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AN INWARD-RECTIFYING K^+ CURRENT IN CLONAL RAT PITUITARY CELLS AND ITS MODULATION BY THYROTROPHIN-RELEASING HORMONE

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SUMMARY

1. Voltage-dependent K^+ currents were recorded in cultured tumour-derived anterior pituitary cells of the rat (GH_3 cells) with the patch clamp technique. An inward-rectifying current is described which is found to be carried by K^+.

2. In isotonic KCl, whole-cell inward K^+ currents were elicited by hyperpolarizing pulses from a holding potential of −40 mV. These inward K^+ currents showed time- and voltage-dependent inactivation at potentials more negative than −60 mV. Inactivation was faster and more complete at larger hyperpolarizations. Recovery from inactivation was also time- and voltage-dependent. It was faster and more complete with more positive potentials. Time course of inactivation and of recovery from inactivation could be fitted by single exponentials.

3. Two results showed that a steady inward K^+ current is present at −40 mV. The holding current at −40 mV was reduced following complete inactivation of the inward K^+ current during strong hyperpolarizing pulses, and the amplitude of maximum inward K^+ current elicited by hyperpolarization increased after depolarizing pre-pulses of 5 s. The resting conductance was estimated to be 20–30% of the maximum inward-rectifying conductance.

4. The inward K^+ current was drastically reduced by Cs^+ and Ba^{2+}, but not by Ni^{2+} and Co^{2+}. Quinidine, 4-aminopyridine and tetraethylammonium chloride blocked the current. In contrast, dendrotoxin was without effect.

5. Thyrotrophin-releasing hormone (TRH) which induces biphasic secretion of prolactin in GH_3 cells consistently reduced the inward K^+ current in the presence of internal Ca^{2+}. This reduction was abolished if the pipette solution contained guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS; 400 μM), confirming the involvement of G-proteins in the signal transduction pathway.

6. TRH shifted the voltage-dependence of inward K^+ current inactivation to less negative potentials resulting in pronounced K^+ current inactivation in the range of the resting potential of these cells (−40 to −60 mV).

7. In intact cells, closing of K^+ channels would result in a depolarization. The existence of an inward-rectifying K^+ current in GH_3 cells which is able to be reduced

by TRH could readily explain the TRH-induced increase in action potential firing underlying the sustained second phase of secretion.

INTRODUCTION

Thyrotrophin-releasing hormone (TRH) induces a biphasic secretion of prolactin in GH_3 cells (clonal rat pituitary adenoma cells) which is accompanied by changes in their electrical activity. The hyperpolarization during phase 1 and the increase in action potential firing during phase 2 reflect various second messenger activities within the cell. It has been well established that binding of TRH to its receptor activates a G-protein, which is coupled to phospholipase C, and therefore induces an elevation of intracellular Ca^{2+} released from internal stores via inositol trisphosphate (IP_3) (reviewed by Gershengorn, 1986, 1989). This rapid transient increase in intracellular Ca^{2+} underlying phase 1 of secretion leads to the opening of Ca^{2+}-activated K^+ channels resulting in a hyperpolarization. The second or sustained phase of prolactin secretion is accompanied by an increase in the frequency of Ca^{2+} action potentials which is assumed to be due to closing of voltage-dependent K^+ channels (Gershengorn, 1986; Ozawa & Sand, 1986). The second phase is suggested to be mediated by diacylglycerol (DAG) activation of protein kinase C. DAG is produced by the generation of IP_3 from phosphatidylinositol bisphosphate (PIP_2), catalysed by an activated phospholipase C. Activation of protein kinase C has been found to result in a phosphorylation of a group of cytoplasmic phosphoproteins (Beretta, Boutterin, Drouva & Sobel, 1989). Some of these proteins might be involved in the modulation of K^+ channel activity. Although some effects of TRH on the outward K^+ current have been described (Dubinsky & Oxford, 1985), they do not explain the slight depolarization underlying the increased frequency of action potentials. The present paper describes an inward-rectifying K^+ current in GH_3 cells, present at the resting potential and able to be reduced by TRH.

Some of the results have been published in abstract form (Bauer & Schwarz, 1990).

METHODS

Cell culture

The GH strains of rat pituitary tumour cells were established in 1968 by Tashjian, Yasumura, Levine, Sato and Parker. In the present study we used the GH_3/B_6 cell line, kindly provided by Dr A. Tixier-Vidal (Collège de France, Paris, France). The cell culture was maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were grown as monolayers in Ham's medium supplemented with 15% horse serum and 2·5% fetal calf serum. The medium was changed every 2–3 days. For the electrophysiological experiments the cells were plated on 35 mm plastic tissue culture dishes (Nunc).

Electrophysiological recordings

Recordings were made from cells 2–6 days after plating, using the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The electrodes were pulled from 1·5 mm diameter borosilicate glass capillaries (Clark Electromedical Instruments, Reading, England) on a L/M-3P-A puller (List-electronic, Darmstadt, FRG), coated with silicone rubber and fire-polished. Filled pipettes had tip resistances of 3–8 MΩ. The patch electrode was connected to a List EPC7 patch clamp amplifier.

Stimulation and data acquisition were carried out with CED 1401 patch clamp software (Cambridge Electronic Design, Cambridge, England). Data were low-pass filtered at 3 kHz and are shown without correction for leakage current and capacity transients. All experiments were performed at room temperature.

Solutions

Two internal solutions were used which differed in the concentration of free Ca^{2+}. (1) The Ca^{2+}-free solution contained (in mM): 130 KCl; 2 $MgCl_2$; 20 ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) titrated with KOH; 10 HEPES. (2) The Ca^{2+}-containing solution consisted of: 140 KCl; 2 $MgCl_2$; 0·7 $CaCl_2$; 1·1 EGTA; 10 HEPES. The amount of free Ca^{2+} in this solution was estimated to be 236 nM at pH 7·3 (calculated from eqn (1) and data given by Abercrombie, Masukawa, Sjodin & Livengood (1981); concentration of EGTA corrected for Mg-EGTA). Considering a contamination of about 10 μM-Ca^{2+}, the Ca^{2+}-free solution contained considerably less than 1 nM-free Ca^{2+}. In most experiments, Ca^{2+}-free isotonic KCl was used as external solution. It contained (in mM): 130 KCl; 4 $MgCl_2$; 20 EGTA; 5 glucose; 5 HEPES. In experiments with less K^+ in the external solution, equimolar amounts of either Na^+, tris(hydroxymethyl)aminomethane-H^+ (Tris), or choline$^+$ replaced the K^+. Tetrodotoxin (TTX, 500 nM) was added to all external solutions to block Na^+ currents. All solutions were buffered to pH 7·3–7·4 using KOH.

Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), quinidine, dendrotoxin (DTX), Ba^{2+}, Cs^+, Co^{2+} and Ni^{2+}, and thyrotrophin-releasing hormone (TRH) were added to the external isotonic KCl solution. Tamoxifen, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) and 1-oleoyl-2-acetyl-glycerol (OAG) were dissolved in dimethyl sulphoxide (DMSO) and diluted before use so that the final DMSO concentration in the external solution was less than 1%. GDPβS was added to the internal solution. DTX was kindly provided by Dr F. Dreyer (Gießen, FRG). All other chemicals were purchased from Sigma (München, FRG).

External solutions were changed by perfusion of the 35 mm plastic culture dish or by superfusion of the cell with solution from a large pipette (15–20 μm tip diameter) positioned about 30 μm from the cell and connected to a pressure ejection system.

