Prolactin induces an inward current through voltage-independent Ca^{2+} channels in Chinese hamster ovary cells stably expressing prolactin receptor

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ABSTRACT

There is still only limited understanding of the early steps of prolactin (PRL) signal transduction in target cells. Recent studies have identified some of the essential first steps: these include the rapid association of the PRL receptor with JAK tyrosine kinases and tyrosine phosphorylation of a number of proteins, including members of the signal transducer and activator of transcription (Stats) family. On the other hand, binding of PRL to its receptor is rapidly followed by calcium influx. However, PRL-induced ionic events and the related ionic channels involved have not been clearly established.

This work was undertaken to characterise the channels responsible for calcium influx and to obtain an insight into their activation processes. Using the patch-clamp technique in the cell-attached configuration, single Ca^{2+} channel currents were recorded following PRL application (10 nM) in Chinese hamster ovary (CHO) cells stably expressing PRL receptor (CHO-E32). Statistical analysis showed that the recorded currents were voltage-independent, with a slope conductance of 16 pS. Although these channels were present in excised patches, the fact that PRL was unable to activate them suggested that a soluble cytoplasmic compo-

nent may be required. Application of the purified inositol phosphate, $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM), to the inside of the excised patch membrane activated the voltage-independent 16 pS Ca^{2+} channel. The open probability (Popen) was enhanced. The inositol phosphates $\text{Ins}(1,2,3,4,5)\text{P}_5$ and $\text{Ins}(1,4,5)\text{P}_3$ did not affect channel activity while InsP_6 (20 μM) had some effect, although less marked than that of $\text{Ins}(1,3,4,5)\text{P}_4$. Using the anion-exchange HPLC technique, we then studied the effects of PRL (10 nM) on the turnover of inositol phosphates (InsPs) in CHO-E32. Our studies showed that PRL induces rapid increases in the production of $\text{Ins}(1,3,4,5)\text{P}_4$ (207% at 30 s), InsP_5 (171% at 30 s), and InsP_6 (241% at 30 s). Conversely, $\text{Ins}(1,4,5)\text{P}_3$ showed a transient decrease at 5 s, accompanied by a concomitant increase in $\text{Ins}(1,3,4,5)\text{P}_4$, suggesting that the former could be transiently phosphorylated to produce the latter. Comparison of the production kinetics of $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$, InsP_5 , and InsP_6 indicated the possibility of additional metabolic routes which have yet to be determined. This study suggests that PRL promotes Ca^{2+} entry through voltage-independent Ca^{2+} channels that may be activated by $\text{Ins}(1,3,4,5)\text{P}_4$ and InsP_6 . *Journal of Molecular Endocrinology* (1998) **21**, 85–95

INTRODUCTION

Prolactin receptors (PRL-R) belong to a recently recognised family including receptors for growth

hormone (GH) and cytokines (Bazan 1990, Kelly *et al.* 1991). Amino acid homologies and structural similarities among members of this receptor class

account for their shared action mechanisms. In recent years, there has been considerable progress in understanding the intracellular signalling mechanisms for the various members of this family. Following binding of prolactin (PRL) to PRL-R, dimerisation of the receptor occurs prior to phosphorylation of a member of the JAK family of tyrosine kinases (JAK2) (Rui *et al.* 1994a). JAK2 activation is followed by tyrosine phosphorylation of a number of cytoplasmic proteins, including members of the mitogen-activated protein (MAP)/Raf kinases and signal transducers and activators of transcription (Stats) families (Buckley *et al.* 1994, Clevenger *et al.* 1994, Gouilleux *et al.* 1994). On the other hand, numerous studies have demonstrated that calcium channels play an important role in receptor signal transduction (Peppelenbosch *et al.* 1991, Boquet *et al.* 1997). In previous work using patch-clamp and microfluorimetric techniques, we analysed the first steps of the PRL-R signal transduction: we first showed a cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) increase associated with hyperpolarisation (Prevorskaya *et al.* 1994, Vacher *et al.* 1994). We also reported a direct stimulation of voltage- and calcium-activated potassium channels (KCa) by PRL (Prevorskaya *et al.* 1995). We found that these KCa channels were regulated through tyrosine phosphorylation/dephosphorylation and that JAK2 tyrosine kinase, constitutively associated with PRL-R, was implicated in the stimulation of KCa channels. These observations explain the PRL-induced hyperpolarisation but not the increase in $[\text{Ca}^{2+}]_i$.

In previous studies we investigated voltage-dependent conductances in Chinese hamster ovary (CHO) cells. We found voltage-dependent Ca^{2+} channels (Skryma *et al.* 1994) but these are not involved in the PRL-triggered Ca^{2+} influx, as, instead of depolarising the cell, PRL induces hyperpolarisation (Prevorskaya *et al.* 1994). Thus, PRL-induced calcium influx occurs through the opening of calcium channels, but the mechanisms responsible for the Ca^{2+} influx and the channels involved have yet to be identified.

An important observation in this research was that PRL-induced Ca^{2+} influx was controlled by an intracellular messenger. Over the past decade, there has been some controversy regarding direct control of Ca^{2+} influx by inositol phosphates (InsPs). Although the activation of PRL-R initiates signalling pathways involving a number of molecules, InsPs involvement has not been reported. Moreover, studies performed in rat node lymphoma cells (Nb2), which are absolutely dependent on lactogenic hormones for proliferation, failed to detect any increase in inositol 1,4,5-trisphosphate

(Ins(1,4,5)P3) following activation of PRL-R. This suggests that Ins(1,4,5)P3 was not involved in the PRL receptor transduction pathway (Gertler & Friesen 1986). In addition, it was inferred that phosphatidylinositol (PtdIns) P2 hydrolysis was not directly coupled to the receptor. A further investigation of the role played by InsPs in PRL transduction signalling and an analysis of earlier modifications induced after receptor activation, appear worthy of interest for the following reasons: recent studies have shown that Ca^{2+} increase occurs within seconds after receptor activation and no study of these early stages of PRL-induced phosphoinositide hydrolysis has yet been performed. As only InsP1, InsP2 and Ins(1,4,5)P3 were measured in previous reports, a knowledge of the more highly-phosphorylated InsPs produced required further experiments. Two forms of PRL-R, (long and short), with different cytoplasmic domains have been cloned. Nb2 cells are provided with a mutant intermediate of PRL-R and there is no evidence that the transduction pathway of the physiological long form of PRL-R is identical. Finally, phosphoinositide metabolites may be involved in the PRL transduction pathway, as PRL has been shown to stimulate the activation of phosphatidyl inositol 3-kinase (PtdIns3-kinase) in Nb2 cells (al-Sakkaf *et al.* 1996).

