Role of Tyrosine Phosphorylation in Potassium Channel Activation

FUNCTIONAL ASSOCIATION WITH PROLACTIN RECEPTOR AND JAK2 TYROSINE KINASE*

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Chinese hamster ovary (CHO) cells, stably transfected with the long form of the prolactin (PRL) receptor (PRL-R) cDNA, were used for PRL-R signal transduction studies. Patch-clamp technique in whole cell and cell-free configurations were employed. Exposure of transfected CHO cells to 5 nM PRL led to the increase of Ca2+- and voltage-dependent K+ channel (KCa) activity. The effect was direct as it was observed also in excised patch experiments. A series of tyrosine kinase inhibitors was studied to investigate the possible involvement of protein tyrosine kinases in KCa functioning and its stimulation by PRL. Genistein, lavendustin A, and herbimycin A decreased in a concentration and time-dependent manner the amplitude of the KCa current in whole cell and the open probability of KCa channels in cell-free experiments. The subsequent application of PRL was ineffective. The protein tyrosine phosphatase inhibitor orthovanadate (1 mM) stimulated KCa channel activity in excised patches, indicating that channels can be modulated in opposite directions by protein tyrosine kinase and protein tyrosine phosphatase. Moreover, in whole cell experiments as well as in excised patch recordings, anti-J AK2 tyrosine kinase antibody decreased the KCa conductance and the open probability of the KCa channels. Subsequent application of PRL was no longer able to stimulate KCa conductance. Immunoblotting studies using the same anti-J AK2 antibody, revealed the constitutive association of J AK2 kinase with PRL-R. Preincubation of anti-J AK2 antibody with the J AK2 immunizing Peptide abolished the effects observed using anti-J AK2 antibody alone in both electrophysiological and immunoblotting studies.

We conclude from these findings that these KCa channels are regulated through tyrosine phosphorylation/dephosphorylation; J AK2 tyrosine kinase, constitutively associated with PRL-R, is implicated in PRL stimulation of KCa channels.

Prolactin (PRL) is a multifunctional pituitary hormone involved in the control of a wide variety of physiological processes in vertebrates, including lactation, reproduction, immune responses, and osmoregulation, as well as cell proliferation (1–3). The PRL receptor (PRL-R) belongs to the cytokine-growth factor receptor superfamily that includes receptors for growth hormone, erythropoietin, numerous hematopoietic interleukins (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, granulocyte colony-stimulating factor, granulomacrophage colony-stimulating factor, and ciliary neurotrophic factor (4, 5). This family of receptors possesses common structural motifs, both external (two disulfide loops and the WXSXW homology box) and internal (proline-rich homology box 1). Recent studies have been marked by considerable progress in understanding the mechanisms of intracellular signaling for the different members of this family, particularly for PRL-R. Most of the data were obtained in the PRL-dependent rat T lymphoma cell line Nb2. It has been shown by several groups that, following binding of PRL to the PRL-R in these cells, dimerization of the receptor occurs (6, 7) prior to phosphorylation of an associated tyrosine kinase (J AK2). This represents the first event in the process of PRL-R signal transduction (8, 9). Other studies demonstrated that PRL stimulation of Nb2 cells induced a concentration- and time-dependent activation of another protein tyrosine kinase, p59fyn, from the Src protein tyrosine kinase family (10). On the other hand, more and more studies demonstrate an important role of ion channels in receptor signal transduction (11–22). Several different mechanisms have been proposed for channel involvement in signal transduction: direct agonist effect on the channel (15, 16), second messenger participation in channel modulation (17–19), and regulation by kinases and phosphatases through channel phosphorylation/dephosphorylation processes (11, 20–22). Moreover channel regulation by phosphorylation has been shown to play a key role in physiological processes such as proliferation and transformation (23–25). Nothing, however, is known about the putative role of channel phosphorylation in cytokine-growth factor receptor superfamily signal transduction and, in particular, in PRL-R signal transduction.

