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Characterization of the Neuropeptide Y-Induced Intracellular Calcium Release in Human Erythroleukemic Cells

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SUMMARY

Human erythroleukemic (HEL) cells, loaded with fura-2, respond to neuropeptide Y (NPY) with a fast and transient increase in intracellular calcium. The Y_1 receptor-specific agonist (Leu-31,Pro-34)-NPY is 4-fold more potent and the carboxyl-terminal fragment NPY13–36 is 150-fold less potent than NPY. Thus, it is concluded that the response is mediated through the activation of a Y_1 type of NPY receptor. HEL cells do not respond to a second addition of NPY but do respond to a further addition of α-thrombin (α-T). However, in a calcium-free medium, prior stimulation with NPY largely inhibits a subsequent response to α-T. Moreover, prior stimulation with α-T in the absence of external calcium completely prevents the response to the addition of NPY, indicating a common effector pathway. The latter is further reinforced by using thapsigargin (TG), which has been shown to deplete the inositol 1,4,5-trisphosphate-dependent calcium pool in other systems. HEL cells preincubated with TG in calcium-free medium fail to respond to either NPY or α-T. Likewise, prior stimulation with NPY or α-T in calcium-free medium significantly inhibits the response to TG. Preincubation of cells with phorbol esters strongly inhibits the NPY-induced release of intracellular Ca^{2+} in HEL cells, an effect that is partially prevented by preincubation of the cells with H7, a protein kinase C inhibitor. However, neither the homologous nor the apparent heterologous desensitization of the NPY receptor can be prevented by H7. It is concluded that NPY releases intracellular Ca^{2+} from an inositol 1,4,5-trisphosphate-sensitive calcium pool, which is restored by external calcium, and that NPY receptor desensitization is protein kinase C independent.

NPY is a 36-amino acid peptide, containing an amino-terminal tyrosine and a carboxyl-terminal tyrosine amide, that was first isolated from porcine brain by Tatemoto *et al.* (1). NPY is widely distributed throughout the mammalian central and peripheral nervous systems (2–4) and has been implicated in a wide variety of biological activities. In the periphery, NPY is coreleased with norepinephrine from sympathetic terminals and may play an important role as a neurotransmitter or as a neuromodulator of the vascular response to sympathetic stimulation (5–7).

The effects of NPY appear to be mediated by at least two receptor subtypes (Y_1 and Y_2), which may activate different intracellular signaling mechanisms. It has been shown that NPY induces the mobilization of intracellular Ca^{2+} in several cell types (8–11) and inhibits adenylate cyclase activity from various cells and tissues (8, 10, 12–15) through pertussis toxin-sensitive GTP-binding protein(s). Furthermore, we have recently shown that the NPY-induced intracellular calcium mobilization in HEL cells is correlated with the formation of inositol phosphates (11), suggesting that inositide metabolism and activation of phospholipase C are coupled to the NPY receptor in these cells. However, the specific receptor subtype responsible for these changes has not been identified. In this study, we have further characterized the NPY-induced calcium release in HEL cells and identified the intracellular calcium pool from which it is mobilized.

Experimental Procedures

Cell culture. HEL cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 15% bovine calf serum. Cells were maintained in suspension at 37°, in 95% air and 5% CO_2, at a density of 1–4 × 10^5 cells/ml.

Intracellular Ca^{2+} measurements. Cells were centrifuged (250 × *g* for 5 min), resuspended (10^6 cells/ml), and washed twice in a buffer containing 120 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM magnesium acetate, 1 mM $CaCl_2$, and 1 mg/ml glucose, at pH 7.4. Fura-2 acetoxymethyl ester was then added to a final concentration of 1 μM, and the cells were incubated in the dark for 1 hr at room temperature. The fura-2-loaded cells were centrifuged, resuspended, further incubated for 30 min in the absence of the fluorescent probe, washed by centrifugation, and finally resuspended in fresh buffer at 10^6 cells/ml. Fluorescence was measured at room temperature in a SLM AMINCO DMX-

ABBREVIATIONS: NPY, neuropeptide Y; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEL, human erythroleukemic; TG, thapsigargin; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PMA, phorbol 12-myristate 13-acetate; α-T, α-thrombin; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate.

1000 spectrofluorometer, with dual excitation at 340 nm and 380 nm and emission recording at 510 nm. Each cuvette (Fisher Ultra-VU styrene) contained 0.5 ml of cell suspension (5×10^5 cells) and buffer up to a total volume of 2.5 ml, with constant stirring. For the experiments in calcium-free medium, the buffer had no calcium and contained 6 mM EGTA. The intracellular Ca^{2+} concentration was calculated from the ratio of the fluorescence at 340 nm/380 nm, with the aid of a computer program, where $[Ca^{2+}] = K(F - F_{min})/(F_{max} - F)$, as described by Grynkiewicz *et al.* (16). K is the apparent dissociation constant for fura-2-Ca^{2+}. F_{max} was obtained by lysis of the cells by addition of digitonin (0.02% final concentration) and F_{min} by subsequent addition of 10 mM EGTA.