This report describes electrophysiological and biochemical measurements performed specifically to characterise the Ca^{2+} membrane channels involved in PRL-induced Ca^{2+} influx and the process by which they are activated. First, we show that PRL activates 16 pS voltage-independent Ca^{2+} channels, also activated by the inositol phosphates Ins(1,3,4,5)P4 and InsP6. Then we show that, in CHO cells stably expressing functional PRL-R, PRL rapidly increases Ins(1,3,4,5)P4, InsP5 and InsP6 levels. Thus, Ins(1,3,4,5)P4 and InsP6 may induce an increase in Ca^{2+} influx following activation of PRL receptors.

MATERIALS AND METHODS

Cell cultures

We used CHO cells transfected with the long form of the rabbit PRL receptor cDNA (CHO-E32), as previously described (Lesueur *et al.* 1990, Bignon *et al.* 1993). In this subclone, an examination of binding affinity showed that the PRL receptor exhibited a $K_a \approx 4 \text{ nM}^{-1}$. These CHO-transfected cells responded to PRL by stimulating the co-transfected milk protein gene promoter, proving that such cells were fully capable of transmitting the

PRL signal and that PRL-R was functional (Lesueur *et al.* 1990, 1991). The cells were grown in Ham's F12 medium (Seromed, Strasbourg, France) supplemented with 10% (v/v) fetal calf serum (Gibco, Grand Island, NY, USA), L-glutamine (2.5 nM, Sigma, L'Isle d'Abeau, Chesnes, France) and 1% penicillin-streptomycin (Gibco). Cells were cultured in Petri dishes (10 cm and 3 cm, Nunc Polylabo, Strasbourg, France). Cells were used 5 days after seeding. The day before the experiments, cells were cultured in serum-free medium to avoid occupancy of PRL receptors by lactogenic factors in serum. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 .

Electrophysiological measurements

For electrophysiological studies cells were bathed in a saline solution containing (in mM): 140 NaCl, 5 KCl, 10 CaCl_2 , 2 MgCl_2 , 0.3 Na_2HPO_4 , 0.4 KH_2PO_4 , 4 Na_2HCO_3 , 5 glucose, 10 Hepes (N-2 hydroxy ethylpiperazine-N'-2-ethanesulphonic acid). Tetrodo-toxin (TTX, 2 μM) was added to the solution to eliminate the Na^+ voltage-activated current. The pH was adjusted to 7.3–7.4 using NaOH, and osmolarity was adjusted to 310–320 mOsm/kg with mannitol.

To avoid intracellular dialysis, whole-cell current was measured using the slow whole-cell configuration with nystatine (100 $\mu\text{g}/\text{ml}$ of final solution) in the pipette to perforate the patch.

To eliminate K^+ currents in whole-cell recordings, we used a pipette solution containing (in mM): 150 N-methylglucamine (NMG) methane sulphate, 2 MgCl_2 , 5 Hepes (pH 7.3 $\pm$ 0.01 with gluconic acid or HCl), 1.1 EGTA (ethyleneglycol bis(b-aminoethyl ether-N,N,N',N'-tetraacetic acid). The pH was adjusted to 7.3–7.4 using gluconic acid and osmolarity was adjusted to 300 mOsm/kg.

For single channel recordings, the patch pipette was filled with a saline solution containing (in mM): 110 CaCl_2 , 2 MgCl_2 , 5 tetraethyl ammonium chloride, 10 Hepes. The pH was adjusted to 7.3–7.4 using NaOH and osmolarity was adjusted to 310 mOsm/kg with mannitol.

In excised-patch membrane experiments, we used symmetrical medium with Ca^{2+} or Ba^{2+} as charge carrier and permeant cation through the channel.

When $[\text{Ca}^{2+}]$ in the range of 10 nM to 10 μM was applied to the inner side of the membrane, the solution was buffered with EGTA (1.1 mM) and $[\text{Ca}^{2+}]_i$ was calculated using the method described by Abercrombie *et al.* (1981). Concentrations of $[\text{Ca}^{2+}] > 1 \mu\text{M}$ were achieved by adding the desired amount of CaCl_2 .

Drugs were applied locally by positive pressure pulses (0.1 bar) in a pipette with the tip (diameter 5–10 μm) positioned 15–20 μm from the cells recorded. This 'pouring pipette' was filled with the same extracellular saline solution as that used in the bath for recordings in cell-attached mode, or with the same solution as that in the patch pipette for measurements in symmetrical conditions. The drugs under investigation were added in appropriate concentrations. A second micropipette filled only with the extracellular saline solution was also positioned near the recorded cell and used to wash out the drugs.

All experiments were carried out at room temperature (21 $\pm$ 1 °C).

The patch electrodes were pulled from capillary glass tubes (GC; Clark Electrode Medical Instruments, Reading, Berks, UK) on a L/U-3P (List Medical, Darmstadt, Eberstad, Germany) puller, coated with Sylgard and fire polished. The final resistance was 2–4 M Ω .

