the hormone ($p < 0\ 001$)

(1 μmol/l light heparin), the control I_K amplitudes did not change significantly compared to the control ones, measured in the absence of heparin (Fig. 5B). In heparin-dialyzed cells TRH also failed to increase I_K densities, even at 10 μmol/l, and even caused a statistically significant decrease of the current amplitudes, which was observed 18 to 25 min after TRH application (Fig 5B). Further decrease in I_K densities was observed 40 min later, in fact, I_K was virtually abolished 60 minutes after the start of dialysis (closed circles) Thus, it was suggested that TRH could activate I_K by releasing calcium from both inositol 1,4,5-trisphosphate (IP_3)-sensitive and ryanodine-sensitive Ca^{2+} stores.

Following the transduction pathway

Figure 6 shows that neither the selective inhibitors of both cAMP-dependent and cGMP-dependent protein kinases (Rp-cAMPS, 10 μmol/l, Rp-cGMPS, 10 μmol/l) nor the non-selective blocker of both protein kinases H-8 (N-[2-methylamino)ethyl]-

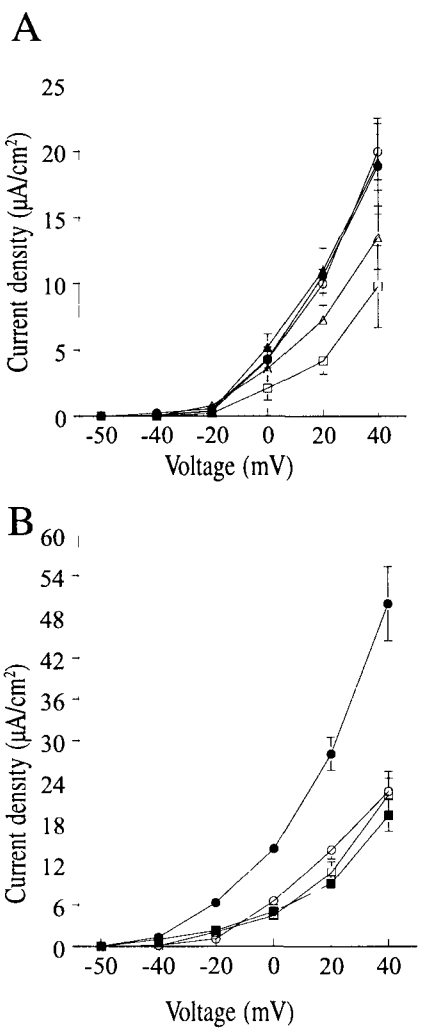

Figure 7. (A) NCDC (50 μmol/l, closed triangles) and U-73122 (10 μmol/l, closed circles) had no effect on its own on the whole cell I_K densities (open circles) However, when added before the application of 1 μmol/l TRH each of them was effectively abolishing the I_K-increasing action of the hormone Current amplitudes were measured 30 min after application of TRH on the background of either NCDC (open squares) or U-73122 (open triangles) A multiple comparison test revealed significant differences only between the values marked with the open squares and those marked with the circles and the closed triangles ($p < 0\ 01$) (B) After the control amplitudes of I_K (open circles) were increased by 0 1 μmol/l TRH (closed circles), 10 μmol/l U-73122 (open squares) or 50 μmol/l NCDC (closed squares) restored the initial current densities within 10–15 min following their addition into the perfusing solution Each point represents mean ± S E M of 5 measurements Voltage pulses were applied from $V_h = -50$ mV potentials between −60 mV and +40 mV in 20 mV increments for 500 ms Significant differences were observed only at the potential of +40 mV between the values marked with the closed circles and the other values ($p < 0\ 001$)

5-isoquinoline-sulfonamide hydrochloride) could affect the I_K-increasing efficacy of TRH. Thus, it was suggested that the cyclic nucleotide-dependent protein kinases were not involved in the observed activation of I_K, triggered by TRH.