RESULTS

Voltage-dependent K^+ currents

Voltage-dependent K^+ currents were recorded in Ca^{2+}-free internal and external solutions to avoid interactions with Ca^{2+} currents (Matteson & Armstrong, 1984) and Ca^{2+}-dependent K^+ (Lang & Ritchie, 1987) and Cl^- (Rogawski, Inoue, Suzuki & Barker, 1988) currents. Na^+ currents (Matteson & Armstrong, 1984) were blocked by the addition of TTX to the external solution. In saline with low (5 mM) K^+ concentration, the cells studied showed a variable inactivation of the outward currents elicited by depolarization from the holding potential of −80 mV, as previously shown by Pappone & Lucero (1988) and Oxford & Wagoner (1989). With hyperpolarizing pulses only leakage current and capacity transients were clearly visible. To measure K^+ tail currents, experiments were performed in external solution with elevated (70 mM) K^+ concentration. In this case, depolarizing pulses elicited inward and outward K^+ currents which changed direction at *ca* −15 mV, close to the calculated Nernst potential for K^+ (Fig. 1*A*). The time course of K^+ tail currents could not be well fitted by single-exponential functions. In addition to a principal fast component with a time constant of several milliseconds, there was at least one additional component with a time constant of up to a few seconds. A pronounced sag in some of these tail currents also hinted at the existence of a current component which was activated by repolarizing the cell to −80 mV and then

inactivated. In agreement with this interpretation, hyperpolarizations elicited an inward current with distinct activation and inactivation kinetics (Fig. 1*B*), indicating that this current is inward-rectifying. The gating kinetics were quicker and steady-state inactivation was more complete with stronger hyperpolarizations.

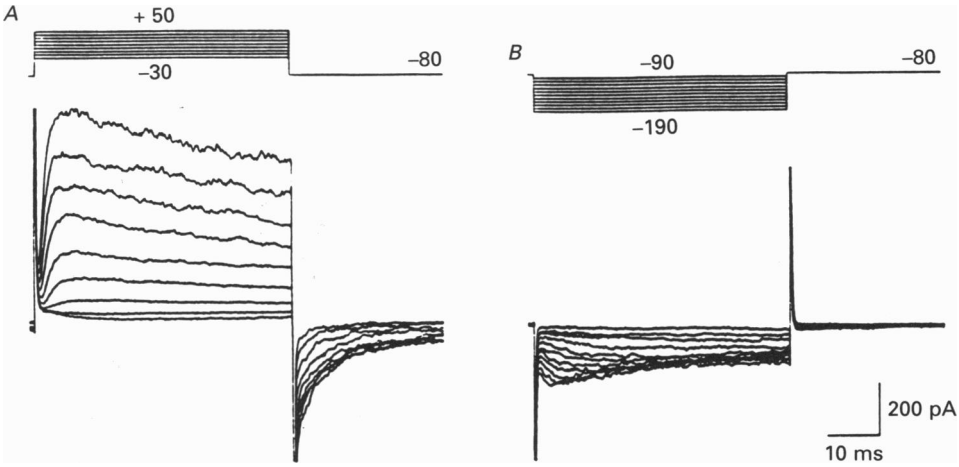

Fig. 1. Voltage-dependent whole-cell K^+ currents recorded with an external solution containing 70 mM-K^+. Pulse programme as indicated above the traces. *A*, depolarizing and *B*, hyperpolarizing pulses elicited outward and inward K^+ currents. Holding potential −80 mV.

An inward-rectifying current was not observed in saline with low K^+ concentration, suggesting that this current is carried by K^+ ions. A number of experiments were performed to check this hypothesis. In all of these experiments, a holding potential of −40 mV was used. This potential is just below the threshold for activation of the voltage-dependent transient outward K^+ current (Oxford & Wagoner, 1989; Pappone & Lucero, 1988) and is within the range of the normal resting potential of these cells (−60 to −40 mV; Ozawa & Sand, 1986).

Analysis of tail currents to determine the reversal potential of the inward-rectifying current was found to be problematic. Upon repolarization after hyperpolarizing pulses, a transient outward-rectifying K^+ current was activated at potentials more positive than −50 mV, within the same potential range where the inward-rectifying K^+ current is suggested to deactivate. Even in experiments where the amplitude of the transient K^+ current was very small, no deactivation of the inward-rectifying current was detected. This suggests an instantaneous rectification of the current upon depolarization, which might be produced by the presence of intracellular Mg^{2+} (Ishihara, Mitsuiye, Noma & Takano, 1989).

To investigate if Na^+ ions contribute to the inward-rectifying current, an external 45 mM-K^+ solution containing 130 mM-Na^+ was exchanged with solutions in which Na^+ was replaced by choline$^+$ (three experiments) or by Tris (three experiments). In all of these experiments, an exchange to the Na^+-free solution produced no alteration of the hyperpolarization-elicited inward current, indicating that the inward-rectifying current is entirely carried by K^+.

In another series of five experiments, the effect of external K^+ concentration on the inward-rectifying current was investigated. The external solution was switched several times from an isotonic KCl solution to a solution containing 57·5 mM-K^+ (K^+ replaced by Na^+). The amplitude of the inward current clearly increased in the isotonic KCl solution, as expected if this current is carried by K^+. This increase (*ca* 50%) was less than the postulated difference in amplitudes calculated on the basis of the 'independence principle' (Hodgkin & Huxley, 1952), but this fact was probably due to saturation, as K^+ concentrations were used which are well above the physiological level. Voltage dependence of activation and inactivation of inward currents elicited by hyperpolarizing pulses were identical in both K^+ solutions. Therefore, the properties of this inward-rectifying K^+ current were investigated in more detail, using isotonic KCl as external solution to maximize the amplitude of the inward K^+ current.

Inward-rectifying K^+ current

Upon changing the external solution to isotonic KCl, the holding current at the potential of −40 mV increased. Hyperpolarizing potential steps revealed a potential-dependent activation and inactivation of the inward-rectifying K^+ current (Fig. 2*A*). Maximum inward current occurred 10–100 ms after the onset of the hyperpolarizing pulse with the shortest latencies at the strongest hyperpolarizations. The time course of inactivation was potential-dependent and could be described by a single exponential. This hints at a single population of K^+ channels underlying the inward

TABLE 1. Time constants of inward-rectifying K^+ current inactivation in GH_3 cells (holding potential −40 mV, $n = 7$) determined at various pulse potentials E

E (mV)	τ_{mean} (ms)	τ_{min} (ms)	τ_{max} (ms)
−80	924	625	1091
−90	715	454	976
−100	502	329	658
−110	296	181	430
−120	203	124	269

current. The time constants of the fitted exponentials decreased with increasing amplitude of the hyperpolarizing pulses (see Table 1). The degree of inactivation can be seen by the difference between peak and steady-state current (Fig. 2*B*). Inactivation occurred at potentials more negative than −60 mV. This potential range is outside the physiological range of membrane potentials in intact cells, probably due to the inactivation characteristics of the inward-rectifying K^+ current and to the small effect of other K^+ currents at these potentials. Following strong hyperpolarizations which completely inactivated the inward-rectifying K^+ current, the holding current was clearly decreased (Fig. 2*A*). This difference in the level of the holding current is suggested to represent the contribution of the inward-rectifying K^+ current to the holding current. Assuming a reversal potential of 0 mV (in symmetrical KCl), the amplitude of the steady-state inward K^+ current at −40 mV corresponded to 32·0 ± 3·8% (mean ± S.E.M., five experiments) of the maximum

specific inward K^+ current ($I_{peak} - I_{ss}$, for pulse potentials E from -120 to -100 mV) activatable from the -40 mV holding potential. As the current already activated at -40 mV immediately starts to inactivate with a strong hyperpolarizing pulse, the difference between the peak current and the level of complete inactivation does not