An axopatch-1D (Axon Instruments, Foster City, CA, USA) patch amplifier, equipped with a 100 G Ω resistance in the head stage, was used for all electrophysiological recordings.

The patch-clamp studies were computer driven using p-clamp program version 5.5.1 and axotape 2.1 (Axon Instruments). The recordings were fed to a digital converter (TL1 DMA interface, Axon Instruments).

Data analysis

Single channel current recordings were low-pass filtered at 2 kHz using an eight-pole Bessel filter (–3 dB) and digitised at 5 kHz for storage and analysis on a PC compatible computer.

Single channel data were analysed after elimination of leak current by subtraction, from each current recording, of recording averages without channel activity. Conductances were calculated from potential-current relations and $\pm$ s.e.m. was given by Kaleidagraph statistical software (Microsoft, USA).

The open probability (Popen) was defined for the Ba^{2+} single channel as the time a channel spent in the open state divided by the duration of the recording. The area under the Gaussian curve in the amplitude histogram was integrated in order to calculate the proportional time a channel spent in a given state. When patches contained more than one channel, Popen was calculated as the total open time divided by the number of channels in the patch and the duration of the data segment analysed (Skryma *et al.* 1994). Data segments of 30 s were analysed for Popen estimates. A criterion of a 50% excursion

between fully open and fully closed states was used to determine the occurrence of an opening or closing event. Open channel amplitude was measured while the current level was above average.

Labelling of inositol lipids

Cells were incubated for 24 h in serum-free medium containing 10 $\mu\text{Ci/ml}$ myo[2- ^3H]inositol (specific radioactivity: 10–20 Ci/mmol; ICN, Orsay, France) at 37 °C. After labelling, following removal of labelled media, cells were rinsed for 1 min with myo[2- ^3H]inositol-free medium before experimental treatment.

Extraction and separation of inositol phosphates

Cells were stimulated for different times with 10 nM PRL. Each stimulation was stopped by adding 1.5 ml of a phenol-saturated solution (pH 6.3) containing 5 mM KH_2PO_4 , 10 mM EDTA and unlabelled InsPs (10^{-5} M) to minimise breakdown of labelled InsPs, as previously indicated (Mouillac *et al.* 1989). The cells were then removed with a cell scraper and collected in polypropylene tubes for InsPs extraction. After 2 min sonication, 1.5 ml ice-cold homogenisation buffer containing chloroform, phenol and isoamyl alcohol (38/24/1) (v/v/v) were added to each sample. After 20 s vortex and centrifugation for 5 min at 6000 *g*, the lower phase was removed and the upper aqueous phase was transferred to another tube and re-rinsed with 1.5 ml chloroform. After 20 s vortex and centrifugation for 5 min at 6000 *g*, the final upper aqueous phase containing water-soluble InsPs was stored at –20 °C prior to separation. All extraction steps were carried out at 4 °C on ice.

Inositol phosphate isomers were separated using high-performance liquid chromatography (HPLC) on a Beckman Gold system using a partisil 10-SAX Interchrom (25*0.46 cm:10 mm; Interchim, Montluçon, France) anion-exchange column as previously described (Wong *et al.* 1988, Dean & Beaven 1989, Nogaro *et al.* 1995). After filtration and centrifugation, the homogenised sample was injected into the column. Briefly, the elution of InsPs was achieved by increasing ammonium formate concentration (pH 3.7 adjusted with orthophosphoric acid) from 0 to 3 M, at a constant flow rate of 1.1 ml/min. Fractions were collected every 24 s to optimise separation of the various InsP isomers. After addition of scintillation liquid, fractions were then vortexed and preserved from daylight for 48 h before counting. Under these

conditions, about 70% of the radioactivity was recovered.

InsPs production study

All substances were identified in our samples by comparison with standards, eluting in the following order: inositol (Ins), glycerophosphorylinositols (GPI), InsP1, InsP2, Ins(1,3,4)P3, Ins(1,4,5)P3, Ins(1,3,4,5)P4, InsP5, InsP6.

To compensate for differences in inositol phosphate isomer levels due to differences in the number of cells harvested, the InsPs values obtained in both controls and stimulated groups (corresponding to cells stimulated by PRL for 5, 10, 30, or 60 s) were normalised by multiplying them by a factor corresponding to the ratio of normal GPI values/an arbitrary chosen GPI value as already described (Nogaro *et al.* 1995). Each experiment was repeated 4 to 6 times.

Statistical analysis

Statistical analysis of the data was performed using the Student's *t*-test for unpaired samples to determine significant differences between control and treated groups. A probability value (*P*) less than 0.05 was considered significant. Results are presented as means ($\pm$ S.E.M.) and expressed as percentage of control value.

Materials

Ovine PRL (oPRL-19) was kindly provided by the NIDDK (National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, MD, USA). Thapsigargin (1 μM) was used to deplete Ca^{2+} intracellular pools (Sigma, France). Radiolabelled inositol phosphates were obtained from the Radiochemical Centre (Amersham, Bucks, UK), and from NEN Research Products (Du Pont de Nemours, Les Ulis, France). Unlabelled InsPs were generously provided by Dr G Mayr (Germany). As additional experiments were needed, Ins(1,3,4,5)P4 was also purchased from Sigma (France).