NCDC (2-nitro-4-carboxyphenyl N,N-diphenylcarbamate, 50 μmol/l) and U-73122 (1-[6[[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), 10 μmol/l), both being inhibitors of phospholipase C (Fig. 7A), but not D-609 (O-(octahydro-4,7-methano-1H-inden-5-yl) ester of carbonodithioic acid, potassium salt, 1 μmol/l), added to the bath before application of TRH (not shown) effectively prevented the effect of TRH on I_K Moreover, both NCDC and U-73122,

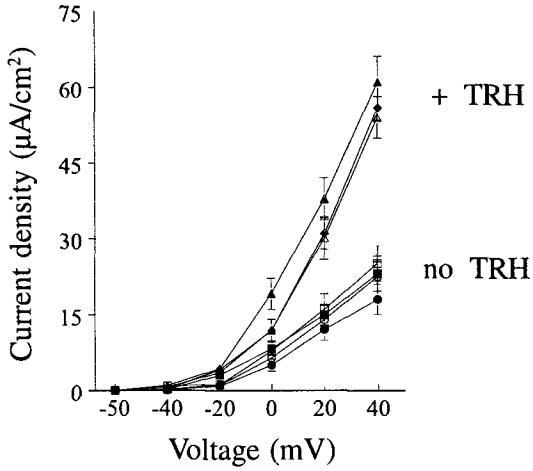

Figure 8. The protein kinase C inhibitors staurosporin (30 μmol/l, open squares) or GF 109203X (100 nmol/l, closed squares) and the activator phorbol-12,13-acetate (0 7 μmol/l, closed circles) caused no significant changes in the voltage dependence of the control I_K densities (open circles) when applied separately *via* the perfusing system In the presence of phorbol-12, 13-acetate (open triangles), staurosporin (closed triangles) or GF 109203X (closed diamonds), 1 μmol/l TRH still increased I_K and the increase was comparable with the one reached in the absence of the drugs Each point represents mean ± S E M of 5 measurements Voltage pulses were applied from $V_h = -50$ mV to potentials between −60 mV and +40 mV in 10 mV increments for 500 ms A multiple comparison test showed significant differences only between cells treated with blockers or activators of PKC and cells treated with TRH ($p < 0\ 001$)

applied separately to the bath after TRH, restored the control I_K densities after they had been augmented by the hormone (0.1 μmol/l) (Fig. 7B).

Protein kinase C seemed not to participate in the I_K-increasing effect of TRH. Neither phorbol ester (phorbol-12, 13 acetate, a protein kinase C stimulator), nor GF 109203X or staurosporine (both of them known as blockers of protein kinase C) were able to influence the TRH-induced activation of I_K (Fig. 8). Therefore, it was suggested that TRH expressed its I_K-increasing effect solely *via* phospholipase C

Discussion

In the present study $BaCl_2$ and acethylcholine were used as contraction agonists to reveal the mechanism of TRH action on contraction. It is well known that Ba^{2+} ions block some types of K channels and could enter the cell through L-type Ca^{2+} channels, thus causing membrane depolarization, which results in contraction (Hille 1992). On the contrary, the contractile effects of acethylcholine are due to its ability to release Ca^{2+} from intracellular sources *via* inositol-1,4,5-triphosphate

triggered Ca^{2+} release. Thus, we found these two contraction agonists suitable to discriminate between membrane-delimited effects of TRH and its intracellular targets of action. The present study demonstrated that TRH affects both types of contractions. This led us to suggest that TRH inhibits the tonic contractions of fundic strips by a mechanism that involves modulation of both the membrane potential and the intracellular Ca^{2+} homeostasis.

The results obtained from the patch-clamp experiments show that TRH exerts dual action on whole-cell ionic currents: inhibition of I_{Ca} and activation of I_K. Given that 90% of the whole cell I_K in fundus cells is carried through Ca^{2+}-activated K^+ channels (Duridanova et al. 1995), the observed enormous increase of I_K densities following TRH administration could be adequately explained with an increase in the Ca^{2+} concentration in the restricted space between the plasma membrane and the superficial sarcoplasmic reticulum (SR). This explanation accounts also for the observed decrease of I_{Ca} amplitudes, which could be attributed to the phenomenon of Ca^{2+}-dependent inhibition of the L-type Ca^{2+} channels (Ganitkevich et al. 1991; Lammel et al. 1991). In favor of this hypothesis was the finding that TRH-induced activation of I_K could be effectively prevented by blocking either the intracellular IP_3-induced Ca^{2+} release with heparin (known as a selective blocker of IP_3-activated Ca^{2+} release channels; Kobayashi et al. 1989) or of the Ca^{2+}-induced Ca^{2+} release with ryanodine (a selective inhibitor of Ca^{2+}-activated Ca^{2+} release channels in SR). These observations suggest that the effects of TRH on ionic currents are obligatory coupled to the intracellular Ca^{2+} homeostasis, and to the SR loading state in particular.

