EVIDENCE FOR A NOVEL SIGNALING PATHWAY*

Yanhe Tong and James Zull‡
From the Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

Lei Yu
From the Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana 46202

Expression of human parathyroid hormone receptor (hPTHR) was obtained in Xenopus oocytes. Receptor function was detected by hormone stimulation of endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) current. This current was blocked by injected, but not by extracellular, EGTA, confirming that the hPTHR activates cytosolic Ca\(^{2+}\) signaling pathways. PTH responses were acutely desensitized but were regained in 6–12 h. Injection of cAMP or analogues had no effect on either responsiveness or desensitization to hPTH. The hPTH response was more sluggish than seen with serotonin 5-hydroxytryptamine (5-HT\(_{2c}\)) receptor. In oocytes co-expressing both hPTHR and 5-HT\(_{2c}\) receptors, homologous desensitization was seen, but cross-desensitization was not observed. Injection of inositol 1,4,5-trisphosphate (InsP\(_3\)) elicited a fast inward current similar to that induced by serotonin, and complete cross-desensitization occurred between the InsP\(_3\) and 5-HT\(_{2c}\) responses. Desensitization by hPTH did not affect responses to either InsP\(_3\) or serotonin, but cells desensitized to injected InsP\(_3\) still responded strongly to PTH. Oocytes did not respond to either cADPR or NAADP\(^{+}\), but NAADP\(^{+}\) and analogues were found to be potent inhibitors of PTH signaling. We suggest that PTH cytosolic Ca\(^{2+}\) signaling in oocytes either involves a novel signaling system or proceeds through a Ca\(^{2+}\)-regulated pathway whose responsiveness is regulated in a novel way.

Parathyroid hormone (PTH)\(^{1}\) is the primary regulator of calcium and phosphate homeostasis in higher animals (1). This 84-amino acid peptide and truncated forms such as the 1–34 or 1–36 fragments initiate the biological actions of PTH through a specific receptor (PTH-R) on the plasma membrane in target tissues, primarily kidney and bone (2–5). Recently, the cDNAs of PTHR from rat, mouse, and human cells have been cloned (6–10), and both transient and stable transfection of PTHR has been described (7, 11, 12).

The transfection experiments demonstrated that the PTHR can activate multiple signaling events in the same cell, including production of cAMP, activation of phospholipase C, and elevation of cytosolic Ca\(^{2+}\) (7, 12). However, this multiple signaling capability is not always present in cells that constitutively express the PTHR. In some systems, a cAMP response is observed in the absence of the cytosolic Ca\(^{2+}\) response (13, 14), while in others the reverse appears to be true (15). In addition, N-terminally truncated forms of PTH that do not activate adenyl cyclase still appear to generate the cytosolic Ca\(^{2+}\) signal (16), and likewise cells in a cultured bone cell line desensitized to PTH with regard to the cAMP response still show a cytosolic Ca\(^{2+}\) signal (17). Thus, the factors that determine signaling in different systems are not understood.

The signaling systems responsible for elevation of cytosolic Ca\(^{2+}\) in response to PTH are also complex. The hormone has been reported to activate Ca\(^{2+}\) influx through a cAMP sensitive plasma membrane channel (18), but elevated cytosolic Ca\(^{2+}\) can also be observed in the absence of influx of extracellular Ca\(^{2+}\) (19). The latter effects have been attributed to generation of InsP\(_3\) through the action of phospholipase C (20, 21). However, some studies suggest that production of InsP\(_3\) is not obligatory for elevation of cytosolic Ca\(^{2+}\) by PTH. It has been reported that parathyroid hormone and thrombin release intracellular calcium from different calcium stores in osteoblast-like UMR 106-H5 rat osteosarcoma cells (22), and no effect of PTH on InsP\(_3\) was found in this study. It has also been reported that full-length hPTH stimulated by hPTH does not increase InsP\(_3\) levels in stably transfected HK-293 cells, while some truncated forms of the receptor do express this activity (23).