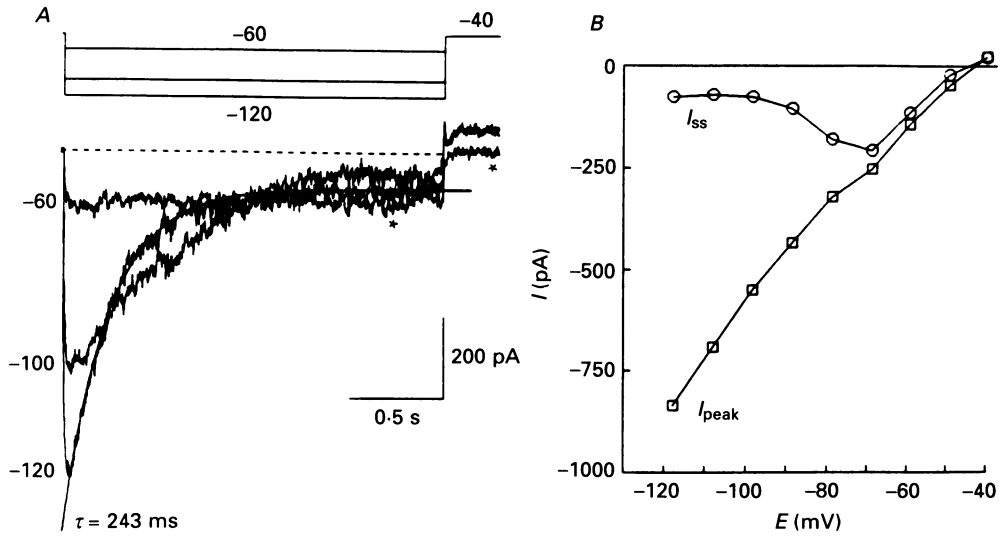

Fig 2. *A*, inward K^+ currents elicited by hyperpolarizing pulses of varying amplitude as shown in the pulse programme at the top. Holding potential -40 mV; external solution: isotonic KCl. A single-exponential function was fitted to the declining part of the current trace during hyperpolarization and is superimposed for a hyperpolarization to -120 mV. The time constant τ of this exponential is 243 ms. The dashed line represents the level of the holding current at -40 mV preceding the pulses. Note that upon repolarization, the holding current only returns to its previous level after the smallest hyperpolarization (*indicates the corresponding current trace, pulse to -60 mV). *B*, current–voltage relationship: the amplitudes of the peak inward currents (I_{peak}) and of the currents at the end of the 2 s hyperpolarizing pulse (I_{ss}) are plotted *versus* test pulse potential.

represent the maximum specific K^+ conductance. Therefore, the value of 32% overestimates the K^+ conductance at -40 mV in relation to the maximum specific K^+ conductance.

A typical inactivation curve of the inward-rectifying current is shown in Fig. 3*B*. Steady-state voltage dependence of inactivation was studied with 5 s pre-pulses to potentials between 0 and -100 mV followed by test pulses to -120 mV. More positive depolarizing pre-pulses were not used to avoid interaction with tail currents of voltage-activated outward K^+ currents. Maximum inward current was activated by the test pulses following depolarizations to potentials between -30 and 0 mV. At membrane potentials between -40 and -60 mV, about 80% of the maximum inward current could be elicited by hyperpolarization. Since time-dependent inactivation of activated K^+ currents did not occur in this potential range (see Fig. 2*B*), the above result suggests that about 20% of the current did not contribute to the peak current due to the fact that this current was already activated at the normal resting potential and immediately started to inactivate upon hyperpolarization

reducing the peak current. As the time course of inactivation was slower than the time course of activation, the current activated at the normal resting potential still contributed to the peak current to some extent. Therefore, this value of about 20% for the steady current between −40 and −60 mV must be regarded as an

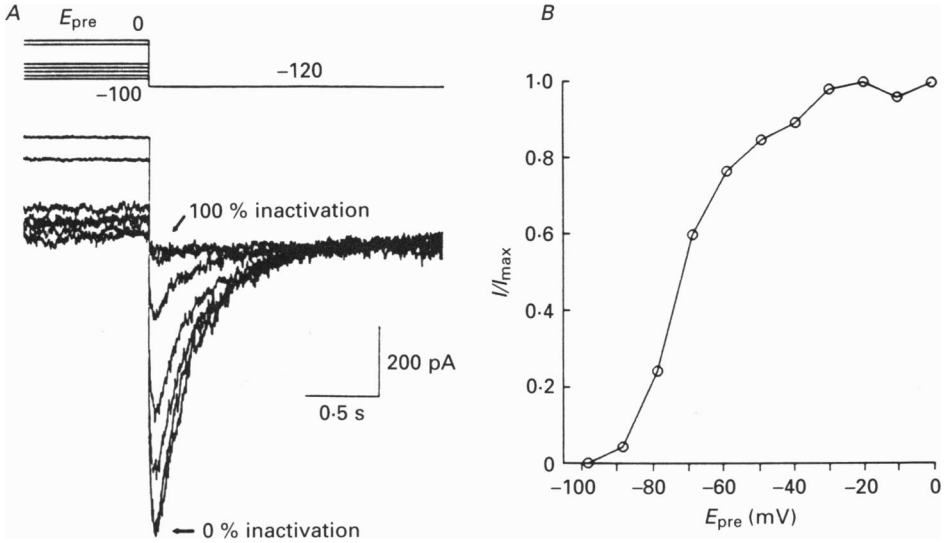

Fig. 3. Inactivation of inward K^+ current produced by a 5 s pre-pulse with varying amplitude (E_{pre}). Holding potential −40 mV. *A*, K^+ currents obtained with the pulse programme indicated above the traces. Maximum inward current (0% inactivation) was elicited by the −120 mV test pulses after depolarizing pre-pulses. No inward current (100% inactivation) could be elicited after a pre-pulse of −100 mV. *B*, ratio of peak inward current to maximum inward current (I/I_{max}) as a function of pre-pulse potential.

underestimation. The above estimations suggest that 20–30% of the maximum inward-rectifying K^+ conductance is activated at −40 mV. Pre-pulses more negative than the resting potential inactivated the inward current and less current could be elicited by the test pulse to −120 mV. At −100 mV pre-pulse potential, inactivation of the inward current was complete. The inactivation time course of the inward currents elicited by the test pulses to −120 mV could also be well fitted by single exponentials. These functions showed no significant difference in their time constants indicating that the same population of K^+ channels was activated, independent of pre-pulse potential and percentage of activatable channels.

In the above experiments, test pulses were applied every 30 s to ensure complete recovery from inactivation, since test pulse application every 5–10 s was found to result in a small reduction of the activatable inward current. To investigate the time course of recovery from inactivation and its voltage dependence, double-pulse experiments were performed (Fig. 4). A 2·5 s hyperpolarizing pulse to −120 mV was applied to completely inactivate the inward-rectifying current. This pulse was preceded by a 3 s pre-pulse to −20 mV to enable measurements of maximum inward current amplitude used as reference. Different holding potentials (−60 to −30 mV)

were then applied for variable durations, and recovery from inactivation was traced with a short test pulse to −120 mV. In Fig. 4*B*, the peak amplitude of the elicited inward current (I_t) in relation to the maximum inward current amplitude (I_{max}) is plotted *versus* duration of the interpulse interval t for differing holding potentials E_h.

