BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 142–147 (1996)
Article No. 0025

Regulation of Single Cl^- Channel Conductance by Insulin and Tyrosine Phosphatase

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Received November 14, 1995

The apical membrane of distal nephron epithelium (A6) had a Ca^{2+}-activated outwardly rectifying Cl^- channel with single-channel conductances of 3 pS for outward current and 0.8 pS for inward current. The single-channel conductance for inward current was dependent on cytosolic Ca^{2+} concentration. Insulin increased the single-channel conductance for the inward current by increasing Ca^{2+} sensitivity about 300-fold. The insulin action was diminished by vanadyl hydroperoxide (vanadate, an inhibitor of protein tyrosine phosphatase (PTP)). Application of protein tyrosine kinase, p60c-src, reversibly diminished the insulin-induced increase in single-channel conductance. Further, the application of PTP to the cytosolic surface of the inside-out patch membrane, like insulin, increased single-channel conductance. PTP-mediated dephosphorylation of the phospho-tyrosine of the channel protein, as a mechanism of intracellular signaling of insulin action, is a novel mechanism for regulating single-channel conductance by modulating Ca^{2+} sensitivity.

Insulin has various effects on cell metabolism, glucose and ion transport (1–4). Activation of the protein tyrosine kinase (PTK) involved in the insulin receptor is the first step and one of the most important signal-transduction pathways in insulin action (5–8). On the other hand, it has been suggested that the functions of ion channels are modulated by protein phosphorylation and dephosphorylation (8,9). However, little information is available about how insulin acts on remote effectors like ion channels in the apical membrane after activating the PTK of the receptor located in the basolateral membrane of the cell. Here we report that insulin increased single-channel conductance of the Ca^{2+}-activated Cl^- channel by dephosphorylating the tyrosine of the channel through activation of protein tyrosine phosphatase (PTP).

MATERIALS AND METHODS

The methods and materials were similar to those used in previous studies (2,10). Briefly, A6 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) in the 68th plating. Cells were maintained in plastic culture flasks at 27°C in an incubator with 4% CO_2 in air. All the experiments were carried out on the 75th–83rd platings. The culture medium was NCTC-109 medium (GIBCO, Grand Island, NY, USA) modified for amphibian cells (NaCl 100 mM, $NaHCO_3$ 20 mM, pH 7.4), in which 10% fetal bovine serum (FBS, GIBCO) and 10,000 μg/ml streptomycin and 10,000 U/ml penicillin (Irvine Scientific, Santa Ana, CA, USA) were added. For the patch-clamp experiments, A6 cells were subcultured for 10–14 days on a permeable support filter (Nunc Tissue Culture Inserts, Roskilde, Denmark) to form a polarized monolayer. In addition, 1 μM aldosterone (Sigma Chemical Co., St. Louis, MO, USA) was added to stimulate transports. Standard patch-clamp techniques were used (2,10,11). Patch pipettes were made from LG 16 glass (Dagan Corporation, Minneapolis, MN, USA) and fired-polished to produce tip diameters of about 0.5 μm. The patch pipette was applied from the apical side; we made a gigaohm seal (>100 gigaohm) on the apical membrane of cells. Single channel currents from cell-attached and excised inside-out patches were obtained at 22–23°C with an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Current signals were recorded on a digital video recorder (HF860D, Sony, Tokyo, Japan) with pulse-code modulation (1-DR-390, Neuro Data Instruments Corporation, New York, NY, USA), and then digitized and analyzed with a continuous-data acquisition program. A 200-Hz low-pass filter was used to demonstrate the single channel current. The bathing and pipette solutions contained 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES with pH of 7.4. The solution of the patch pipette contained 50 μM amiloride to