These studies indicate a need for further examination of signaling by the PTHR under conditions where different signals can be studied independently in the same cell system. The functional expression of the PTHR in Xenopus oocytes appears to offer that possibility. Specifically, the frog oocyte has a Ca\(^{2+}\)-activated Cl\(^{-}\) channel that is directly utilized to detect cytosolic Ca\(^{2+}\) changes (24–27) but no endogenous cAMP-activated channels. Also, the ability for direct manipulation of cytosolic contents provide a flexible system that may be used to further the PTH signaling system. In this paper, we demonstrate its use for examination of cytosolic Ca\(^{2+}\) signaling.

EXPERIMENTAL PROCEDURES

Materials—Human parathyroid hormone receptor cDNA in pcDNA I vector was generously provided by Dr. G. V. Segre (Massachusetts Institute of Technology, Cambridge, MA). Human parathyroid hormone (1–34) and 8-(4-chlorophenylthio)-cAMP were purchased from Peninsula Laboratories (Belmont, CA). 8-Br-cAMP, 8-bromo-cAMP; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; 5-HT, serotonin (5-hydroxytryptamine); 4-AP, 4-aminopyridine; TEA, tetraethylammonium; cADPR, cyclic adenosine diphosphate ribose; NAADP\(^{+}\), nicotinic acid adenine dinucleotide phosphate.
Fig. 1. Functional expression of human parathyroid hormone receptor in Xenopus oocytes. Whole cell voltage clamp analysis of oocytes microinjected with cDNA or mRNA of the human parathyroid hormone receptor. A, membrane current traces recorded at a holding potential of −80 mV, inward current is downward. 3 μl of 2.43 × 10^{-5} M hPTH (1–34) was directly added to the recording chamber with a capacity of about 500 μl so that the final concentration of hPTH in the recording chamber was 10^{-6}–10^{-7} M. Applying hPTH to un.injected oocytes, no response was observed (top trace). When hPTH-injected oocytes were stimulated with hPTH, inward currents were induced (middle and bottom trace). After the first hPTH stimulation, the oocyte was washed with ND96 with 1.8 mM CaCl₂. When a second hPTH was applied about 1 min after the washout, no response was elicited (middle trace). When the second hPTH stimulation was applied several hours after the washout, the response was partially recovered (bottom trace). B, bar graph showing hPTH-mediated membrane currents and homologous desensitization. hPTH did not induce noticeable membrane currents in uninjected oocytes (control). Following the first hPTH stimulation, the oocyte was stimulated with hPTH, inward currents were induced (middle and bottom trace). When the second hPTH stimulation was applied 5 min after the washout, the response was partially recovered (bottom trace). The response slowly recovered after several hours (257.5 ± 85.7 nA, n = 4). ** indicates a significant difference from the control value (p < 0.01). Upon washout of hPTH, very little response could be induced by a second application of hPTH within 5 min, indicating complete homologous desensitization. The response slowly recovered after several hours (257.5 ± 85.7 nA, n = 4). ** indicates a significant difference from the 5-min washout value (p < 0.01).
RESULTS

Functional Expression of Human Parathyroid Hormone Receptor in X. laevis Oocytes—To express the hPTHR in Xenopus oocytes, a DNA clone for the hPTHR (10) was used. Aliquots of cDNA of the hPTHR were microinjected into the nucleus of defolliculated X. laevis oocytes. Alternatively, synthetic cRNA was in vitro transcribed from the cDNA clone and microinjected into the cytoplasm of Xenopus oocytes. 3–4 days after cRNA or cDNA injection, stimulation with 1–34 hPTH elicited an inward current in hPTHR-injected oocytes but not in un.injected oocytes (Fig. 1A). This response was always a strong homologous desensitization, as shown by a lack of response from the second hPTH stimulation following washing out the bath hPTH used in the initial stimulation (Fig. 1A, middle trace, and Fig. 1B). The responsiveness of hPTHR-expressing oocytes slowly recovers, with the size of the inward current showing a partial recovery after 6 h (Fig. 1A, bottom trace, and Fig. 1B) and a complete recovery after 12 h (data not shown). The hPTHR was consistently expressed in oocytes, whether DNA or RNA was injected.