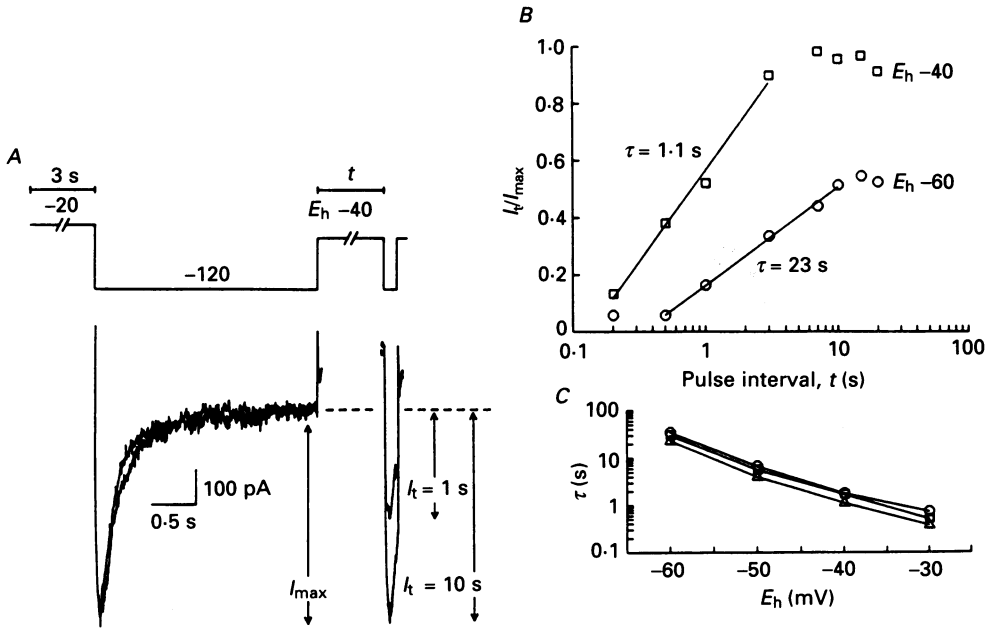

Fig. 4. Time- and potential-dependent recovery from inactivation. *A*, double-pulse experiment. A −120 mV pulse of 2·5 s duration preceded by a 3 s depolarizing pulse to −20 mV elicited a maximum peak inward current (I_{max}) which totally inactivated. Recovery from inactivation was measured with a 150 ms test pulse applied after the interpulse interval t. Holding potential E_h −40 mV in this experiment. *B*, ratio of I_t to I_{max} (linear scale) as a function of the interpulse interval t (log scale) at two different holding potentials E_h. Lines represent the linear regression of I_t/I_{max} on log(t) determined from the indicated range of data. Time constants τ of corresponding single exponentials are given. *C*, time constant τ of recovery from inactivation (log scale) as function of E_h (linear scale). Data of three experiments. Temperature 21 °C.

Recovery was faster and more complete with more positive holding potentials. After a potential-dependent delay, the elicited inward current exponentially increased with time. This phase could be described by single exponentials as indicated by the regression lines in the semilogarithmic plot in Fig. 4*B*. Time constants of recovery from inactivation were potential-dependent and increased from less than 1 s at −30 mV to more than 20 s at −60 mV (Fig. 4*C*).

Pharmacology

The inward K^+ current was reversibly suppressed by external application of Ba^{2+} (5 mM, seven experiments) and Cs^+ (5 mM, four experiments). The block by Ba^{2+} (Fig. 5) and Cs^+ was incomplete and the residual current showed typical kinetics following

application of open channel blockers (Standen & Stanfield, 1978). Addition of 5 mM-Ni^{2+} (four experiments) or 5 mM-Co^{2+} (five experiments) to the external solution did not change the inward K^+ current. Externally applied K^+ channel blockers like quinidine (2 mM, five experiments), TEA (10 mM, five experiments) and 4-AP

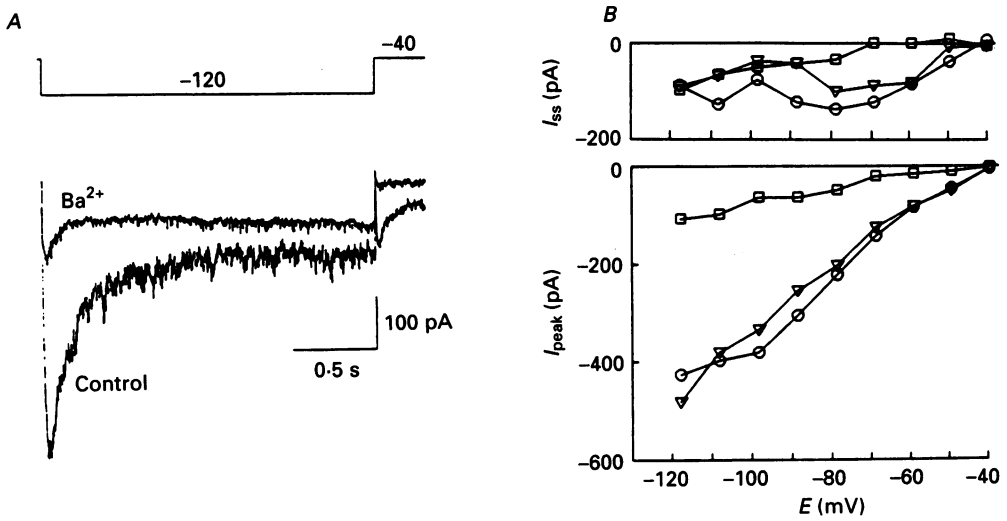

Fig. 5. Reversible block of K^+ inward current by 5 mM-Ba^{2+} in the external solution. *A*, current traces elicited by a hyperpolarizing pulse to −120 mV before (control) and after (Ba^{2+}) changing the external solution. *B*, steady-state (upper graph) and peak (lower graph) inward currents as functions of pulse potential before application, in the presence, and after removal of 5 mM-external Ba^{2+}. ○, control; □, 5 mM-Ba^{2+}; ▽, wash.

(10 mM, six experiments) totally blocked the inward current. DTX (142 nM, four experiments) was without any effect. Figure 6 demonstrates the effect of most of these drugs on the inward K^+ current in a single experiment. The fact that the inward K^+ current is not sensitive to DTX is consistent with the result that DTX (up to 600 nM added to the external solution containing 70 mM-K^+, five experiments) also failed to block the voltage-activated outward K^+ currents (data not shown). In all of these experiments the holding current was diminished if the hyperpolarization-elicited inward current was blocked. This is suggested to be at least partially due to a block of the specific inward K^+ current which has been shown to be present at the holding potential of −40 mV.