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0006-291X/96 $12.00

block amiloride-blockable sodium channels that were frequently observed in the apical membrane of A6 cells (12). Further, to block K^+ channels that were observed in some cases, we also added 2 mM $BaCl_2$ to the pipette solution. Vanadyl hydroperoxide (vanadate), an inhibitor of PTP, was prepared as Bianchini *et al.* have previously described (13). Briefly, equimolar concentrations of sodium orthovanadate and H_2O_2 (10 mM) were mixed, then incubated for 15 min at 22°C. To remove residual H_2O_2, 200 μg/ml of catalase was then added. Insulin was obtained from Sigma. Lavendustin A was obtained from BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA). PTP and catalase were obtained from Boehringer Mannheim GmbH (Mannheim, Germany); sodium orthovanadate was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA); c-src kinase (p60c-src) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA); PKA catalytic subunit was obtained from Sigma; okadaic acid was obtained from Research Biochemicals International (Natick, MA, USA). The data show the mean ± SD. Where SD bars are not visible, they are smaller than the symbol. The *t*-test and ANOVA were used for statistical analysis as appropriate and the p value < 0.05 was considered significant. When the p value was less than 0.05, it is marked by *.

RESULTS

The apical membrane had an outwardly rectifying Cl^- channel whose single-channel conductances for outward and inward currents were respectively 3 and 0.8 pS (Figs. 1A-a and B). The reversal potential was around the resting apical membrane potential in cell-attached patches. Insulin (1 μM) applied from the basolateral side increased the amplitude of the inward current, but did not affect the amplitude of the outward current (Figs. 1A-b and B). Namely, insulin increased the single-channel conductance for inward currents from 0.8 to 2.9 pS, resulting in a linear current-voltage relationship (Fig. 1B). The single-channel conductance depended on cytosolic Ca^{2+}; the insulin-induced increase in single-channel conductance was due to an increase of 300-fold in the sensitivity to cytosolic Ca^{2+} (Fig. 1C).

We tested whether vanadate (13,14) could have any effect on the single-channel conductance (Fig. 2). Vanadate (100 μM, an inhibitor of PTP) by itself had no effect on the basal single-channel conductance in the cell-attached condition (Fig. 2A). However, treatment with vanadate diminished the insulin action on single-channel conductance (Fig. 2A). This observation indicates that some part of the insulin action on the single-channel conductance is mediated through activation of PTP. To confirm the effect of PTP on the single-channel conductance, we directly applied PTP (25 mU/ml), a well-characterized 34 kD-fragment containing the catalytic domain, to the cytosolic surface of inside-out patch membrane that contained the Cl^- channel at 1μM cytosolic Ca^{2+}. No significant inward current could be observed in the inside-out patch without application of PTP at 1μM cytosolic Ca^{2+} (Control in Fig. 2B-a), while about 0.3 pA inward currents were observed in the same inside-out patch when cytosolic Ca^{2+} concentration was increased to 1 mM (data not shown). After application of PTP inward currents were observed even at 1 μM cytosolic Ca^{2+} (PTP in Fig. 2B-a). Namely, application of PTP increased the single-channel conductance to those reached with insulin (Fig. 2B-b).

Finally, to study whether the increased single-channel conductance of the insulin-activated Cl^- channel is reversibly decreased by phosphorylation of tyrosine, we applied p60c-src (12.5 U/ml), a well-characterized PTK (9,15) and ATP (2 mM) directly to the cytosolic surface of inside-out patch membrane containing the insulin-activated Cl^- channel. The single-channel conductance of 3 pS at 1 μM cytosolic Ca^{2+} through the insulin-activated Cl^- channel was diminished by the addition of src kinase and ATP (Fig. 3). However, when the cytosolic Ca^{2+} was increased to 1 mM this diminished conductance at 1 μM cytosolic Ca^{2+} increased to a level similar to that before application of p60c-src (a PTK) and ATP, suggesting that the application of src kinase with ATP decreases Ca^{2+} sensitivity. Only the application of ATP could not diminish the single-channel conductance of the insulin-activated Cl^- channel, suggesting that the patch membrane does not have membrane-associated PTK (data not shown) and the diminution of the single-channel conductance is caused by phosphorylation through PTK but not by ATP itself.