The signal transduction mechanism for the full-length PRL receptor has been studied using a CHO line stably transfected with the cDNA of the long form of rabbit mammary PRL-R (26). These CHO-transfected cells responded to PRL by stimulating the cotransfected milk protein gene promoter (27), proving that such cells are fully capable of transmitting the PRL signal and that PRL-R is functional. In a series of studies using patch clamp and microfluorimetric techniques, we analyzed the first steps of the PRL-R signal transduction pathway: an increase in intracellular Ca2+ (28, 29) and direct stimulation of calcium-and voltage-activated potassium channels (KCa) by PRL (29). These observations suggested the existence of a regulatory complex involving a protein kinase tightly associated with KCa.
channels and PRL-R. Furthermore, by immunoblotting studies we presented evidence for the tyrosine phosphorylation of this type of PRL-R, the association of JAK2 tyrosine kinase with the receptor, as well as changes in tyrosine phosphorylation of a number of cytoplasmic proteins (30).

In this article, we report on the very first steps in PRL-R-mediated signal transduction at the plasma membrane level: we demonstrate an endogenous large conductance $K_{\text{Ca}}$ channel as the primary ionic event triggered by PRL. These $K_{\text{Ca}}$ channels are constitutively regulated through tyrosine phosphorylation/dephosphorylation. We also show that JAK2 tyrosine kinase, associated with PRL-R, is implicated in the stimulation of $K_{\text{Ca}}$ channels by PRL.

**EXPERIMENTAL PROCEDURES**

Cell Culture—We used CHO cells transfected with PRL-R-cDNA (CHO E3) as described previously (31). Different subclones were challenged for PRL binding, and one of them (E32), exhibiting the highest binding capacity (12% specific binding versus 4% for E3) was used in these experiments. The PRL receptor in E32 clone has a $K_{d}$ of $10.8 \times 10^{-9} \text{M}^{-1}$ which is higher than that of the parental E3 clone (32), but the same number of sites (about 9000). The cells were grown in Ham's F-12 medium (Seromed, Strasbourg, France) containing 10% (v/v) fetal calf serum. Cells were harvested by trypsinization and counted. Cells were maintained at 37 °C in a humidified atmosphere gassed with 95% air, 5% CO$_2$. In order to avoid occupancy of PRL receptors by lactogenic factors contained in the serum of the culture medium, 6–24 h before the experiments cells were transferred into a serum-free medium (32). This medium was derived from the GC3 medium described by Gasser et al. (33) and is a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Seromed) supplemented with nonessential amino acids (Life Technologies, Inc.), insulin (Sigma; 80 milliunits/ml), amino acids (Life Technologies, Inc.), insulin (Sigma; 80 milliunits/ml). Glutamine (Sigma, 2.5 mM), and transferrin (Life Technologies, Inc., 10 mg/ml).

Electrophysiological Recordings—The cultures were viewed under phase contrast with a "Leitz-Diavert" (Leitz, Germany) inverted microscope equipped by examining the record for a "giga seal" (Germany) micromanipulators. Grounding was through a silver chloride-coated silver wire inserted into an agar bridge (4% agar in electrode solution). An Axopatch-1D amplifier (Axon Instruments, Inc., Foster City, Ca) was used for tight seal, whole cell, and cell-free voltage clamping. Stimulus control and data acquisition and processing were carried out with a PC computer AT-80386 (Tandon, Moorpark, CA), fitted with a Labmaster TL-1 interface, using Pclamp 5.5.1 software (Axon Instruments, Inc., interface and software). Electode offset was balanced before forming a "giga seal." Leakage and capacitive current subtraction protocols were composed of four or five hyperpolarizing pulses, one-fourth or one-fifth pulse, respectively, and were applied from a holding potential before test pulses eliciting active responses. During data analysis, leak data were subtracted from the raw data. Series resistances were compensated and were calculated before and after compensation. Recordings were stopped by washing E32 cells three times with cold buffer (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na$_2$VO$_3$ (pH 7.5)). Immediately thereafter, cells were in lysis buffer (20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% glycerol, 1% Brij 96, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$, and 5 mM meroatin plus 2 mM leupeptin (pH 7.5) and left 30 min at 4 °C. After centrifugation at 15,000 rpm for 10 min, prolactin receptor complexes were immunoprecipitated with anti-JAK2 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) and harvested with protein G-Sepharose beads. After extensive washes, immune complexes were eluted by boiling in SDS sample buffer (0.0625% Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% beta-mercaptoethanol). Samples were loaded onto an 8% Laemmli gel, and after completion of the run, gels were transferred. Densitometric analysis of proteins transferred to nitrocellulose was performed after staining with Coomassie blue. The membranes were then probed with the indicated antibodies in blocking buffer for 1 h (S46 anti-JAK2 A and herbimycin A were from Life Technologies, Inc. Antibody anti-JAK2 B and AK2 2 immunizing peptide were purchased from Upstate Biotechnology, Inc., New York, UK). Immune complexes were detected by enhanced chemiluminescence (ECL).