RESULTS

PRL rapidly induced an inward Ca^{2+} current

Whole-cell current was measured in response to direct application of PRL (10 nM) to the cell recorded. In a previous study, we demonstrated that the very first step in PRL-R signal transduction

at the plasma membrane level consisted of the activation of a KCa^{2+} conductance (Prevarskaya *et al.* 1994, 1995). To record only inward currents, K^+ currents were eliminated in these electrophysiological measurements by using NMG-substituted solutions (see experimental procedures). TTX ($2\text{ }\mu\text{M}$) was added to the bath medium to block Na^+ voltage-activated currents present in the cells (Skryma *et al.* 1994). In the fast whole-cell configuration, PRL (10 nM) induced a barely detectable inward current (approximately 5 pA) which, moreover, diminished very quickly, suggesting that intracellular components were required for its activation (Fig. 1A). To avoid intracellular dialysis, nystatin was used in the patch pipette in the perforated or slow whole-cell configuration (see experimental procedures). Under these recording conditions, PRL (10 nM) rapidly induced an inward current of 22 pA at a clamped voltage of -45 mV (Fig. 1B), close to the membrane resting potential of CHO cells ($-45 \pm 5\text{ mV}$), in a majority of cells analysed ($n=8/13$). In contrast, PRL (10 nM) did not produce any effect in non-transfected CHO (CHO-K1) cells ($n=0/9$). Experiments were carried out to characterise the ionic nature of this current. First, we used the Ca^{2+} channel blockers cobalt (Co^{2+} , 5 mM) and nifedipine ($1\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$). Co^{2+} completely blocked the PRL-induced inward current. Nifedipine reduced the inward current only at the highest concentration used ($100\text{ }\mu\text{M}$) ($n=6$). Removal of Na^+ from the bath medium and its replacement by choline did not affect the PRL-induced inward current. We also attempted to determine the reversal potential (E_{rev}) of the activated whole-cell current. The holding potential was stepped from -60 mV to $+60\text{ mV}$, in 10-mV steps, and the PRL-induced inward current amplitude was measured. Figure 1C illustrates the relationship between the holding potential and the mean PRL-induced current in whole cells ($n=4$). According to the data, the PRL-induced current reversal potential was close to $+40\text{ mV}$. However, as shown in Fig. 1C, we never obtained a clear reversed potential at $+60\text{ mV}$.

We initially attempted to record single channels in membrane patches in the outside-out configuration, but PRL (10 , 20 , 100 nM) failed to activate the channel directly (data not shown).

To avoid dialysis of the intracellular components suspected to be required, we used the cell-attached configuration of the patch-clamp technique. In the cell-attached configuration, -10 mV was applied to the patch pipette. The patch of membrane under the pipette was thus clamped at about $-35 \pm 5\text{ mV}$, as the resting membrane potential of CHO cells was estimated to be $-45 \pm 5\text{ mV}$. When all types of ion

FIGURE 1. PRL activated an inward current in CHO-E32 cells. Application of PRL (10 nM , 10 s) to a fast whole-patched cell induced an inward current (A). This current was more marked in a slow whole-cell configuration using nystatin ($100\text{ }\mu\text{g/ml}$) in the patch pipette (B). Membrane potential was clamped at -45 mV in both A and B. Recordings were made with a $1\text{ G}\Omega$ headstage and a 2 kHz filter. The amplitude of the PRL-induced current at different membrane potentials, recorded under the same conditions as in B, is reported in C. Mean of four experiments. The straight line is a linear regression to the points.

channels were suppressed except those for calcium (see Materials and Methods), PRL (10 nM) application induced the activation of single calcium channels. Popen for control experiments was very low (Fig. 2A ($n=7/11$)). Single channel events were recorded 5 to 40 s after the wash-out of PRL. The potential amplitude relationship of this PRL-activated calcium channel showed a slope conductance of 16 ± 0.7 pS ($n=7$).

CHO cells, like other non-excitable cells, are characterised by a capacitative Ca^{2+} influx following depletion of endoplasmic reticulum Ca^{2+} stores. We therefore studied the effect of Ca^{2+} store depletion on channel activity. Although in other experiments thapsigargin (1 μM) has been found to be fully effective in releasing Ca^{2+} , it was unable to activate the 16 pS Ca^{2+} channels in the cell-attached configuration described above (data not shown).

Ins(1,3,4,5)P4 opened the 16 pS Ca^{2+} channel

To explore further a putative involvement of InsPs in the PRL-induced Ca^{2+} influx, we studied the effects of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ on membrane channels, using the excised inside-out patch configuration. $\text{Ins}(1,4,5)\text{P}_3$ (0.2, 2 and 20 μM , $n=11$) produced no effect (data not shown). When $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) was applied to the inside of the patch membrane in an inside-out free patch configuration (under symmetrical conditions in which both the bath (external) and recording pipette (internal) solutions contained 110 mM Ca^{2+}), it activated a 16 pS conductance calcium channel (Fig. 3A). When Ca^{2+} was replaced by Ba^{2+} in both the external and internal solutions, the slope conductance of the evoked $\text{Ins}(1,3,4,5)\text{P}_4$ single-channel current was 21 pS. Figure 3A shows current-voltage slopes for both symmetrical solutions of Ca^{2+} and Ba^{2+} ($n=3$ to 7).

Various configurations of the patch-clamp technique were used for further study of these channels. The results presented below were obtained in the presence of Ba^{2+} instead of Ca^{2+} .

The voltage-dependence of the channel was studied. We recorded the calcium single channel current at different clamped voltages (from -60 mV to $+60$ mV, $n=7$) in an inside-out excised patch configuration. $\text{Ins}(1,3,4,5)\text{P}_4$ was applied to the inside of the membrane at each membrane clamped potential. The opening time did not show any significant variation. The curve in Fig. 3B shows the Popen of the $\text{Ins}(1,3,4,5)\text{P}_4$ -activated calcium channel current. Thus, these results indicate that this single channel current is not voltage-dependent. Ca^{2+} channels, recorded in an outside-out free patch configuration, had the same

FIGURE 2. Action of PRL (10 nM) and $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) on single Ca^{2+} channels of CHO-E32 cells. (A) In the cell-attached configuration, single Ca^{2+} channels were activated when PRL (10 nM) was applied to the outside of the cell. The patch pipette contained the same medium as the external solution. Control activity recovered 20 s after the end of PRL application. Pipette potential was maintained at -10 mV. (B) Application of $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) to an excised inside-out patch, at a pipette potential of -40 mV, activated single Ca^{2+} channels. Initial single-channel activity recovered after a rapid wash-out. The experiments were carried out at room temperature (20 ± 1 $^{\circ}\text{C}$) with a 100 G Ω headstage and filtered at 1 kHz.