How is this coupling achieved? In most of the tissues studied TRH receptors act either through the adenylyl cyclase- or through the phospholipase C-signaling systems (for review, see O'Leary and O'Connor 1995). Our data demonstrate that in the guinea-pig fundus, TRH receptors are coupled to the phospholipase C $\rightarrow$ IP_3 transduction pathway: the inhibition of either phospholipase C or of the IP_3-induced Ca^{2+} release abolished the TRH-induced I_K activation. The receptor occupation is thought to result in an increase of intracellular IP_3 concentrations and subsequent IP_3-induced release of Ca^{2+} from the SR. Calcium ions thus released could in turn trigger the release of Ca^{2+} *via* the ryanodine-sensitive channels. In support of this suggestion is the observed blockade of the TRH effect by ryanodine after long-lasting exposure of the cells to this drug.

In our previous papers we reported the existence of a vectorial Ca^{2+} release mechanism in smooth muscle cells of guinea pig fundus (Duridanova et al. 1995, 1996), similar to that described by Chen and van Breemen (Chen and van Breemen 1992; van Breemen et al. 1995) in vascular smooth muscle. This mechanism suggests that, in tonically contracting smooth muscles under basal conditions, intracellularly stored Ca^{2+} ions are liberated predominantly in the direction of the plasmalemma due to physiological asymmetry of superficially located cisternae of the SR in very close proximity to the outer membrane. This asymmetry also involves a non-uniform distribution of Ca^{2+} release channels of both types and SR Ca^{2+}-ATPases between the superficially and more deeply located compartments of the SR Ca^{2+} stores

(Duridanova et al. 1996). It is thought that this accounts for the observed phenomena of K^+ current activation and relaxation following the application of NO-donors or cyclic GMP analogs, known to activate the SR Ca^{2+}-ATPase (Duridanova et al 1996).

The present data imply that the signal transduction pathway triggered by the occupation of the TRH receptor links the IP_3 synthesis with the vectorial Ca^{2+} release mechanism. That is why the final effect of TRH on both $BaCl_2$- and Ach-induced contractions is inhibitory, which most probably results from a preferential release of SR Ca^{2+} towards the plasmalemma. This directional release is followed by an activation of I_K and a long-lasting inhibition of I_{Ca}. Thus, the TRH-induced changes in the intracellular Ca^{2+} homeostasis are restricted mainly to the regulation of plasmalemmal conductivity. Such phenomena have been described in other tissues as well (Den Hertog 1981; Duridanova et al. 1997).

In conclusion, the data presented indicate that TRH can modify the contractile response of the guinea-pig gastric fundus to excitable neurotransmission This modulation is realized by simultaneous suppression of I_{Ca} and stimulation of I_K caused by intracellular calcium release in the direction of the plasma membrane, *via* the phospholipase C – IP_3 signaling pathway. Calcium ions thus released activate Ca^{2+}-sensitive K^+ conductance and suppress the currents through the L-type Ca^{2+} channels. Both effects result in hyperpolarization of the plasma membrane and in inhibition of tonic contractions Given that the local concentration of TRH in the stomach wall is usually in the micro molar range (Konagaya et al 1998a,b) the modulatory action of TRH on gastric contractions may play a significant role in the physiology and pathophysiology of the stomach.

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References

Bond E F , Heitkemper M M , Gruver M K (1992) Mediation of thyrotropin-releasing hormone induced gastric motility increases in developing rats Eur J Pharmacol **217,** 127—135

Chen Q , van Breemen C (1992) Function of smooth muscle endoplasmic reticulum In Advances in Second Messenger and Phosphoprotein Research (Ed J W Putney), pp 335—350, Raven Press, New York

Den Hertog A (1981) Calcium and the alpha action of catecholamines on guinea pig *taenia caeci* J Physiol (London) **316,** 109—125

Dolva L O , Hanssen K F , Aadland E , Sand T (1983) Thyrotropin-releasing hormone immunoreactivity in the gastrointestinal tract of man J Clin Endocrinol Metab **56,** 524—529

Duntas L , Malfertheiner P (1993) Thyrotropin releasing hormone functions and importance in the gastrointestinal tract and pancreas Leber Magen Darm **23,** 67—71 (*in German*)