Membrane Stripping and Rehybridization—In order to rehybridize the membranes with other antibodies, they were stripped 30 min at 60 °C in 62.5 mM Tris (pH 6.7). 2% SDS, 100 mM beta-mercaptoethanol. After extensive washes, the membranes were then processed as described above.

**RESULTS**

As was demonstrated by our previous studies (35), a 210-kDomsion K$^{+}$ conductance, dependent on voltage and intracellular Ca$^{2+}$, was revealed by patch-clamp experiments in...
CHO cells. To check whether the KCa channels could be phosphorylated, we studied the effect of ATP, which serves as a substrate for protein kinase, on the activity of the KCa channels in CHO cells. Experiments using Mg-ATP (10–100 M), applied to the cytoplasmic side of the membrane, showed an increase in the open probability of the channel, proving that a protein kinase is tightly associated with it. Fig. 1 shows the very low KCa channel open probability values in the absence of ATP in the internal solution and the much higher values in the presence of ATP. The open probability values in the absence of ATP were so low that it was impossible to carry out the statistical analysis required to establish the effect of PRL on KCa channel activity under these conditions. In whole cell experiments without ATP in the patch pipette, no effect of PRL on KCa total current was observed. ATP hydrolysis was required for channel modulation, because the nonhydrolyzable ATP analog, AMP-PNP, was ineffective (not shown). As, on the one hand, tyrosine kinase was found to be a primary target of PRL in PRL-R signal transduction (8, 9) and, on the other hand, the KCa channels stimulated by PRL were found to be associated with protein kinase, we assumed the existence of a PRL-R-KCa channel-tyrosine kinase regulatory complex. To investigate whether the KCa channels stimulated by PRL may be modulated by an endogenous tyrosine kinase, we examined the effects of different tyrosine kinase inhibitors on KCa channel activity, using both whole cell and cell-free modes of the patch-clamp technique. In these studies we used two types of experiments differing in the duration of drug application.

Short Application of the Drug for Periods Varying from 15 s to Several Minutes—Application was performed from an additional pipette directly on the cell membrane (in whole cell patch-clamp configuration) or pieces excised from the membrane, containing one or more ion channels (excised-patch configuration).

Bath application (3 min) of the protein tyrosine kinase inhibitor genistein (36, 37) caused a progressive reduction in the KCa currents (Fig. 2A). Further application of PRL was ineffective. Genistein concentrations lower than 50 M were ineffective under these experimental conditions (n = 6). After genistein was washed out, KCa currents gradually recovered, indicating that the depression was reversible. Genistin, an analog of genistein that lacks protein tyrosine kinase inhibitory activity (36), had no effect on KCa current (n = 4; Fig. 9A).

We also tested two structurally distinct protein tyrosine kinase inhibitors: herbimycin A (9, 38) and lavendustin A (36, 39). Herbimycin A (1.5 M) and lavendustin A (10 M) depressed KCa currents to 57 ± 8% (n = 8) and to 54 ± 5% (n = 6) of control, respectively. Fig. 2B shows an example of KCa current inhibition induced by 3-min application of 10 M lavendustin A and current-voltage relationships for this effect, where current amplitudes were plotted at different test potentials. KCa currents were evoked by 160-ms test pulses from a holding potential of -40 to +10 mV.

Preincubation of All Cells by Addition of the Drug to the Bath Solution for a Time Varying from 30 min to Several Hours—The concentration dependence of current depression caused by three tyrosine kinase inhibitors is shown in Fig. 2C for cells preincubated with the drugs for 6 h. Under these conditions, even when cells were pretreated with low concentrations of inhibitors (n = 5 for cells treated with 10 M of genistein, n = 4 for 100 nm of herbimycin, and n = 5 for 1 M of lavendustin), the application of 5 nm PRL on these cells was ineffective (data not shown).