Impact of Calcium for the Human Parathyroid Hormone Receptor-mediated Current—To determine whether the hPTH-induced current in oocytes requires cytosolic Ca\(^{2+}\), 500 pmol of Ca\(^{2+}\) chelator EGTA was microinjected into hPTH-expressing oocytes. This gives a final concentration of EGTA at ~0.5 mM for oocytes with an average volume of 1 μl. Stimulation with hPTH (1–34) after EGTA injection produced no responses (Fig. 2A, middle trace, and Fig. 2B), indicating that the hPTH-induced currents were completely eliminated by intracellular EGTA injection. However, the addition of 1 mM EGTA to the calcium-free ND96 superfusion solution had no effect on hPTH-induced currents (Fig. 2A, bottom trace, and Fig. 2B). This showed that the hPTH-induced response in Xenopus oocytes is dependent on intracellular Ca\(^{2+}\) but not on extracellular Ca\(^{2+}\).

The Inward Current is Mainly Carried by the Chloride Ion—The ionic selectivity of hPTH-induced currents were determined using different channel blockers. There was no effect on hPTH-induced membrane currents when in the bath solution Ca\(^{2+}\) was chelated with EGTA and, with the inclusion of Cd\(^{2+}\) as the Ca\(^{2+}\) channel blocker and 4-AP and TEA as the K\(^{+}\) channel blocker. The membrane current could be completely suppressed by using an external ND96 solution containing 1 mM niflumic acid, a Cl\(^{-}\) channel blocker (Fig. 3A, upper trace, and Fig. 3B). However, the membrane current was also eliminated by clamping the oocytes at the holding potential of −25 mV (data not shown). Together, these results indicate that the hPTH-induced current is mainly carried by the Cl\(^{-}\) ion.

Human Parathyroid Hormone-induced Response Is Independent of CAMP—It has previously been reported that PTH can activate production of CAMP in Xenopus oocytes injected with mRNA extracts from target cells that contain the PTHR message (32). Also, in some systems, CAMP has been reported to be important for the PTH response. However, in this study, the presence of 1 mM cAMP had no effect on the hPTH-induced response (data not shown).
to mediate desensitization to PTH by a protein kinase A-dependent process (33, 34). Therefore it was of interest to examine whether cAMP has any effect on the cytosolic Ca\(^{2+}\) response to PTH in our system. Intracellular injection of cAMP and cAMP analogues, 8-Br-cAMP, 8-CPT-cAMP, and 2',3'-cAMP, did not induce any membrane current in either control oocytes not expressing the hPTHr (data not shown) or hPTHr-expressing cells (Fig. 4). These same hPTHr-expressing cells

**FIG. 4. Human parathyroid hormone receptor-induced current is not mediated by cAMP.** Oocytes expressing the hPTHr were voltage-clamped at −80 mV. Microinjection of cAMP or its analogues 8-Br-cAMP, 8-CPT-cAMP, and 2',3'-cAMP did not induce any inward current. hPTH was subsequently applied to confirm the oocytes did have hPTH-induceable response.
can produce an inward current by subsequent hPTH application, demonstrating that they are not desensitized to PTH by cAMP, and suggesting that the desensitization process in oocytes is not mediated by protein kinase A.