FIGURE 3. Potential dependency of $\text{Ins}(1,3,4,5)\text{P}_4$ -activated Ca^{2+} channel conductance. The potential–current relationship was obtained in voltage clamp from -80 mV to $+80$ mV, in 10 -mV increments. The curve slope indicates a conductance of 21 pS or 16 pS in symmetrical solutions containing 110 mM Ba^{2+} (solid circles) or 110 mM Ca^{2+} (open circles) respectively. $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) was continuously present on the inside of the patch membrane. Means of 5 to 7 values are shown for each potential and standard errors are shown for all points (A). Popen (Po) calculated at various clamped potentials does not show any correlation with potential. Po was calculated as described (Skryma *et al.* 1994) from continuous, long recordings (30 s) for 3 to 5 patches. Means and standard errors are illustrated for each potential (solid circles). Po for one patch (open circles) shows the variation in one Ca^{2+} channel according to potential (B).

conductance as in cell-attached or inside-out free patch configurations. When $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) was present continuously in the patch pipette on the inside of the membrane, application of cobalt (5 mM) to the clamped patch at $+50$ mV induced a complete blockade of single Ca^{2+} channel activity (Fig. 4A). Nifedipine (100 μM) blocked the single Ca^{2+} channel, but not completely ($n=5$) (Fig. 4B). Apparent Popen of $\text{Ins}(1,3,4,5)\text{P}_4$ -activated Ca^{2+} channels is shown before, during, and after ejection

FIGURE 4. Effects of inorganic (cobalt, 5 mM), and organic (nifedipine, 100 μM) calcium channel blockers on $\text{Ins}(1,3,4,5)\text{P}_4$ -activated channel. Using outside-out patch configuration, with $\text{Ins}(1,3,4,5)\text{P}_4$ (2 μM) in the patch pipette solution, the application of Co^{2+} (5 mM) (A) blocked single-channel activity completely. Nifedipine (100 μM) (B) had only a partial blocking effect. Potential was clamped at $+50$ mV for A and $+60$ mV for B. Recovery occurred at the end of Co^{2+} or nifedipine application. Apparent Popen (Po) is shown in (C) before, during and after the application of Co^{2+} (5 mM).

of Co^{2+} (5 mM) to an outside-out patch membrane (Fig. 4C). $\text{Ins}(1,3,4,5)\text{P}_4$ (2 and 20 μM), applied to the external side of the free patch membrane to test the specificity of its effect on the Ca^{2+} channel, had no effect (data not shown).

We tested the effects of $\text{Ins}(1,4,5)\text{P}_3$ (0.2 , 2 and 20 μM , $n=11$), $\text{Ins}(2,3,4,5)\text{P}_4$ (2 and 20 μM , $n=9$), $\text{Ins}(1,2,3,4,5)\text{P}_5$ (2 and 20 μM , $n=6$) and InsP_6 (20 μM , $n=4$) on this single 16 pS voltage-independent Ca^{2+} channel. Only InsP_6 had a similar, although less marked, effect on channel activity. When InsP_6 (20 μM) was applied to the inside of a -40 mV clamped excised patch of membrane, the 16 pS Ca^{2+} channel was activated and remained open for up to 12% of the total recording time (data not shown).

Effects of PRL on InsP metabolism

The above observations prompted us to analyse the possible involvement of InsPs in the PRL-induced

FIGURE 5. Time-dependent changes in levels of various inositol phosphate isomers in the CHO cell line in response to PRL (10 nM). After 24-h labelling and 1-min rinsing in normal medium, cells were stimulated with PRL for various times (5, 10, 30, 60 s) and then the reaction was terminated by addition of a phenol-saturated solution. Inositol phosphate isomers were extracted from cells and separated in anion-exchange HPLC. Fractions eluted were collected and InsP1 (A), InsP2 (B), Ins(1,4,5)P3 (C), Ins(1,3,4,5)P4 (D), InsP5 (E) and InsP6 (F) levels were counted. Results are expressed as percentage of control value. The values shown are the means $\pm$ S.E.M. for at least four combined experiments and a significant difference ($P < 0.05$) between stimulated and control samples is denoted by an asterisk. Two asterisks indicate a highly significant difference ($P < 0.01$).

Ca^{2+} influx. Therefore we performed anion exchange HPLC separation of InsPs on 6×10^6 CHO-E32 cells incubated in the presence of 10 nM PRL for different times: 5, 10, 30 and 60 s.

Figure 5 shows the time-course of InsP levels following PRL stimulation. Results are expressed as percentage of control value. InsP1 production did not change, while InsP2 decreased (Fig. 5A,B). There was an initial slight decrease in Ins(1,4,5)P3 after 5 s stimulation with PRL, associated with a

concomitant increase in Ins(1,3,4,5)P4 (Fig. 5C,D). There was no significant change in Ins(1,3,4)P3 (not shown). However, the major changes were large increases in Ins(1,3,4,5)P4, InsP5 and InsP6 (Fig. 5D,E,F). The maximum increase in Ins(1,3,4,5)P4 reached 207% of control at 30 s, InsP5 reached 171% of control at 30 s while InsP6 reached 241% of control at 30 s and 236% at 60 s. Ins(1,3,4,5)P4 and InsP5 showed parallel kinetics. While levels of Ins(1,3,4,5)P4 and InsP5 decreased at 60 s, InsP6 remained high. The conditions for labelling the CHO cells with [^3H]myo-inositol resulted in steady-state labelling of InsP₁₋₄, but this state was not completely reached for InsP5 and InsP6 (Menniti *et al.* 1990). Thus, although significant changes in InsP5 and InsP6 levels were observed in stimulated cells compared with controls, these results should be considered with caution.