Duridanova D B , Gagov H S , Petkov G V , Shkodrov G B , Boev K K (1995) Cyclic GMP-induced activation of potassium currents by sarcoplasmic reticulum Ca^{2+} pump-dependent mechanism Gen Physiol Biophys **14,** 139—151

Duridanova D B, Gagov H S , Boev K K (1996) Ca^{2+}-induced Ca^{2+} release activates K^+ currents by a cyclic GMP-dependent mechanism in single gastric smooth muscle cells Eur J Pharmacol **298,** 159—163

Duridanova D B , Nedelcheva M D , Gagov H S (1997) Oxytocin-induced changes in single cell K^+ currents and smooth muscle contraction of guinea-pig gastric antrum Eur J Endocrinol **136,** 531—538

Feng H S , Lynn R B , Han J , Brooks F P (1990) Gastric effects of TRH analogue and bicuculline injected into dorsal motor vagal nucleus in cats Amer J Physiol **259,** 321—326

Ganitkevich V Ya , Shuba M F , Smirnov S V (1991) Inactivation of calcium channels in single vascular and visceral smooth muscle cells of the guinea-pig Gen Physiol Biophys **10,** 137—161

Grasso S , Buffa R , Martino E , Bartalena L , Curzio, Salome E (1992) Gastrin (G) cells are the cellular site of the gastric thyrotropin-releasing hormone in human fetuses and newborns A chromatographic, radioimmunological, and immunocytochemical study J Clin Endocrinol Metab **74,** 1421—1426

Harada N , Chijiwa Y , Akiho H , Okabe H , Nawata H (1996) Direct inhibitory effect of thyrotropin-releasing hormone on isolated cecal circular smooth muscle cells of guinea pig Digestion **57,** 79—82

Heymann-Monnikes I , Tache Y , Trauner M , Weiner H , Garrick T (1991) CRF microinjected into the dorsal vagal complex inhibits TRH-analog and kainic acid-stimulated gastric contractility in rats Brain Res **554,** 139—144

Hille B (1992) Calcium channels, potassium channels and chloride channels In Ionic Channels of Excitable membranes (Second Edition), pp 83—140, Sinauer Associates Inc , Sunderland, Massachusetts

Hokfelt T , Tsuruo Y , Ulfhake B , Cullheim S , Arvidsson U , Foster G A , Schultzberg M , Schalling M , Arborelius L , Freedman J (1989) Distribution of TRH-like immunoreactivity with special reference to coexistence with other neuroactive compounds Ann N Y Acad Sci **553,** 76—105

Horita A (1998) An update on the CNS actions of TRH and its analogues Life Sci **62,** 1443—1448

Jarvinen A , Paakkari I (1991) TRH-induced tachyphylaxis present in the smooth muscle but not in the cardiorespiratory effects Pharmacol Toxicol (Copenhagen) **68,** 270—275

Kaneko H , Mitsuma T , Morise K , Uchida K , Furusawa A , Maeda Y , Nakada K , Adachi K (1992) Effect of serotonin on the immunoreactive thyrotropin-releasing hormone concentrations of the rat stomach Digestion **53,** 149—156

Klootwijk W , Sleddens-Linkels E , de Boer R , Jansen C A , Autar R , de Herder W W , Boeve I R , Visser T J , de Greef W J (1997) Renal clearance of the thyrotropin-releasing hormone-like peptide pyroglutamyl-glutamyl-prolineamide in humans J Clin Endocrinol Metab **82,** 3068—3073

Knoblach S M , Kubek M J (1997) Increases in thyrotropin-releasing hormone messenger RNA expression induced by a model of human temporal lobe epilepsy effect of partial and complete kindling Neuroscience (Oxford) **76,** 85—95

Kobayashi S , Kitazawa T , Somlyo A V , Somlyo A P (1989) Cytosolic heparin inhibits muscarinic and alpha-adrenergic Ca^{2+} release in smooth muscle physiological role of inositol 1,4,5-trisphosphate in pharmacomechanical coupling J Biol Chem **264,** 17997—18004

Konagaya T , Kusugami K , Yamamoto H , Kaneko H , Nagai H , Mitsuma T (1998a) Effect of histamine on thyrotropin-releasing hormone and somatostatin secretion in rat stomach Hepatogastroenterology **445,** 567—572