The Response to PTH Is More Sluggish Than That of a Receptor Coupled to the Phospholipase C Pathway—Xenopus oocytes have been used widely to study membrane receptors that couple to the phospholipase C signaling pathway (24), since stimulation of such a receptor expressed in oocytes gives a robust inward Cl\textsuperscript{−} current. To compare the hPTHR-induced response to that of a system that utilizes the InsP\textsubscript{3} pathway, we also expressed a cloned serotonin receptor, the 5-HT\textsubscript{2C} receptor, that couples to the phospholipase C pathway (36). Two parameters were analyzed for time course studies of receptor-mediated membrane currents, the latency of onset and the start-peak time (Fig. 5A). The response profiles from the two receptors are very distinct from each other, with the 5-HT response being a fast and robust one, and the hPTH response showing a longer latency and a slower kinetics for start-peak time (Fig. 5, A and B). Specifically, application of 5-HT produces an almost immediate current, whereas hPTH-induced current does not commence until about 13 s after the hormone is added (Fig. 5B, top panel). The 5-HT current also develops quickly, with an average start-peak time at 4 s, whereas hPTH-induced current takes about 7 s to develop (Fig. 5B, bottom panel). These differences are statistically significant, and they suggest that the two receptors may use different signaling pathways.

On average, the chloride current responses to serotonin are also larger than those to PTH. However, this quantitative difference cannot be interpreted since it could be due to one or a combination of several variables including the efficiency of expression of the receptor message, the stability of the injected nucleic acid messages, the stability of the expressed receptor in the cell membrane, the efficiency of coupling to second messenger systems, and the sensitivity and size of responsive calcium compartments.

The relative levels of receptor expression were examined in ligand binding studies. We found that the levels of expression of hPTH receptor and 5-HT\textsubscript{2C} receptors were roughly comparable. The radioligand binding studies showed 2.2 \times 10^{9} and 4.2 \times 10^{9} receptors/oocyte for hPTH and 5-HT\textsubscript{2C} receptors, respectively. Thus, it is unlikely that the qualitative and quantitative differences between the PTH and the 5-HT\textsubscript{2C} systems are related to different levels of receptor expression.

Examination of Desensitization Properties for the Human Parathyroid Hormone Receptor and the 5-HT\textsubscript{2C} Receptor—G protein-coupled pathways often display marked desensitization, operationally defined as a reduced response to continued or repeated stimulation of agonists. The response induced by both the hPTHR and 5-HT\textsubscript{2C} receptor show strong homologous desensitization, as the second stimulation by the same agonist produced little or no membrane current (Fig. 6A). It has been well characterized that 5-HT\textsubscript{2C} receptor uses inositol trisphosphate as the second messenger to induce calcium release from

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**Fig. 5.** Human parathyroid hormone receptor-mediated current has a long latency of onset and a slow kinetics. A, diagram showing the measurement of the latency time and the start-peak time. Top trace shows an hPTH-induced response. As a comparison, the bottom trace shows a serotonin (5-HT)-induced membrane current in an oocyte injected with the RNA for 5-HT\textsubscript{2C} receptor. Oocytes were clamped at −80 mV. B, relative time courses of the latency of onset and the start-peak time between hPTH- and 5-HT\textsubscript{2C}-induced responses. Data are presented by mean ± S.E.; n is for numbers of oocytes used. The latency of onset for hPTH, 12.8 ± 0.8, n = 52; for 5-HT\textsubscript{2C}, 0.6 ± 0.06, n = 14. The start-peak time for hPTH, 6.9 ± 0.5, n = 52; for 5-HT\textsubscript{2C}, 3.9 ± 0.3, n = 14. * (top graph) indicates a significant difference for the latency time between the 5-HT response and hPTH response (p < 0.01). * (bottom graph) indicates a significant difference for the start-peak time between the two groups (p < 0.01).
receptor were coexpressed in oocytes. When hPTH application has caused a complete homologous desensitization, 5-HT stimulation was still effective in releasing Ca\(^{2+}\) (Fig. 6B). The results suggest that the 5-HT\(_{2c}\) receptor and the hPTH receptor cause intracellular calcium release through distinct pathways.

**Fig. 6. hPTH and 5-HT responses do not cross-desensitize each other.** Oocytes were voltage clamped at \(-80\) mV. A, membrane current traces of first and second 5-HT (upper trace) or hPTH (lower trace) stimulation, showing that activation of each receptor induces complete homologous desensitization. B, membrane current trace induced by 5-HT and hPTH in a single oocyte coinjected with 5-HT\(_{2c}\) and hPTH receptor cDNA. After the cell is completely desensitized to hPTH stimulation, 5-HT still elicited an inward current, suggesting that the two receptors are coupled to different signaling pathways.