Control experiments were also performed with native CHO cells (CHO-K1) not transfected with PRL-R. Under the same conditions as for CHO-E32, PRL stimulation (10 nM) of InsPs production was measured only at 30 s, since, as reported above, major changes in Ins(1,3,4,5)P4, InsP5 and InsP6 occurred at that time. InsP4 ($91 \pm 10.5\%$ of control, $P = 0.279$), InsP5 ($95 \pm 11.8\%$ of control, $P = 0.276$) and InsP6 ($101 \pm 1.5\%$ of control, $P = 0.125$) production did not significantly change after PRL stimulation compared with controls (4 experiments).

CHO cells, like a variety of cell lines, contain purinoceptors linked to PLC activation associated with production of Ins(1,4,5)P3 and subsequent mobilisation of intracellular calcium (Iredale *et al.* 1992). To validate further the above protocol, we stimulated CHO-E32 with ATP (10 μM) at 30 s and then investigated changes in the production of InsP. As expected, we found a large increase (10 times, $n = 4$) in Ins(1,4,5)P3 production at 30 s while Ins(1,3,4,5)P4, InsP5, and InsP6 were unchanged (results not shown).

DISCUSSION

Despite intensive studies, the mechanism of action of PRL in target cells is still not completely understood. Agreement has now been reached over several steps in the transduction pathway, including receptor dimerisation (Rui *et al.* 1994a), JAK kinase activation (Rui *et al.* 1994b), and tyrosine phosphorylation of several proteins (Tourkine *et al.* 1995, Waters *et al.* 1995). The rapid increase in intracellular Ca^{2+} levels is also among the early steps of the transduction signal generally accepted

for PRL-R (Villalba *et al.* 1991, Vacher *et al.* 1994) as well as for GH receptor (Ilondo *et al.* 1994, Billestrup *et al.* 1995).

In this work using patch-clamp techniques, we show that PRL rapidly induces an inward current. In the fast whole-cell configuration, the current was not easily recorded, as it diminished very quickly. It was more easily recorded in the slow whole-cell configuration which prevented dialysis of intracellular components, suggesting that the PRL-induced current was controlled by an intracellular messenger. In accordance with this view, channel activation could not be obtained in membrane patches devoid of cytoplasm. PRL (10 nM) failed to activate the channel directly, so single channel recordings were performed in the cell-attached mode. PRL activated a 16 pS channel which may account for Ca^{2+} influx. This channel which was blocked completely by Co^{2+} (5 mM) and partially by nifedipine (100 μM), was not voltage-dependent. We showed that the channel was permeable to Ca^{2+} and Ba^{2+} , and permeability to other ions, mainly at depolarised potentials, cannot be excluded. We have already reported the existence of a voltage-dependent Ca^{2+} channel in CHO cells. This channel has properties similar to the L-type channel of excitable cells (Skryma *et al.* 1994). It does not participate in the PRL-induced increase in intracellular Ca^{2+} as, instead of depolarising, CHO-E32 cells transiently hyperpolarise following PRL administration (Prevarskaya *et al.* 1994). The hyperpolarisation results from the activation of K^{+} channels. Membrane hyperpolarisation associated with Ca^{2+} influx has already been reported in other systems. The hyperpolarisation-induced Ca^{2+} influx appears to be mediated by a specific class of small-conductance Ca^{2+} channels (2–3 pS), whose open probability drastically increases at hyperpolarised potentials (–60, –70 mV) (Peppelenbosch *et al.* 1991). We have not identified these small conductance channels in our CHO cell line. This may be due to the fact that they may not have been resolved under our recording conditions due to their very small amplitude. The Ca^{2+} channel reported in this study has a much higher conductance (16 pS) and is voltage independent.

We show that binding of PRL to its receptor is followed by a rapid activation of phosphatidylinositol metabolism in CHO cells stably transfected with the long form of the PRL receptor. Conversely, studies performed on rat node lymphoma cells (Nb2), which are absolutely dependent on lactogenic hormones for proliferation, failed to detect any increase in inositol phosphates (InsP1, InsP2, Ins(1,4,5)P3) following activation of PRL-R. This suggests that the phosphatidylinositol metabolism

was not involved in the transduction pathway of the PRL signal (Gertler & Friesen 1986). These early experiments are not in contradiction with the present observations for the following reasons: first, Gertler and Friesen studied only InsP1, InsP2, InsP3, while our study focused on a rapid accumulation of Ins(1,3,4,5)P4, InsP5 and InsP6; secondly, the experiments were performed 2–5 to 5 min after interaction of PRL with the receptor, so the rapid modifications reported in our study were probably not observed. Finally, the PRL-R in Nb2 cells is truncated by the deletion of 198 amino acids in the middle of its cytoplasmic domain. A recent study has assigned critical roles for specific receptor domains regulating different PRL-stimulated events (Lebrun *et al.* 1995). In Nb2 cells, PRL is able to induce tyrosine phosphorylation of JAK2 but we have not yet found any evidence that this pathway is required for the activation of the phosphatidylinositol metabolism.

We show that the binding of PRL to its receptor is followed, in CHO cells, by a rapid accumulation of Ins(1,3,4,5)P4, InsP5 and InsP6. Ins(1,3,4)P3 was not significantly modified. We observed a rapid, transient decrease (5 s) in Ins(1,4,5)P3, associated with a concomitant increase in Ins(1,3,4,5)P4. Thus, Ins(1,4,5)P3 may be transiently phosphorylated by an InsP-3-kinase to produce Ins(1,3,4,5)P4. Conversely, the Ins(1,3,4,5)P4 peak at 30 s would not seem to involve this metabolic pathway, as Ins(1,4,5)P3 did not show any variation at that time. Its decrease at 60 s has yet to be explained.