To examine the contribution of protein kinase A and serine/threonine phosphatase to single-channel conductance, we investigated the effect of protein kinase A (PKA) and okadaic acid, an inhibitor of serine/threonine phosphatase. The application of catalytic subunits of PKA (10 μ/ml)

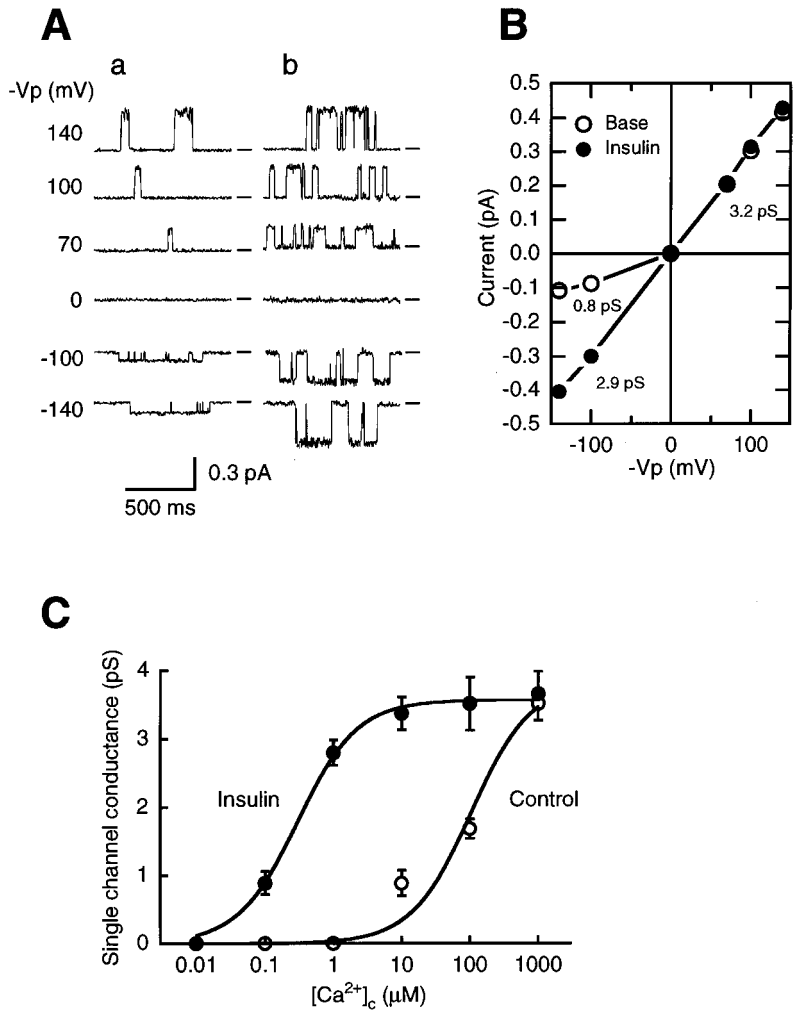

FIG. 1. Single 3 pS Cl^- channel activity(A),the current–voltage relationship (B), and the dependency of single channel conductance on cytosolic Ca^{2+} concentration (C) with and without insulin (1 μM) treatment. (**A**) Single channel currents were recorded at various applied potentials in cell-attached patches obtained from the cell before (a, base) and after addition of 1 μM insulin (b, insulin). At $-Vp = 100$ mV, the intracellular potential of the patch membrane, referred to as the intra-pipette potential (extracellular potential), was 100 mV more positive than that of the resting membrane potential. The closed level of the single channel within the patch is marked with a horizontal dash at the right of each trace. Upward deflection indicates outward current across the patch membrane. The currents in Fig. 1A-a (base) were recorded from a cell not treated with insulin. The currents in Fig. 1A-b (insulin) were recorded from the same cell about 10 min after the addition of 1 μM insulin from the basolateral side. (**B**) The current–voltage relationship was obtained from cell-attached patches formed on cells 10 min after treatment with 1 μM insulin ($n = 5$; closed circles) and without insulin treatment (base, $n = 6$; open circles). (**C**) The effect of insulin on cytosolic Ca^{2+} dependency of single channel conductance for the inward current of the Cl^- channel in inside-out patches. The single channel conductance (code conductance) shown here was measured at a holding potential of −100 mV in inside-out patches ($n = 5$). The curves drawn here were obtained from the best fit to the observed values, based on the assumption that the number of binding sites of cytosolic Ca^{2+} to the channel is one. The maximum values of the single channel conductance at a holding potential of −100 mV in inside-out patches