The role of InsP\(_3\) in signaling by the PTH receptor—It has previously been proposed that the cytosolic Ca\(^{2+}\) signal for PTH is activated by InsP\(_3\) (20). However, some studies indicate that this may not be the case in every system (23), and the existence of InsP\(_3\)-insensitive calcium compartments that are activated by PTH has been proposed (22). Our kinetic and desensitization data also suggest that possibility, and further experiments were designed to test it more directly by studies with microinjection of InsP\(_3\). Strong activation of the Cl\(^-\) channel was observed upon initial injection of InsP\(_3\), but no response was observed with a second injection (Fig. 7, top trace). Thus, either the InsP\(_3\) receptor is desensitized or the Ca\(^{2+}\) pools, which respond to injected InsP\(_3\), are emptied. 5-HT and InsP\(_3\) also showed cross-desensitization as expected (Fig. 7, middle two traces), since they share the same calcium release pathway. However, in hPTH-desensitized cells, InsP\(_3\) injection still elicited an inward current, and in cells that were unresponsive to injected InsP\(_3\), a strong signal was still observed upon addition of PTH (Fig. 7, bottom two traces).

Since the PTHR can activate Ca\(^{2+}\) compartments that are insensitive to injected InsP\(_3\), we examined whether PTH signaling might proceed through the cADPR pathway (44–46) or the recently described NAADP\(^{+}\) pathway (51). However, we observed no effect of injection of these compounds on the Cl\(^-\) current in Xenopus oocytes. In addition, as expected from the above results, inhibitors that block both InsP\(_3\) and cADPR signaling systems were found to have no affect on the PTH responses in oocytes (data not shown). However, unexpectedly we found that NADP\(^{+}\), thio-NADP\(^{+}\), and the NADP\(^{+}\) analogue, 3-acetylpyridine-ADP\(^{+}\), are all strong inhibitors of the cytosolic Ca\(^{2+}\) response to PTH in oocytes (Fig. 8). This inhibition persists for a few hours, but the hormone response is eventually regained. In control experiments, injection of these compounds alone had no impact on cytosolic Ca\(^{2+}\), or on oocyte responsiveness to serotonin.

**DISCUSSION**

As with other hormone receptor systems that have been expressed in oocytes (24–27), this work shows that increases in cytosolic Ca\(^{2+}\) can be detected by activation of an endogenous Cl\(^-\) channel. Identification of the PTHR-stimulated signal as a Cl\(^-\) current was accomplished by its inhibition with niflumic acid and by elimination of the signal through clamping the voltage at \(-25\) mV, the known resting potential for Cl\(^-\) in this system. The Ca\(^{2+}\) dependence for this current is demonstrated by its inhibition with injected EGTA, and a lack of inhibition by external EGTA establishes that the signal is not mediated by extracellular Ca\(^{2+}\). The results are consistent with earlier demonstrations that the PTHR activates increases in cytosolic Ca\(^{2+}\) in wild-type and in transfected cells (7, 12, 15, 16).

Earlier studies indicated that PTH can activate increases in cytosolic Ca\(^{2+}\) by a cAMP-mediated stimulation of a plasma membrane channel in some cells (18). This effect is dependent on extracellular Ca\(^{2+}\) and thus appears unlikely to be responsible for the signals we observe. The PTHR expressed in oocytes does activate production of cAMP (32), and thus a lack of any detectable affect of injected cAMP or its nonhydrolyzable analogues represents additional evidence that our signals are not mediated through this plasma membrane channel. The apparent lack of any effect of cAMP on PTH responses or desensi-
zation are also of interest since protein kinase A has been implicated in the down-regulation of PTHR in some cultured cell systems (33, 34). Further work should also examine the possible role of protein kinase C in this system, since it has also been implicated in down-regulation of PTHR (37).