The kinetics of InsP peaks thus suggests two metabolic pathways, both leading to a transient increase in Ins(1,3,4,5)P4. At 1 min, InsP6 remained high while InsP5 began to decrease and Ins(1,3,4,5)P4 returned to basal levels. We therefore propose that PRL binding to its receptor was rapidly followed by a transient production of Ins(1,3,4,5)P4. These data indicate a possible rapid phosphorylation of Ins(1,4,5)P3 to Ins(1,3,4,5)P4 but this does not represent a major part of the increase in the level of Ins(1,3,4,5)P4.

The increase in Ins(1,3,4,5)P4 at 30 s probably involves PtdIns(3,4,5)P3. Thus, production of Ins(1,3,4,5)P4 may result from the hydrolysis of PtdIns(3,4,5)P3. This, in turn, would imply stimulation of PtdIns3-kinase. A recent work has shown that PtdIns3-kinase is involved in PRL signalling in Nb2 cells (al-Sakkaf *et al.* 1996). Moreover, we have also reported accumulation of PtdIns(3,4,5)P3 following PRL stimulation under the same conditions as in the present study (Ratovondrahona *et al.* 1998).

The exact role of PtdIns3-kinase in intracellular signalling is not known and may differ from one cell type to another. PtdIns3-kinase catalyses phosphorylation of PtdIns, PtdIns4P and PtdIns(4,5) P2 to produce PtdIns3P, PtdIns(3,4) P2 and PtdIns(3,4,5)P3 respectively (Auger & Cantley 1991). However, we do not know how PtdIns(3,4,5)P3 is metabolised and, to our knowledge, a metabolic pathway from PtdIns(3,4,5)P3 to Ins(1,3,4,5)P4 has not yet been described.

An important result of these experiments is the identification of a class of Ca^{2+} channel responsible, at least partially, for the PRL-activated Ca^{2+} entry in CHO-E32 cells. As already stated, activation is not direct, but mediated through the activation of a cytoplasmic messenger. In this work, we showed that Ins(1,3,4,5)P4, directly ejected on a CHO membrane patch, activated a 16 pS Ca^{2+} channel identical to that activated by PRL in a cell-attached patch. This strongly suggests that PRL activated this channel through Ins(1,3,4,5)P4 or InsP6, since this other inositol phosphate was also effective, although to a lesser extent. A direct demonstration of Ca^{2+} channel activation by Ins(1,3,4,5)P4 has also been reported in endothelial cells (Lüchhoff & Clapham 1992).

To summarise, the signal transduction mechanism for PRL receptor was investigated using a CHO line transfected with cDNA of the long form PRL receptor. We showed that, in these transfected cells, PRL rapidly triggered an influx of Ca^{2+} through a two step process: (1) PRL directly activated K^{+} channels, leading to membrane hyperpolarisation and thus increasing the gradient for Ca^{2+} ions (Prevarskaya *et al.* 1994), and (2) through the production of a cytoplasmic or diffusible second messenger which probably opened Ca^{2+} channels, allowing Ca^{2+} influx. In addition, we showed that this Ca^{2+} influx was due to activation of voltage-independent Ca^{2+} channels, with a conductance of 16 pS. As activation was not direct but due to a second messenger, these channels should be classified as second messenger-operated channels. Our observations suggest that the second messenger may be Ins(1,3,4,5)P4 or InsP6, as the levels of these molecules increased following PRL binding to its receptor.

It remains to be shown whether endogenous PRL-R activates the same transduction pathway, and whether there are differences in transduction pathways according to the type of target cell. Thus, other studies will be required before this transduction pathway, or part of it, can be generalised to other systems or target cells with endogenous PRL-R.

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REFERENCES

- Abercrombie RF, Maszucka LM, Sjodin RA & Linvengood D 1981 Uptake and release of ^{45}Ca by *Myxicola axoplasm*. *Journal of General Physiology* **78** 413–429.
- Auger KR & Cantley LC 1991 Novel polyphosphoinositides in cell growth and activation. *Cancer Cells* **3** 263–270.
- Bazan JF 1990 Structural design and molecular evolution of a cytokine receptor superfamily. *Proceedings of the National Academy of Sciences of the USA* **87** 6934–6938.
- Bignon C, Daniel N & Djiane J 1993 β -Galactosidase and chloramphenicol acetyltransferase assays in 96-well plates. *Biotechniques* **15** 243–245.
- Billestrup N, Bouchelouche P, Allevato G, Ilondo M & Nielsen JH 1995 Growth hormone receptor C-terminal domains required for growth hormone-induced intracellular free Ca^{2+} oscillations and gene transcription. *Proceedings of the National Academy of Sciences of the USA* **92** 2725–2729.
- Boquet G, Barakat L, Paly J, Djiane J & Dufy B 1997 Involvement of both calcium influx and calcium mobilisation in growth hormone-induced $[\text{Ca}^{2+}]_i$ increases in Chinese hamster ovary cells. *Molecular and Cellular Endocrinology* **131** 109–120.
- Buckley AR, Rao YP, Buckley DJ & Gout PW 1994 Prolactin-induced phosphorylation and nuclear translocation of MAP kinase in Nb2 lymphoma cells. *Biochemical and Biophysical Research Communications* **204** 1158–1164.
- Clevenger CV, Torigoe T & Reed JC 1994 Prolactin induces rapid phosphorylation and activation of prolactin receptor-associated RAF-1 kinase in a T-cell line. *Journal of Biological Chemistry* **269** 5559–5565.
- Dean NM & Beaven MA 1989 Methods for the analysis of inositol phosphates. *Analytical Biochemistry* **183** 199–209.
- Gertler A & Friesen HG 1986 Human growth hormone-stimulated mitogenesis of Nb2 node lymphoma cells is not mediated by an immediate acceleration of phosphoinositide metabolism. *Molecular and Cellular Endocrinology* **48** 221–228.
- Gouilleux F, Wakao H, Mundt M & Groner B 1994 Prolactin induces phosphorylation of Tyr694 of Stat 5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO Journal* **13** 4361–4369.
- Ilondo MM, De Meyts P & Bouchelouche P 1994 Human growth hormone increases cytosolic free calcium in cultured TM-9 lymphocyte: a novel mechanism of growth hormone transmembrane signaling. *Biochemical and Biophysical Research Communications* **202** 391–397.
- Iredale PA, Martin KF, Hill SJ & Kendall DA 1992 Inositol 1,4,5-trisphosphate generation and calcium mobilisation via activation of an atypical P2 receptor in the neuronal cell line N1E-115. *British Journal of Pharmacology* **107** 1083–1087.
- Kelly A, Djiane J, Postel-Vinay MC & Edery M 1991 The prolactin/growth hormone receptor family. *Endocrine Reviews* **12** 235–251.
- Lebrun JJ, Ali S, Ullrich A & Kelly PA 1995 Proline-rich sequence-mediated JAK2 association to the prolactin