TRH effects on the inward-rectifying K^+ current

Given the existence of an inward-rectifying K^+ current in GH_3 cells, the most interesting question was whether this current could be modulated by TRH. To answer this question, whole-cell experiments were carried out where inward currents were elicited by hyperpolarizing test pulses of constant amplitude and at a constant interval. If the pulse duration was sufficiently long to inactivate the inward K^+ current, reproducible control currents were obtained with an interstimulus interval of 30 s. TRH was applied to the bath solution only if repeated inward current responses

had the same amplitude and time course of inactivation. The time between establishing the whole-cell configuration and TRH application ranged from 1 to 7 min. No changes in the response to TRH were observed with different application times. In Ca^{2+}-free internal and external solution, application of TRH had either no

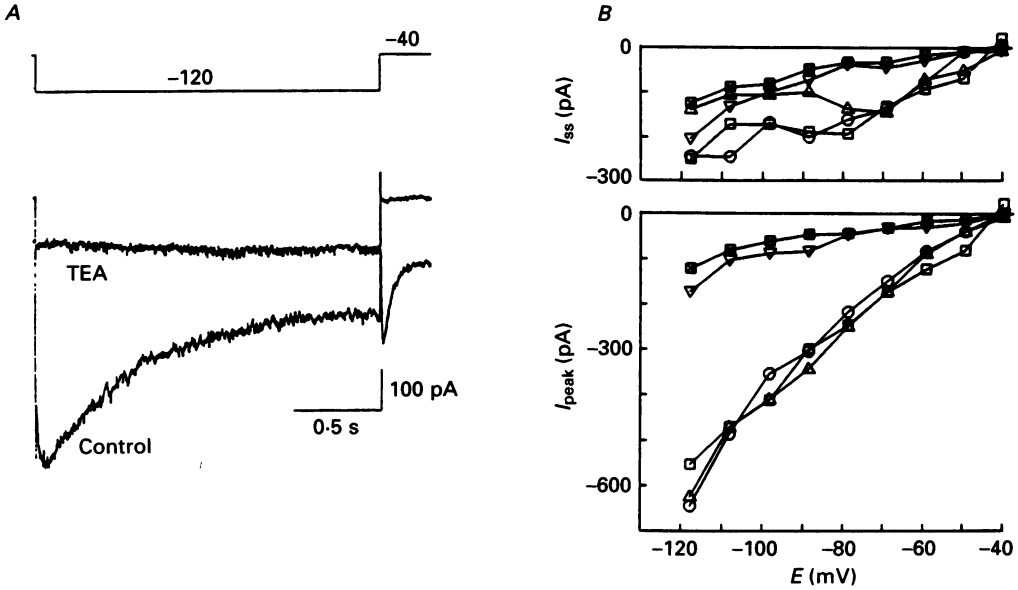

Fig. 6. Effect of K^+ channel blockers on K^+ inward current. *A*, 10 mM-TEA completely blocked the inward current elicited by a hyperpolarizing pulse to −120 mV. *B*, steady-state and peak inward currents as functions of pulse potential. The inward currents are blocked by TEA (10 mM) and 4-AP (10 mM), but not by DTX (142 nM). ○, control; □, 142 nM-DTX; ▽, 10 mM-4-AP; △, wash; ⊠, 10 mM-TEA.

effect or produced only a slight and transient reduction of the inward current (Fig. 7*A*; one example out of five experiments). This current reduction appeared *ca* 30 s after TRH application and lasted for about 1 min. Considering the important role of the Ca^{2+}-dependent protein kinase C in the signal cascade, these experiments were repeated with Ca^{2+} buffered to 240 nM in the internal solution. No difference was observed in the control current responses, confirming that this concentration of free Ca^{2+} was too low to activate Ca^{2+}-dependent K^+ channels (> 300 nM at negative potentials, Ritchie & Lang, 1989). A typical effect of TRH on the recorded current responses is shown in Fig. 7*B*. A few seconds after TRH application the amplitude of the hyperpolarization-elicited inward current increased. This increase, however, is regarded to be the result of an increase in the holding current (continuation of relevant traces are indicated by symbols). This effect can be explained by the opening of the voltage-independent small Ca^{2+}-activated K^+ channel (SK channel, Ritchie & Lang, 1989) during phase 1 of the cellular TRH response. Although the internal solution was Ca^{2+}-buffered with EGTA, Ca^{2+} was probably released from the endoplasmic reticulum resulting in a transient increase of the cytosolic Ca^{2+} concentration which exceeded the threshold for activation of the SK channel

(> 300 nM, Ritchie & Lang, 1989). This period of increased holding current lasted about 10 s, and was shorter than the hyperpolarization during phase 1 (30–40 s, measured in current clamp mode), probably due to the buffering effect of EGTA. Within this period, the amplitude of the K^+ inward current elicited by the

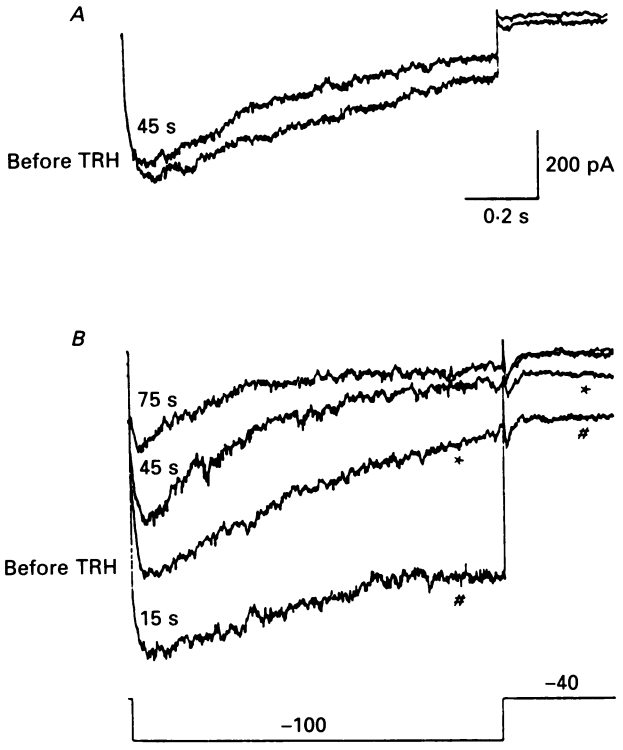

Fig. 7. Effect of 1 μM-TRH on the voltage-dependent K^+ inward current. *A*, measurement without internal free Ca^{2+} ($\ll$ 1 nM) and *B*, with free internal Ca^{2+} of about 240 nM. Currents were elicited by a constant test pulse to −100 mV before and at indicated times after TRH application. Identical symbols denote corresponding traces before and after the end of the impulse.

hyperpolarizing test pulse to −100 mV was increased, because during the pulse to −100 mV, 2·5 times the current at −40 mV would flow through open voltage-independent SK channels. After the closing of the Ca^{2+}-activated K^+ channels, the reaction of the inward-rectifying K^+ channels to application of TRH could be measured separately. TRH consistently reduced the K^+ inward current. In contrast, a small transient inward current which was often activated upon repolarization to −40 mV from a strong hyperpolarizing pulse was not changed (see tail currents of Fig. 7*B*). The amplitude of the inward-rectifying K^+ current decreased with time to about 30 % of the initial peak current amplitude 1–3 min after TRH application. The holding current was also reduced by TRH, suggesting that TRH also induces a closing of K^+ channels which are open at the holding potential. In physiological saline, this would result in a slight depolarization of the membrane potential. The

reduction of inward current was irreversible even after short applications of TRH, indicating a possible wash-out of cytoplasmic factors which normally restore the properties of the channel. This interpretation is supported by Dufy, Jaken & Barker (1987) who reported an irreversibility of increased action potential activity during phase 2 in whole-cell recordings without cell extracts in the patch pipette.