Cell-free experiments demonstrated that the effects of PRL...
and protein tyrosine kinase inhibitors were not mediated by intracellular processes, as they could be also observed in detached patches. Fig. 3 shows K⁺ channel activity stimulation by PRL and its inhibition by genistein (n = 7/9 patches). PRL (5 nM) caused an increase in the open probability of the channels (Fig. 3B), displaying the half-maximum increase in the open probability within 3.6 ± 1.3 min. The open probability of the channel after the addition of PRL was not constant, but oscillated between lower and higher open probability values (Fig. 3B). Subsequent addition of 100 μM genistein inhibited this K⁺ channel activity almost completely within 7 ± 2 min (Fig. 3B). The amplitude histograms (Fig. 3C) for K⁺ channels in control (mean = 8.48 ± 0.12 pA) and in the presence of PRL (mean = 8.34 ± 0.18 pA) demonstrate that PRL does not activate additional conductances. Moreover, prolactin did not stimulate K⁺ channel activity in the presence of 30 nM CTX, a KCa channel inhibitor in CHO cells (20), indicating that PRL activated the CTX-sensitive KCa channels and no other type of outward channels (not shown). As in whole cell experiments, genistein was ineffective (Fig. 9B). Application of 1.5 μM herbimycin A also decreased channel open probability without affecting single-channel conductance (n = 4/5 patches, Fig. 4). The subsequent addition of PRL was ineffective.

As protein tyrosine kinase inhibitors in high concentrations are known to be able to inhibit not only protein tyrosine kinase but also protein kinase C and protein kinase A kinases in some cell types (36), we checked the putative involvement of protein kinase C and protein kinase A in the mechanisms studied. We tested both activators and inhibitors of protein kinase C and protein kinase A (10–8 μM phorbol 12-myristate 13-acetate application, as protein kinase C activator; 10–6 μM phorbol 12-myristate 13-acetate 24-h incubation, as protein kinase C inhibitor; 250 μM phloretin, as protein kinase C inhibitor; 2 μM forskolin and 1 mM 8-bromo-cAMP, as protein kinase A activators) on K⁺ channel activity and on the stimulated effect of PRL. None of these drugs had any effect. A protein kinase C biochemical assay (40) was also carried out. 50–100 μM genistein had no effect in these studies.

The possibility that these channels may be regulated by protein tyrosine phosphatase as well as by protein tyrosine kinase was investigated by application of the protein tyrosine phosphatase inhibitor, sodium orthovanadate (41), to the cytoplasmic side of the cell-free patch. Orthovanadate (1 mM) increased the open probability of the channels in a time-dependent manner (Fig. 5). On average, activity increased by 198 ± 27% within 5 min (n = 6/9 patches). Fig. 5B shows the duration histograms for the channel prior to application of orthovanadate, then 2 and 8 min afterwards, respectively. In the presence of orthovanadate, channel openings were longer, as indicated by an increase in the relative number of events and the time constant to for the open state.