This work is the first demonstration of the cytosolic Ca²⁺ response to PTH in Xenopus oocytes expressing the PTHR. Earlier work with mRNA extracts from target cells for PTH demonstrated that the receptor can be expressed in oocytes, utilizing the cAMP response to measure receptor function (32, 38). Thus, the two primary signaling responses to PTH have now been demonstrated in this system. This is significant since cell systems that express the PTHR do not always show both responses. For example, some cultured bone cells show elevated CAMP in response to PTH, but no detectable increases in cytosolic Ca²⁺ (14), while cultured keratinocytes show a cytosolic Ca²⁺ response but no elevation in CAMP (15). Also, some forms of PTH can generate one response but not the other (16), and in one study, cells that were desensitized with regard to the CAMP response still elicited a cytosolic Ca²⁺ response (17).

The most interesting results from this work are the apparent lack of involvement of InsP₃ in PTHR signaling and the inhibition of signaling by NADP⁺. The most direct interpretation of our data with regard to the role of InsP₃ is that cytosolic Ca²⁺ signaling by PTHR in oocytes is not mediated by this second messenger. This interpretation is supported by several observations. First, oocytes that cannot respond to injected InsP₃ still show a strong response to added PTH. Second, cells whose InsP₃-sensitive Ca²⁺ stores appear to be depleted by activation of coexpressed 5-HT receptors still respond to added PTH. Third, the response to PTH is consistently slower than that to 5-HT, suggesting that signaling is through a different route, and fourth, the PTH response is strongly inhibited by NADP⁺ and related compounds, none of which have any apparent chemical or biological relationship to InsP₃.

An alternative interpretation is that frog oocytes possess Ca²⁺ compartments that are not accessed either by injected materials or by InsP₃ generated by other receptors. Such compartments could be activated by cAMP or InsP₃ released in localized sites near the PTHR, while remaining unresponsive to the injected materials or to increased concentrations of these second messengers generated by activation of other receptor systems. Although we know of no evidence for such compartments, this possibility cannot be excluded at this time. Thus, we cannot unequivocally conclude that InsP₃ is not the signal for cytosolic Ca²⁺ increases triggered by the PTHR in the frog oocyte. However, at the minimum, our studies demonstrate the existence of unique Ca²⁺ compartments in oocytes that are responsive to the PTH receptor. The unique nature of these compartments is either their unresponsiveness or their inaccessibility to presently known signals and their sensitivity to NADP⁺.

Although InsP₃ is probably the most widely studied signal for cytosolic Ca²⁺, three alternative second messenger candidates are known: cADPR (41–46), sphingosine-phosphate (47–50), and NAADP⁺ (51). None of these have yet been shown to be
The inhibition of PTHR signaling by NADP⁺ has not been described previously. This effect is not observed with the serotonin receptor, which is known to utilize the InsP₃ pathway, and it thus provides further evidence that InsP₃ may not be the signal for the PTH system. It is of interest that the most sensitive assay for PTH is based on a procedure that requires activation of glucose-6-phosphate dehydrogenase and production of NADPH (52). The biochemical rationale for this assay is not understood, but our results with NADP⁺ now provide further impetus for examination of the possible role of this enzyme in biochemical actions of PTH. As indicated in Fig. 8, the inhibition of cytotoxic Ca²⁺ signaling is acute and persists for hours following injection of NADP⁺. This effect could be the result of direct interaction of NADP⁺ with some regulatory element in the PTH signaling pathway or a more general effect of a significant alteration of the NADP⁺/NADPH ratio in the cytoplasm and its subsequent impact on the oxidation/reduction or catabolic/anabolic state of the cell. It may be that the signaling by PTHR is modulated by metabolic state since this hormone is known to produce both anabolic and catabolic affects on different cell types under different conditions. Further study of the regulation of signaling by the PTHR in the frog oocyte system may shed light on these possibilities.

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Yanhe Tong, James Zull and Lei Yu

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