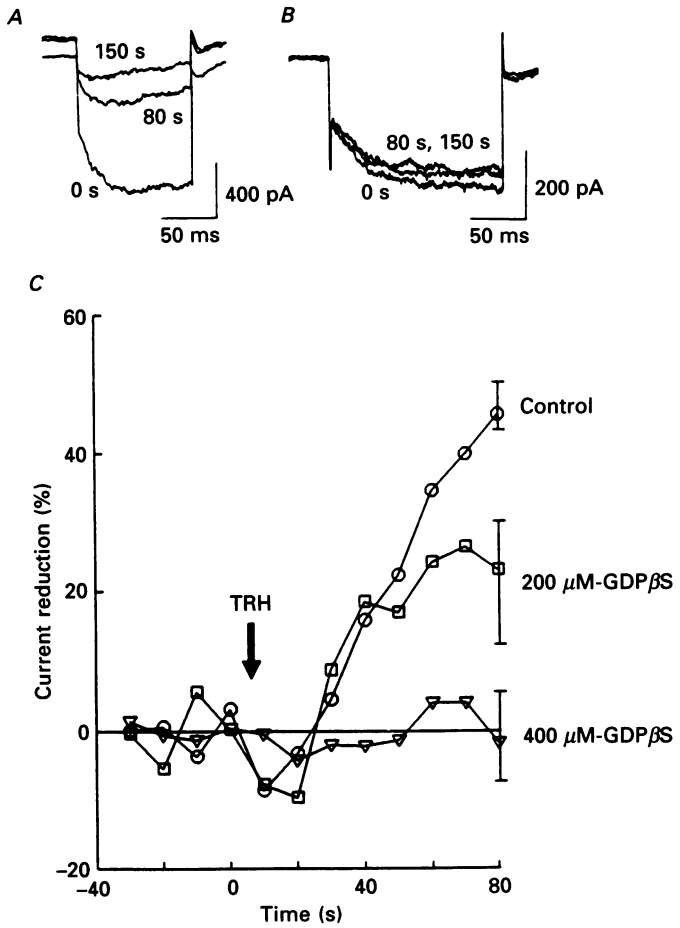

Fig. 8. Time course of the TRH effect and its block by GDPβS. TRH was applied at least 3 min after rupture of the cell membrane. *A* and *B*, current responses to voltage steps from a holding potential of -40 mV to -100 mV immediately before (0 s) and after (80 s and 150 s) application of 1 μM-TRH; *A*, control and *B*, 400 μM-GDPβS in the pipette solution. *C*, reduction of peak inward current after TRH application (arrow) without GDPβS (control; ○), and with 200 μM (□) or 400 μM (▽) GDPβS. Each curve is the average of three experiments. 0% current reduction corresponds to the mean inward current before TRH application. For $t = 80$ s, minimum and maximum values are indicated.

Since TRH is known to act via at least one G-protein (Gershengorn, 1989), we investigated whether the TRH-induced reduction of the inward-rectifying K^+ current is also mediated by activation of G-proteins through experiments with GDPβS (an inhibitor of G-protein activation) in the pipette solution. Shorter

hyperpolarizing pulses of 100–150 ms duration were used to avoid inactivation of the inward current, allowing measurements every 10 s. If TRH was applied to a cell at least 3 min after the establishment of the whole-cell configuration, GDPβS (400 μM) completely inhibited the TRH-induced reduction of the inward K^+ current (Fig. 8). Even 200 μM-GDPβS diminished the effect of TRH on the hyperpolarization-elicited inward current, whereas the activation of the Ca^{2+}-dependent K^+ channels seemed to be unaltered. With 400 μM-GDPβS in the pipette, the holding current was also not able to be changed by TRH. In some experiments where TRH was applied only 1–2 min after rupture of the cell membrane, GDPβS did not block the current response to TRH. This can be explained by the fact that GDPβS needs some minutes to diffuse into the cell and to bind to G-proteins. Therefore GDPβS might work more efficiently at the lower concentration of 200 μM after a prolonged exposure.

Preliminary experiments have been carried out to investigate the involvement of protein kinase C in the modulation of the inward current. OAG (250 μM), an activator of protein kinase C, slightly reduced the hyperpolarization-activated inward current in only two out of six experiments. Also bath-application of the protein kinase C inhibitors tamoxifen (100 μM, two experiments) and H7 (100 μM, two experiments) about 5 min prior to the TRH application did not block the TRH effect on the inward current. In contrast, longer (20–120 min) pre-treatment with tamoxifen (200 μM, six experiments) resulted in a pronounced reduction of the hyperpolarization-elicited inward K^+ current, and no additional current reduction could be induced by TRH (two experiments). In the same cells, the outward K^+ current activated upon depolarization appeared normal.

Voltage dependence of the TRH effect

Since our experiments have shown that the inward K^+ current had its largest amplitude following depolarizing pre-pulses, an extended pulse programme was used to enable an estimation of the portion of K^+ current which remained activatable after TRH application. Experiments were performed where test pulses from the holding potential of -40 mV alternated with test pulses that were preceded by a 3 s depolarizing pulse to 0 mV. Figure 9 demonstrates that the TRH-induced reduction of the inward K^+ current was partly antagonized by a preceding depolarization. Before TRH application, no distinct difference existed between peak inward current amplitudes elicited by test pulses with or without a preceding pre-pulse. Following TRH application, reduction of current amplitude was less pronounced with a preceding depolarization. This effect of the pre-pulse decreased slowly over more than a minute as seen in the continuous decay of the K^+ inward current amplitude elicited by three subsequent test pulses from the holding potential. This suggests that TRH reduced the K^+ current in a voltage-dependent way. With preceding depolarization, the current was only reduced by 40–50%, compared with 70–80% reduction in the absence of preceding depolarizations (data of three experiments).

As the TRH-induced reduction of the inward K^+ current did not reverse, it was possible to compare potential-dependent inactivation of this current before and after application of TRH. The effect of TRH on the inward current was checked by repeated test pulses as shown in Fig. 8. Inactivation curves were not determined until the amplitude of the inward current had declined to a constant level. Figure 10

shows the results of four experiments. The ratio of the peak current amplitude to maximal peak current amplitude is plotted against the potential of the 2 s pre-pulse. In the control experiments, pronounced current reduction due to time- and voltage-dependent inactivation occurred only at potentials more negative than the normal

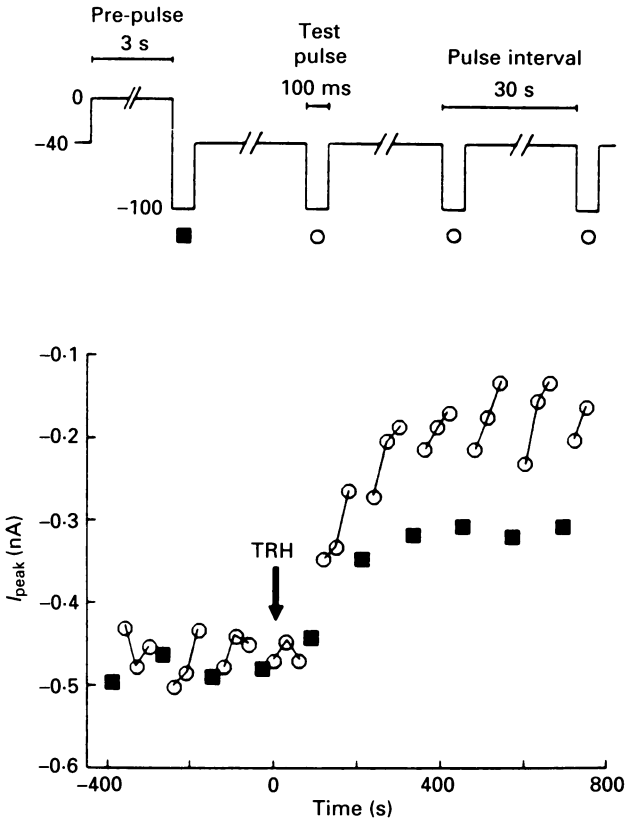

Fig. 9. The reduction by 1 μM-TRH of the peak inward K^+ current can be partially antagonized by a depolarizing pre-pulse. A 3 s pre-pulse to 0 mV was followed by four subsequent test pulses to −100 mV. The intervals between test pulses were 30 s (see pulse protocol). $t = 0$ indicates the last data point before application of TRH (arrow). ■, with pre-pulse; ○, without pre-pulse.