The preceding results strongly support the conclusion that the functioning of PRL-stimulated KCa channels is modulated by protein tyrosine kinases and protein tyrosine phosphatases and thus regulated by constitutive tyrosine phosphorylation/dephosphorylation. It has already been shown that tyrosine-phosphorylated PRL-R is associated with JAK2 kinase (30). Additional immunoblotting experiments were carried out to find out if this association was constitutive. Solubilized proteins from E32 cells incubated with or without 500 ng/ml of prolactin were immunoprecipitated with anti-PRL-R antibody.
46 and analyzed for the presence of JAK2 in the complex. As shown in Fig. 6A, a protein of 130 kDa was detected in the blot hybridized with anti-JAK2, corresponding to JAK2 kinase. This protein was revealed in the presence or absence of stimulation by ovine PRL, showing that this protein is constitutively associated with the PRL receptor. A rehybridization of the same blot with S46 shows the presence of the same amount of receptor in each line. In Fig. 6B, we demonstrate the specificity of the recognition of JAK2 by the antibody. Cell extracts were immunoprecipitated with anti-JAK2 antibody or anti-PRL-R antibody and subjected to SDS-PAGE and Western blotting. Each blot was incubated either with anti-JAK2 antibody or anti-PRL-R antibody 46 or the peptide corresponding to the amino acid residues 758–776 of murine JAK2 (JAK2 immunizing peptide). In JAK2 immunoprecipitates we observed that a protein of 130 kDa was specifically displaced by the presence of the peptide. Nonspecific bands were always present following incubation in the presence of the peptide. The same finding was observed in 46 immunoprecipitates. The 130-kDa band was not revealed in the presence of anti-JAK2 antibody. Within approximately 10 min the current was almost completely inhibited (n = 5). Subsequent application of PRL was ineffective (Fig. 7). Anti-JAK2 antibody applied to the cytoplasmic side of the membrane in inside-out studies decreased the open probability of the channel to 73 ± 18% (n = 4/4 patches) of control (Fig. 8). Subsequent application of PRL did not stimulate channel activity (Fig. 8). When anti-JAK2 antibody was preincubated with a peptide corresponding to the amino acid residues 758–776 of murine JAK2 (JAK2 immunizing peptide) and this mixture applied to the cytoplasmic side of the membrane, the decrease in K+ current in the whole cell experiments (Fig. 7) and in the open probability of the channel in inside-out experiments (Fig. 8)
anism of KCa channel activation by PRL and to identify the patch-clamp technique in order to unravel the mechanism clearly understood. In the present study, we applied the activation (29). The underlying mechanisms are, however, not

transduction. KCa channels are constitutively regulated long form of PRL-R, is the primary ionic event in PRL-R signal transduction. KCa channels are constitutively regulated through tyrosine phosphorylation/dephosphorylation. Stimulation of the channels by PRL possibly occurs through phosphorylation of protein tyrosine residues of the channel or of one or more associated proteins. The observation that KCa channel stimulation by PRL does not occur in the presence of anti-J AK2 kinase antibody suggests that at least one of the kinases involved in the channel stimulation by PRL may be J AK2 tyrosine kinase.

Although the association of PRL-R with J AK2 (8, 9, 30), J AK1 (42), or Fyn (10) tyrosine kinases has already been clearly demonstrated, the cascade of ionic events induced by PRL and the nature of ion channels involved has not yet been studied. Our earlier studies have characterized a membrane hyperpolarization, caused by KCa channel stimulation, and Ca\(^{2+}\) influx among the first detectable responses to PRL-R activation (29). The underlying mechanisms are, however, not very clearly understood. In the present study, we applied the patch-clamp recording technique in order to unravel the mechanism of KCa channel activation by PRL and to identify the nature of the associated protein kinase.

were not observed. As the anti-J AK2 antibody was obtained from Upstate Biotechnology, Inc. in rabbit serum, we checked the effect of non-immune rabbit serum on KCa conductance as a control. Rabbit serum at the same dilution (1:1000) was ineffective in both whole cell and inside-out experiments (not shown). An immune serum antibody (anti-rat PRL antibody) was used as an additional control. At the same dilution it was also ineffective.

Fig. 9 presents a summary of the effects of all the drugs studied on normalized peak currents, obtained by whole cell experiments (Fig. 9A) and normalized P_o, obtained by excised patch experiments (Fig. 9B).

**DISCUSSION**

We conclude from these findings that the activation of KCa channels by PRL in CHO cells, transfected with cDNA of the long form of PRL-R, is the primary ionic event in PRL-R signal transduction. KCa channels are constitutively regulated through tyrosine phosphorylation/dephosphorylation. Stimulation of the channels by PRL possibly occurs through phosphorylation of protein tyrosine residues of the channel or of one or more associated proteins. The observation that KCa channel stimulation by PRL does not occur in the presence of anti-J AK2 kinase antibody suggests that at least one of the kinases involved in the channel stimulation by PRL may be J AK2 tyrosine kinase.

In our experiments ATP (10–100 \(\mu M\)) increased the open probability of the KCa channel, therefore showing that a protein kinase is involved in the regulation of channel activity. Moreover, PRL was unable to stimulate this activity when ATP was absent from the internal solution, demonstrating that kinase phosphorylation is needed for channel stimulation by PRL. Thus, we concluded that protein kinase is closely associated with KCa channel and PRL-R in a regulatory complex.