Konagaya T , Kusugami K , Yamamoto H, Nishio Y , Kaneko H , Nagai H , Mitsuma T (1998b) Effect of intragastric administration of beta-endorphin on thyrotropin-releasing hormone and somatostatin release into gastric lumen of rats J Gastroenterol **33,** 27—31

Krowicki Z K , Hornby P J (1993) Opposing gastric motor responses to TRH and substance P on their microinjection into nucleus raphe obscurus of rats Amer J Physiol **265,** 819—830

Lammel E , Deitmer P , Noack T (1991) Suppression of steady membrane currents by acetylcholine in single smooth muscle cells of the guinea-pig gastric fundus J Physiol (London) **432,** 259—270

Mitra R , Morad M (1985) Ca^{2+} and Ca^{2+}-activated K^+ currents in mammalian gastric smooth muscle cells Science (Washington, D C) **229,** 269—272

Morley J E , Steinbach J H , Feldman E J , Solomon T E (1979) The effects of thyrotropin-releasing homone on the gastrointestinal tract Life Sci **24,** 1059—1066

Nagai H , Morise K , Mitsuma T , Furusawa A , Kaneko H , Uchida K , Yamamoto H (1995) Effect of cold-restraint stress on immunoreactive thyrotropion-releasing hormone and immunoreactive somatostatin in the rat stomach J Gastroenterol **30,** 142—148

O'Leary R , O'Connor B (1995) Thyrotropin-releasing hormone J Neurochem **65,** 953—963

Paakkari I , Jarvinen A , Vonhof S , Mannisto P T , Cohen L A , Labroo V M , Feuerstein G (1990) Selective cardiorespiratory activity of an iodinated analog of thyrotropin-releasing hormone (TRH) Peptides **11,** 939—944

Parkman H P , Reynolds J C , Ogorek C P , Kreider M S (1993) Thyrotropin-releasing hormone an inhibitory regulatory peptide of feline lower esophageal sphincter Amer J Physiol **264,** 522—527

Petkov G V , Boev K K (1996) Cyclopiazonic acid-induced changes in contractile activity of smooth muscle strips isolated from cat and guinea-pig stomach Eur J Pharmacol **318,** 109—115

Prasad C (1987) Thyrotropin- releasing hormone In Handbook of Neurochemistry (Ed A Lajtha), pp 175—200, Plenum Press, New York

Przewlocka B , Labutz D , Mika J , Lipkowski A , van Luijtelaar G , Coenen A Lason W (1998) Protective effects of TRH and its analogues in chemical and genetic models of seizures Pol J Pharmacol Pharm **49,** 373—378

Scarpignato C , Alberici M , Bertaccini G (1981) Effect of TRH on pentagastrin-induced gastric acid secretion Res Commun Chem Pathol Pharmacol **33,** 223—233

Schubert R (1996) Multiple ligand-ion solutions A guide for solution preparation and computer program understanding J Vasc Res **33,** 86—98

Shkodrov G B (1995) Computer program for performing whole-cell voltage-clamp experiments Comput Methods Programs Biomed **48,** 241—246

Shparkovskii I A , Kozak N V (1989) The action of thyroliberin on the myoelectric activity of the stomach in the skate and the cod Fiziol Zh SSSR Im I M Sechenova **75,** 837—844

Smith J R , La Hann T R , Chesnut R M , Carino M A , Horita A (1977) Thyrotropin-releasing hormone stimulation of colonic activity following intracerebroventricular administration Science (Washington, D C) **196,** 660—662

Tachibana M , Takeuchi K , Okabe S (1995) Role of sympathetic nervous system in gastric functional changes induced by thyrotropin-releasing hormone in rats Jpn J Pharmacol **67,** 157—164

Tanaka K , Ogawa N , Asanuma M , Kondo Y (1997) Thyrotropin-releasing hormone prevents abnormalities of cortical acetylcholine and monoamines in mice following head injury Regul Pept **70,** 173—178

Tonoue T , Furukawa K , Nomoto T (1979) The direct influence of thyrotropin-releasing hormone on the smooth muscle of rat duodenum Life Sci **25,** 2011—2016

Van Breemen C , Chen Q , Laher I (1995) Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum Trends Pharmacol Sci , **16,** 98—105

Wan R Q , Noguera E C , Weiss S R (1998) Anticonvulsant effects of intra-hippocampal injection of TRH in amygdala kindled rats NeuroReport **9,** 677—682

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