obtained from insulin-untreated (control) and insulin-treated cells were 3.8 pS and 3.6 pS, respectively. Cytosolic Ca^{2+} concentrations ($K_{1/2}$) inducing the half-maximum amplitude of channel conductance obtained from control and insulin-treated cells were 103 μM and 0.3 μM, respectively. Insulin treatment was done by the addition of insulin (1 μM) from the basolateral side for 10 min before access of the patch pipette to the cells and making the inside-out patches. The data show the mean ± SD.

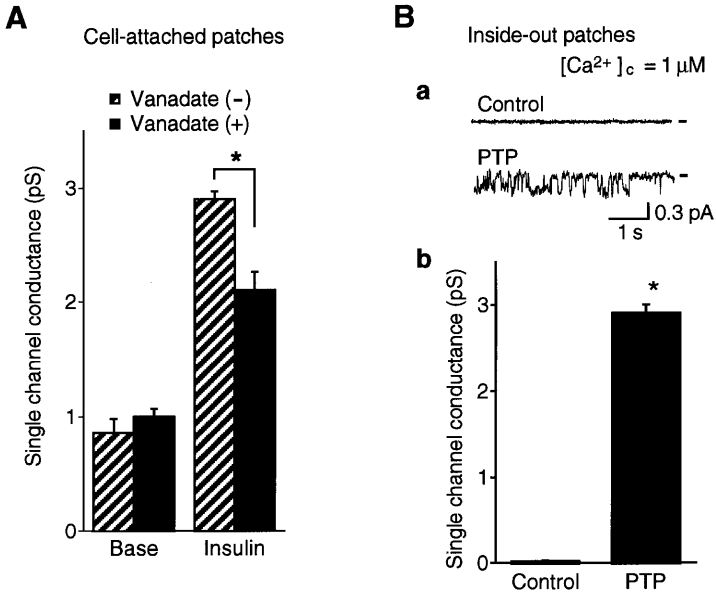

FIG. 2. The effect of a PTP inhibitor (vanadate) and PTP on the Cl^- channel. **(A)** The insulin action on the single channel conductance in cells with and without vanadate pretreatment. The insulin action on the single channel conductance was measured 20 min after its application. The vanadate (100 μM) action on the single channel conductance was measured 30 $\sim$ 50 min after its application. The insulin action with pretreatment of vanadate was measured at 20 min after insulin application subsequently to 20 min preincubation with vanadate; vanadate was present even after application of insulin. **(B) a,** The upper trace (Control) shows a single channel current in an inside-out patch obtained from a cell without insulin treatment in a bathing solution containing 1 μM Ca^{2+} (cytosolic Ca^{2+}) at a holding potential of −100 mV. The lower trace shows a single channel current obtained from the same inside-out patch as the upper trace 5 min after the application of PTP (25 mU/ml) directly to the cytosolic surface of the patch membrane; **b,** the statistical values of the single channel conductances without insulin treatment at 1 μM cytosolic Ca^{2+} (Control) and at 5 min after application of PTP (25 mU/ml; PTP). The data represent the mean ± SD ($n = 4$).

and ATP (2 mM) to the cytosolic surface of inside-out patch membrane caused no change in the insulin-increased single-channel conductance. The treatment with okadaic acid (1 μM, 60 min) did not affect the single-channel conductance or block the insulin action on single-channel conductance in cell-attached patches (data not shown).