It was recently demonstrated that in murine fibroblast cell lines, transfected with Ras or Raf plasmids, the KCa, CTX-sensitive channel is up-regulated by oncogenic p21\(^{ras}\) and that this regulation appears to be due toraf kinase-dependent induction of channel expression (43). The application of either epidermal growth factor or platelet-derived growth factor to nontransfected cells caused a time-dependent induction of KCa channels, obviously, through activation of endogenous cellular p21\(^{ras}\). Epidermal growth factor induction of the KCa channel was blocked by the tyrosine kinase inhibitors lavendustin A (1 \(\mu M\)) or genistein (50 \(\mu M\)). However, application of genistein to cells transfected by oncogenic ras had no effect on KCa current density, indicating that genistein had no direct inhibitory effect on the KCa channel. These results suggest that ras regulates the KCa channels through serine/threonine kinase and not through protein tyrosine kinase. Our experiments using tyrosine kinase inhibitors show a distinct mechanism of KCa channel regulation in CHO cells transfected with cDNA of the long form of PRL-R; the direct regulation of KCa channels by tyrosine kinase (as this activity was inhibited directly in whole cell and single channel experiments by three distinct tyrosine kinase inhibitors) and modulation of this activity by PRL (as PRL was no longer able to stimulate KCa channel activity when the cells were treated with protein tyrosine kinase inhibitors). Experiments using protein kinase C and protein kinase A activators and inhibitors showed that these kinases are not involved in the channel regulation mechanisms.

For epidermal growth factor receptor, which has intrinsic tyrosine kinase activity (44), the first event is activation of voltage-independent Ca\(^{2+}\) channels defined as direct receptor-operated channels (12). This in turn causes the activation of Ca\(^{2+}\)-dependent K\(^+\) channels, sensitive to charybdotoxin (12, 13), resulting in delayed membrane hyperpolarization and leading to the activation of a second class of hyperpolarization-sensitive Ca\(^{2+}\) channels (14). We did not observe the Ca\(^{2+}\) conductance activation prior to KCa channel activation (29). Conversely, our results demonstrate that the first ionic event in PRL-R signal transduction is KCa channel activation, since this activation is observed in excised patches. Based on the observed inhibitory effects of protein tyrosine kinase inhibitors on the
activity of PRL-stimulated K$_{Ca}$ channels, we propose that tyrosine kinase is involved in the positive regulation of these channels. The direct tyrosine phosphorylation of the delayed rectifier K$^+$ channel has also been proposed for the m$_3$ muscarinic acetylcholine receptor (11). This tyrosine kinase regulation is obviously an essential link in PRL signal transduction as it was recently found that the tyrosine kinase inhibitor herbimycin A was able to block a substantial portion of the prolactin signal to the milk protein gene promoter, β-lactoglobulin (30). In Nb2 cells it was shown that herbimycin A could also abolish the JAK2 kinase and receptor phosphorylation (9).