resting membrane potential (Fig. 10*A*, see also Figs 2 and 3). After the TRH response, the peak inward current continuously decreased with more negative pre-pulse potential (Fig. 10*B*). Even with a −70 mV pre-pulse potential, the inward K^+ current nearly completely inactivated. Half-maximal current reduction was achieved between −70 and −80 mV in the control experiments and at about 0 mV following TRH application. Rescaling the mean data (before and after TRH application, Fig. 10*C*) reveals a TRH-induced shift of the inactivation curve to less negative potentials. More positive pre-pulses could possibly enhance the maximum hyperpolarization-activated inward current after TRH. Considering the nearly linear voltage dependence of the inward current amplitude in the negative voltage range near zero,

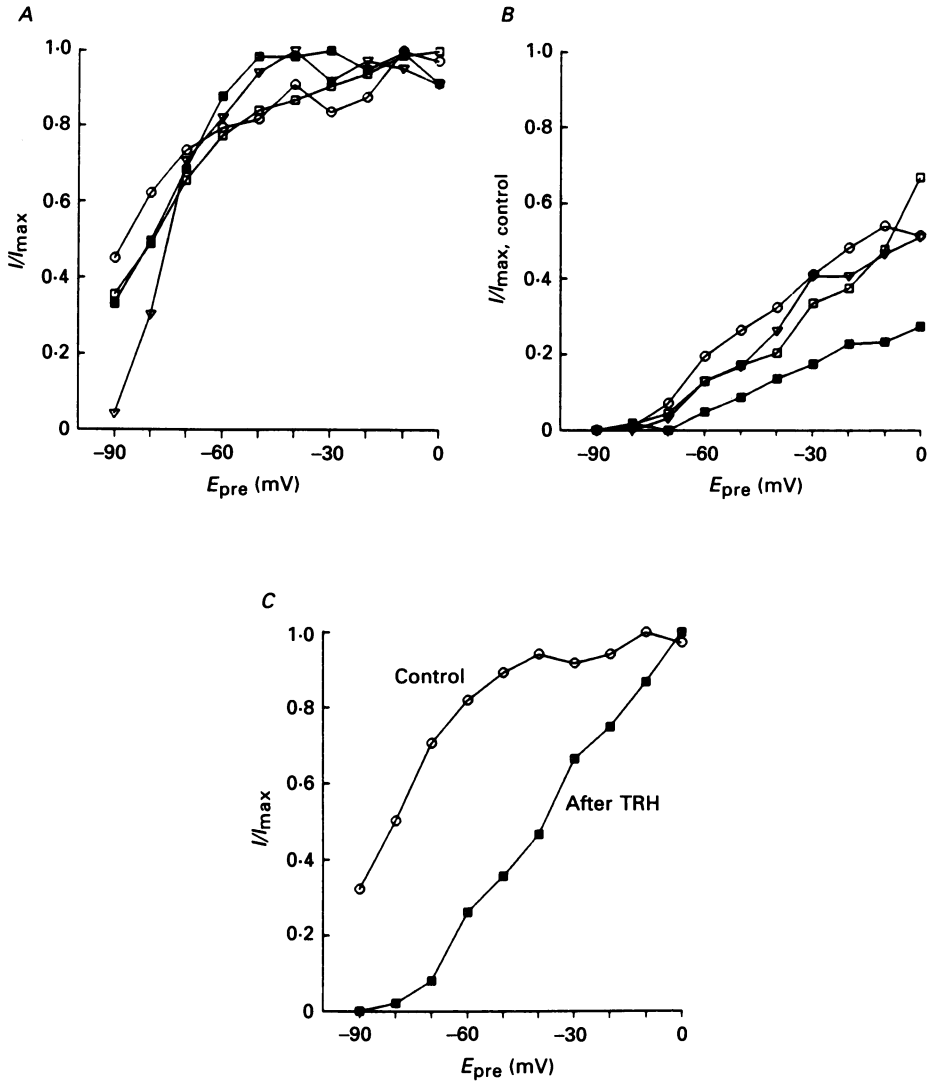

Fig. 10. TRH changed the voltage-dependent inactivation of peak K^+ inward current. *A*, ratio of peak inward current amplitude (*I*) to maximal peak inward current amplitude (I_{max}) as function of 2 s pre-pulse potentials of various amplitudes (E_{pre}). Inward currents elicited with a −120 mV test pulse, data of four cells. *B*, peak K^+ inward currents (*I*) recorded after application of TRH (1 μM) related to the maximal peak inward current measured before TRH application ($I_{max,control}$). Same pulse protocol and cells as in *A*. *C*, the normalized averaged inactivation curves demonstrate a shift to less negative potentials after TRH application (the inactivation curve after TRH was calculated as $I/I_{max,TRH}$).

it cannot be ruled out that the TRH-induced current inhibition could be completely removed by extreme positive pre-pulses.

DISCUSSION

The inward-rectifying K^+ current

This paper describes an inward-rectifying K^+ current in GH_3 cells and its modulation by TRH. In a variety of cell types, a number of anomalous or inward rectifiers has been found (reviewed by Rudy, 1988), differing greatly in their gating behaviour. A potential-dependent time course of activation is described for many inward rectifiers (Hagiwara, Miyazaki & Rosenthal, 1976). Some of these inward rectifiers also show potential-dependent inactivation, although the inward currents may decline with time as a result of a more or less potential-dependent block by Na^+, Ca^{2+}, Ba^{2+} or Mg^{2+} (Hagiwara, Miyazaki, Moody & Patlak, 1978; Ohmori, 1978), or by depletion of K^+ ions in the external solution (Adrian, Chandler & Hodgkin, 1970). A true potential-dependent inactivation of an inward rectifier has been demonstrated in heart cell membranes by Sakmann & Trube (1984) on the single-channel level. Inactivation by strong hyperpolarization as an intrinsic property of the underlying channel is also assumed for the inward-rectifying current described in the present paper. Neither depletion of extracellular K^+ nor accumulation of intracellular K^+ is likely to be the reason for the declining current. In spite of a complete inactivation of the inward-rectifying current during strong hyperpolarizations, an additional small, fast activating and inactivating K^+ inward current often appeared upon repolarization. This additional current is probably a contribution of the inactivating K^+ current described by Oxford & Wagoner (1989), which recovered from inactivation during large hyperpolarizing pulses and would be activated at potentials slightly more positive than -40 mV.