- receptor is required but not sufficient for signal transduction. *Journal of Biological Chemistry* **270** 10664–10670.
- Lesueur L, Edery M, Paly J, Clarck J, Kelly P & Djiane J 1990 Prolactin stimulates milk protein promoter in CHO cells cotransfected with prolactin cDNA. *Molecular and Cellular Endocrinology* **71** 7–12.
- Lesueur L, Edery M, Ali S, Paly J, Kelly A & Djiane J 1991 Comparison of long and short forms of the prolactin receptor on prolactin induced milk protein gene transcription. *Proceedings of the National Academy of Sciences of the USA* **88** 824–828.
- Lüchtkoff A & Clapham D 1992 Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+} -permeable channel. *Nature* **355** 356–358.
- Menniti FS, Oliver KG, Nogimori K, Obie JF, Shears SB & Putney JW 1990 Origins of myo-inositol tetrakisphosphates in WRK1 cells: correlation with intracellular calcium mobilisation. *Biochemical and Biophysical Research Communications* **159** 953–960.
- Mouillac B, Balestre MN & Guillon G 1989 Transient inositol(1,4,5)P₃ accumulation under vasopressin stimulation in WRK1 cells: correlation with intracellular calcium mobilisation. *Biochemical and Biophysical Research Communications* **159** 953–960.
- Nogaro M, Radallah D, Guillon G & Fournier B 1995 HPLC analysis of inositol phosphate isomers induced by neuroparsine in rectal epithelial cells of the African locust: role of external calcium. *Insect Biochemical and Molecular Biology* **25** 559–567.
- Peppelenbosch MP, Tertoolen LGJ & De Laat SW 1991 Epidermal growth factor-activated calcium and potassium channels. *Journal of Biological Chemistry* **266** 19938–19944.
- Prevarskaya N, Skryma R, Vacher P, Daniel N, Bignon C, Djiane J & Dufy B 1994 Early effects of PRL on ion conductances in CHO cells expressing PRL receptor. *American Journal of Physiology* **267** C554–C562.
- Prevarskaya N, Skryma R, Vacher P, Daniel N, Djiane J & Dufy B 1995 Role of tyrosine phosphorylation in potassium channel activation. *Journal of Biological Chemistry* **270** 24292–24299.
- Ratovondrahona D, Fournier B, Odessa MF & Dufy B 1998 Prolactin stimulation of phosphoinositide metabolism in CHO cells stably expressing the PRL receptor. *Biochemical and Biophysical Research Communications* **243** 127–130.
- Rui H, Lebrun JJ, Kirken RA, Kelly PA & Farrar WL 1994a JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerisation. *Endocrinology* **135** 1299–1306.
- Rui H, Kirken RA & Farrar WL 1994b Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *Journal of Biological Chemistry* **269** 5364–5368.
- al-Sakkaf KA, Dobson PRM & Brown BL 1996 Activation of phosphatidylinositol 3-kinase by prolactin in Nb2 cells. *Biochemical and Biophysical Research Communications* **221** 779–784.
- Skryma R, Prevarskaya N, Vacher P & Dufy B 1994 Voltage-dependent Ca^{2+} channels in Chinese hamster ovary (CHO) cells. *FEBS Letters* **349** 289–294.
- Tourkine N, Schindler C, Larose M & Houdebine LM 1995 Activation of Stat factors by prolactin, interferon γ , growth hormones and a tyrosine phosphatase inhibitor in rabbit primary mammary epithelial cells. *Journal of Biological Chemistry* **270** 20952–20961.
- Vacher P, Tran Van Chuoi M, Paly J, Djiane J & Dufy B 1994 Short term effect of prolactin on intracellular calcium in Chinese hamster ovary cells stably transfected with prolactin receptor complementary deoxyribonucleic acid. *Endocrinology* **134** 213–218.
- Villalba M, Zabala MT, Martinez-Serrano A, De la Colina R, Satrustegui J & Garcia-Ruiz 1991 Prolactin increases cytosolic free calcium concentration in hepatocytes of lactating rats. *Endocrinology* **129** 2857–2861.
- Waters MJ, Daniel N, Bignon C & Djiane J 1995 The rabbit mammary gland prolactin receptor is tyrosine-phosphorylated in response to prolactin *in vivo* and *in vitro*. *Journal of Biological Chemistry* **270** 5136–5143.
- Wong NS, Barker C, Shears SB, Kirk CJ & Michell RH 1988 Inositol 1,2-cyclic 4,5-trisphosphate is not a major product of inositol phospholipid metabolism in vasopressin-stimulated SRK1 cells. *Biochemical Journal* **252** 1–5.

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