In our study the effects of PRL and protein tyrosine kinase inhibitors were observed in excised-patch experiments, indicating that the effects are not controlled by cellular metabolism, but are direct and that protein tyrosine kinase remains closely associated with K$_{Ca}$ channel activity. In this context it was of interest to check the effect of anti-JAK2 antibody on K$_{Ca}$ channel activity. The effectiveness of using antibodies in patch-clamp experiments was previously shown by Schweizer et al. (45). When anti-JAK2 antibody was introduced into the patch pipette in whole cell experiments, the K$_{Ca}$ conductance was almost completely inhibited within an average of 15 min, and PRL was no longer able to stimulate K$_{Ca}$ conductance. Anti-JAK2 antibody also decreased the open probability of K$_{Ca}$ channels when it was applied to the cytoplasmic side of the membrane in the inside-out patch mode. In immunoblotting experiments JAK2 kinase was revealed in the presence or absence of stimulation by PRL, showing that it is constitutively associated with the PRL-R. On the other hand, electrophysiological studies using anti-JAK2 antibody showed that this kinase is also constitutively associated with the K$_{Ca}$ channel. The results with JAK2 immunizing peptide, demonstrating the suppression of the effects observed using anti-JAK2 antibody alone in both electrophysiological and immunoblotting studies, show that the effects of the anti-JAK2 antibody are specific. The ability of the constitutively active kinases to stimulate cellular responses has previously been shown for MAP and phosphatidylinositol 3-kinase (46). On the other hand, there is growing evidence for the existence of a protein tyrosine kinase activation mechanism that functions indirectly by second messengers. For example, in the brain, membrane depolarization, which causes an increase in intracellular Ca$^{2+}$ levels, increases protein tyrosine kinase (47) and protein mitogen-activated protein kinase (48) activity. Extracellular signals (in our case PRL) appear to stimulate the activity of protein tyrosine kinase, but it may also be regulated by other factors (e.g. membrane potential and intracellular Ca$^{2+}$). This constitutive activity of JAK2 kinase is the reason for K$_{Ca}$ channel inhibition by the anti-JAK2 antibody in the absence of PRL stimulation. Therefore, our results demonstrate the functional involvement of JAK2 kinase in constitutive K$_{Ca}$ channel activity and the stimulation of these channels by PRL. However, it was shown by immunoblotting experiments that genistein (100–500 μM) was not able to inhibit the JAK2 kinase phosphorylation induced by growth hormone but it blocked the tyrosyl phosphorylation of other proteins (intermediary tyrosine kinases) (49, 50). This fact may be explained by the various examples of interactions at the level of the same protein; many phosphoproteins are phosphorylated at the same or at distinct residues by more than one protein kinase: in the case of tyrosine hydroxylase, the nicotinic acetylcholine receptor, synapsin 1, or Ca$^{2+}$-channel of the L type (47). This multi-site protein phosphorylation appears to be the rule rather than the exception (47). Therefore, we cannot completely exclude the possible involvement of other tyrosine kinases in K$_{Ca}$ channel functions.

The fact that the channel was activated by the protein tyrosine phosphatase inhibitor orthovanadate in excised patches suggests that the channel can be modulated in opposite directions by protein tyrosine kinase and protein tyrosine phosphatase. This modulation may be due to phosphorylation/dephosphorylation of the channels themselves or of regulatory protein(s) associated with these channels and the PRL receptor. The protein tyrosine phosphatase endogenous to CHO cells was observed in most excised patches and thus may be a member of the transmembrane class of protein tyrosine phosphatase (51, 52) or of the cytoplasmic protein tyrosine phosphatase which possess an SH2 domain or another means of association with membrane-bound proteins (53, 54). These studies suggest that tyrosine phosphorylation/dephosphorylation systems can modify K$_{Ca}$ channels rapidly, producing flexible changes in PRL-R signaling. Similar types of complexes that contain regulatory protein kinases and phosphatases have previously been shown for a variety of different ion channels (20–22, 55–57) and for K$_{Ca}$ channels in particular (20, 21, 56, 57). But the participation of such protein tyrosine kinase modulation of K$_{Ca}$ channels in PRL signal transduction has never been shown. To our knowledge this study provides the first example of the regulation of K$_{Ca}$ channels by PRL and protein tyrosine kinase in cell-free preparations. Our study is in accordance with a hypothesis previously proposed (57) about such submembrane complexing of ion channel proteins with modulatory enzymes like kinases and phosphatases as a common means by which cells achieve highly localized regulation of ion channel function by otherwise ubiquitous biochemical processes. Of particular interest is the finding that the modulation described here is due to a constitutive JAK2 tyrosine kinase activity. These results take on added significance because the PRL receptors were expressed in a heterologous system. However, remarkably, the endogenous JAK2 tyrosine kinase and endogenous K$_{Ca}$ channels in the host cell associate closely with heterologously expressed PRL-R and this complex retains its ability to be stimulated by PRL in a detached membrane patch.

As, on the one hand, PRL is known to stimulate the process of proliferation (58–61) and, on the other hand, K$_{Ca}$ channels have also been shown to be involved in the control of cell proliferation (25, 43, 62), the mechanism of PRL stimulation of K$_{Ca}$ channels through tyrosine phosphorylation, presented in this work, could provide a clue to understanding the regulation of cell proliferation by PRL.

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