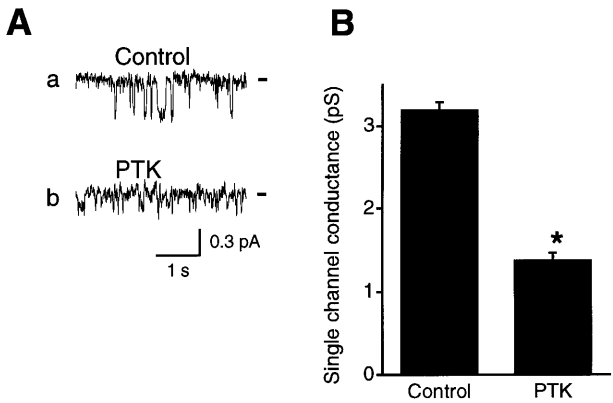

FIG. 3. Effects of PTK (p60c-src) on the insulin-activated Cl^- channel conductance. **(A)** Actual traces obtained in an inside-out patch from an insulin-treated cell before **(a)** and after **(b)** PTK application. A holding potential was −100 mV. **(B)** Single channel conductances in inside-out patches obtained from cells treated with insulin at 1 μM cytosolic Ca^{2+} before (Control) and 10 min after application of PTK (12.5 U/ml) with 2 mM ATP.

Taken together, these observations suggest that insulin could act on the single-channel conductance by activating PTP, which in turn dephosphorylates the Cl^- channel. Furthermore, dephosphorylation of the channel's (or channel-associated protein's) tyrosine increases the single-channel conductance by increasing sensitivity to cytosolic Ca^{2+}, which suggests that tyrosine involved in the channel protein or channel-associated protein plays a role as a sensor for cytosolic Ca^{2+} concentration and that its sensitivity is regulated by phosphorylation and dephosphorylation.

DISCUSSION

It is well known that one of the most important key enzymes in the signal transduction of the insulin action is PTK, which is involved in the insulin receptor (5,16–18). Insulin induces the autophosphorylation of the receptor's tyrosine that results from the activation of PTK (17,18). The activated PTK involved in the insulin receptor does not directly affect effector molecules located in the apical membrane, but rather has a high affinity for potential cytoplasmic substrates and the ability to phosphorylate them (17). Further, the activated substrates can bind to various effector molecules, including intracellular PTPs (16,19,20). Recently, the insulin-receptor substrate-1 (IRS-1), which is a cytoplasmic protein that has a high affinity for the kinase-activated insulin receptor, has been reported (16,17,21). SHPTP2, a PTP that contains two SH2 domains and interacts with IRS-1 after insulin stimulation, has been also reported as an important effector molecule for insulin (20–22).

The interaction of the insulin receptor with cytosolic proteins like IRS-1 may enable post-receptor signaling to affect remote effectors such as ion channels located in the apical membrane apart from the basolateral membrane where the insulin receptor is located. A possible explanation for the observations of the present study is that insulin activates cytoplasmic PTP, such as SHPTP2, through activation of IRS-1, and the increase in PTP activity causes the dephosphorylation of phospho-tyrosine in the Ca^{2+}-activated Cl^- channel. This dephosphorylation of the channel protein augments its sensitivity to the cytosolic Ca^{2+}, altering its single-channel conductance. Dephosphorylation-induced modulation of the sensitivity to cytosolic Ca^{2+} may be the mechanism that regulates ion transports by the Ca^{2+}-activated channel; further, the identification of intermediate molecules such as IRS-1 in the post-receptor pathway may provide more details about insulin signaling for the regulation of ion channels and transport by activation of PTP.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid from the Kidney Foundation of Canada and partially by a grant-in-aid from the Medical Research Council (Group Grant in Lung Development, Project # 9) to Yoshinori Marunaka. Yoshinori Marunaka is a scholar of the Medical Research Council of Canada. This paper was prepared with the assistance of Editorial Services, The Hospital for Sick Children, Toronto, Ontario, Canada.

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