Another current type which is important for modulating the membrane resting potential of a cell is the M current (Adams, Brown & Constanti, 1982). This type of current can be clearly distinguished from the K^+ current described in this paper. Both types of current are present in the range of the resting potential (Adams *et al.* 1982), but the inward currents described in the present paper showed clear activation before they inactivated with stronger hyperpolarizations. The fact that the amplitude of the inward current was maximal and nearly constant with depolarizing pre-pulses to potentials between -30 mV and 0 mV suggests that the channels do not open to any significant degree at these potentials. It is difficult to study this current at more positive potentials since interaction with the outward-rectifying currents occurs, and specific K^+ channel blockers which allow separation of these voltage-dependent currents have still not been found. DTX, the only K^+ channel blocker tested, which did not affect the inward-rectifying current, was also unable to block the outward-rectifying current.

In GH_3 cells, a 55 pS K^+ channel is described which is stimulated by muscarinic receptor activation or by somatostatin and opens at negative potentials (Yatani, Codina, Sekura, Birnbaumer & Brown, 1987). This channel is suggested to mediate hyperpolarization of the cell and thus inhibition of secretion after application of somatostatin which acts antagonistic to TRH. Two main arguments suggest that the

55 pS K^+ channel is not identical to the channel underlying the inward-rectifying K^+ current. (1) GDPβS in the pipette did not alter the hyperpolarization-elicited inward K^+ current, but inhibits the somatostatin-activated GTP-dependent current (Yatani *et al.* 1987). (2) The somatostatin-induced K^+ current in human pituitary tumour cells does not inactivate with more negative pulse potentials (Yamashita, Shibuya & Ogata, 1986, 1988), in contrast to the voltage-dependent inward-rectifying current in GH_3 cells. These cell types seem to be comparable as somatostatin is found to produce similar electrical responses in these cells (Dufy, Mollard, Dufy-Barbe, Manciet, Guerin & Roger, 1988).

The mode of action of TRH

Our results demonstrate that the modulation of the inward-rectifying K^+ current by TRH is mediated by a G-protein. The nature of this G-protein is not yet identified. It might be the same as the cholera-toxin-sensitive G-protein that activates the phosphatidylinositol turnover in GH_3 cells (Yajima, Akita & Saito, 1988). It is also possible that this channel is modulated via other cholera-toxin-sensitive G-proteins or perhaps via a pertussis-toxin-sensitive G-protein. Recently it has been shown by Offermanns, Schultz & Rosenthal (1989) that TRH also activates a pertussis-toxin-sensitive G-protein in GH_3 cells, which is suggested to be a G_i-type G-protein, different from G_{i3}.

Just as unclear as the type of G-protein is the further signal cascade leading to the inhibition of the inward-rectifying K^+ current. Direct coupling of the channel to the G-protein cannot be excluded, although the Ca^{2+} dependence of this modulation seems contradictory. The Ca^{2+} dependence hints at the involvement of the Ca^{2+}-dependent protein kinase C (Takai, Kishimoto, Iwasa, Kawahara, Mori & Nishizuka, 1979) which is shown to enhance action potential frequency in GH_3 cells only in the presence of a minimum of internal free Ca^{2+} (Dufy *et al.* 1987). This fits with the secretion model of Ozawa & Sand (1986) and also with the more recently presented model of Gershengorn (1989). Both models propose that the increased Ca^{2+} influx during phase 2 of secretion is mediated by an activation of the protein kinase C. This suggestion is based on results of several studies which show that an activation of protein kinase C mimics the phase 2 TRH effect. Tumour-promoting phorbol esters have been shown to enhance action potential frequency in GH_4C_1 cells (Østberg, Sand, Bjøro & Haug, 1986) and GH_3 cells (Dufy *et al.* 1987; Gammon, Oxford, Allen, McCarthy & Morell, 1989). Recent publications, though, give hints that TRH and phorbol ester might work in a different way to induce secretion (Gordeladze, Bjøro, Østberg, Sand, Torjesen, Haug & Gautvik, 1988; Gordeladze, Bjøro, Torjesen, Østberg, Haug & Gautvik, 1989) and that protein kinase C is not essential for hormone-induced secretion (French, Moor, Lussier & Kraicer, 1989; Boyd & Wallis, 1989). Villarroel, Marrion, Lopez & Adams (1989) found that protein kinase C is not involved in the normal bradykinin signal transduction, although activation of protein kinase C mimics bradykinin-induced inhibition of the M current in phaeochromocytoma cells. This would fit with the results of our preliminary experiments where inhibitors and activators of protein kinase C failed to alter the effect of TRH on the inward current. However, more experiments are necessary to exclude that these results are due to diffusion effects in the whole-cell mode as

described by Dufy *et al.* (1987). TRH also activates adenylate cyclase in GH_4C_1 cells (Gordeladze *et al.* 1988), and it is not excluded that cyclic AMP is an essential factor in the modulation of the inward-rectifying current by TRH. The GH cells possess receptors for the vasoactive intestinal peptide (VIP), which is shown to act via cyclic AMP (Bjøro, Østberg, Sand, Gordeladze, Iversen, Torjesen, Gautvik & Haug, 1987). In GH_3 cells, VIP has been found to reduce a current which is activated upon hyperpolarization and which is suggested to be a K^+ current (Hedlund, Dufy & Barker, 1988). This interpretation would support a role of cyclic AMP in the modulation of the inward-rectifying current.

The possible role of the inward-rectifying K^+ current in generating phase 2 of secretion

The results indicate that the inward-rectifying K^+ current is modulated by TRH in a Ca^{2+}-dependent way. Control inward currents elicited in Ca^{2+}-free and Ca^{2+}-containing internal solutions showed no difference in the range of amplitudes or in activation and inactivation behaviour, indicating that no additional current type contributes to the inward currents elicited in Ca^{2+}-containing solution (except during phase 1 of the TRH action). Therefore it is concluded that effects of TRH are on the inward-rectifying K^+ current.

The modulation of the inward rectifier by TRH can readily explain the depolarization leading to increased frequency of action potentials. In physiological saline, open channels would mediate a small K^+ outward current at the resting potential. TRH-induced reduction of this outward current would consequently result in a moderate depolarization. The depolarization produced by substance P in rat magnocellular cholinergic neurones has been shown to result from a comparable reduction in an inward-rectifying K^+ current (Stanfield, Nakajima & Yamaguchi, 1985).

The voltage dependence of the TRH-induced inward K^+ current reduction might explain why in spontaneously active cells, the TRH-induced depolarization during phase 2 is sometimes very small. The long-lasting Ca^{2+} action potentials could act in a manner similar to depolarizing pre-pulses and produce a partial release of the TRH block. After repolarization, the threshold for generating an action potential would be reached faster because TRH leads to a reinforced inactivation of the inward-rectifying K^+ channel in the range of the resting potential. In this way, action potential frequency could be increased without an underlying sustained depolarization. This is supported by the observation that a phase 2 depolarization is more pronounced in initially silent cells or in normal lactotrophs as has been reported by Dufy *et al.* (1988). Normal pituitary lactotrophs show little or no spontaneous activity (Ingram, Bicknell & Mason, 1986; Lingle, Sombati & Freeman, 1986).

Although the existence of an inward-rectifier in GH_3 cells and its modulation by TRH is demonstrated, there might exist other ion channels which are modulated by TRH via the same or different signal pathways and which together ensure the increased Ca^{2+} influx during phase 2 of secretion. Candidates for this function are Ca^{2+} channels, as has been shown for the peptide LHRH (Rosenthal, Hescheler, Hinsch, Spicher, Trautwein & Schultz, 1988), or Ca^{2+}-dependent K^+ channels, although a direct modulation of these channels by TRH could not be demonstrated in GH_3 cells (Ritchie & Lang, 1989).

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