Materials. HEL cells were obtained from National Institute of General Medical Sciences Human Genetic Mutant Cell Repository Institute for Medical Research (Camden, NJ). Cell culture medium was obtained from GIBCO (Grand Island, NY), bovine calf serum from HyClone Laboratories, Inc. (Logan, UT), L-glutamine solution from JRH Biosciences (Lenexa, KS), fura-2 acetoxymethyl ester from Molecular Probes (Eugene, OR), TG from LC Services Corporation (Woburn, MA), and H7 and PMA from Sigma (St. Louis, MO). α-T was a generous gift from Dr. John Fenton II (Albany, NY). Porcine NPY was obtained from Dr. D. Klapper (University of North Carolina at Chapel Hill) and further purified in-house on a C_4 Vydac preparative column, using a gradient of acetonitrile (10% to 40% in 30 min) containing 0.1% trifluoroacetic acid. (Leu-31,Pro-34)-NPY and NPY13–36 were synthesized in-house and purified in the same way as porcine NPY. All other reagents were of the highest purity available.

Results

HEL cells, loaded with fura-2, responded to NPY with a fast and transient increase in intracellular Ca^{2+} concentration from a resting level of approximately 40 nM to approximately 300–400 nM (Fig. 1A). The dose-response curves, measuring peak increases in intracellular Ca^{2+} concentration induced by NPY (ED_{50} = 1 nM), (Leu-31,Pro-34)-NPY (ED_{50} = 0.25 nM), and NPY13–36 (ED_{50} = 150 nM), are shown in Fig. 1B. According to the nomenclature proposed by Wahlestedt *et al.* (17) and Sheikh *et al.* (18), these results would indicate that the response was mediated through a Y_1 type of NPY receptor. The relative potency of these peptides to induce intracellular Ca^{2+} release in HEL cells is similar to their potency to displace radiolabeled NPY binding from three different neuroblastoma cell lines expressing the Y_1-type receptor, as observed by Fuhlendorff *et al.* (19). The IC_{50} values reported were 2.1–7.9, 3.8–7.9, and ≫100 nM for NPY, (Leu-31,Pro-34)-NPY, and NPY13–36, respectively. In contrast, the IC_{50} values obtained in three other cell lines, expressing the Y_2-type receptor, were 0.16–0.56, 140–320, and 2–11 nM for NPY, (Leu,Pro)-NPY, and NPY13–36, respectively.

Fig. 2 shows that a second exposure of the cells to a maximal dose of NPY failed to induce any further release of intracellular Ca^{2+}, despite the fact that the fluorescence had returned to basal levels. The cells exposed to NPY were, nevertheless, still responsive to another agonist, α-T. However, a subsequent addition of α-T to these cells could not elicit the release of intracellular Ca^{2+}, suggesting that the NPY and the α-T receptor/effector pathways had undergone homologous desensitization. The same results were obtained when the addition of the agonists was reversed, by adding first α-T and then NPY (data not shown). In the absence of extracellular calcium (6 mM EGTA), however, prior stimulation of the cells with α-T prevented the response to the further addition of NPY (Fig. 3), suggesting a common effector pathway (intracellular Ca^{2+}

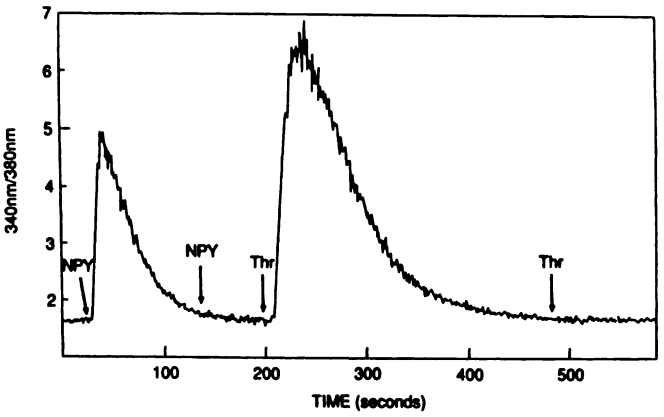

Fig. 2. Homologous desensitization for the release of intracellular Ca^{2+} induced by NPY or α-T. NPY was added at a concentration of 100 nM. After the second addition of NPY, 10 nM α-T (*Thr*) was added. The results represent a typical experiment repeated several times and are expressed as the ratio of fluorescence at 340/380 nm (see Experimental Procedures).

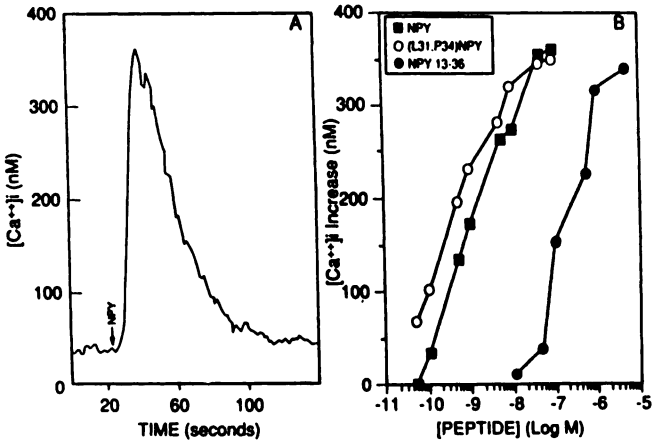

Fig. 1. NPY-induced release of intracellular Ca^{2+} in fura-2-loaded HEL cells. A, Time course for the effect of 100 nM NPY. B, Dose-response curves for the effects of NPY, (Leu-31,Pro-34)-NPY, and NPY13–36. The calcium concentration was calculated from the ratio of fluorescence at 340/380 nm, as described in Experimental Procedures.

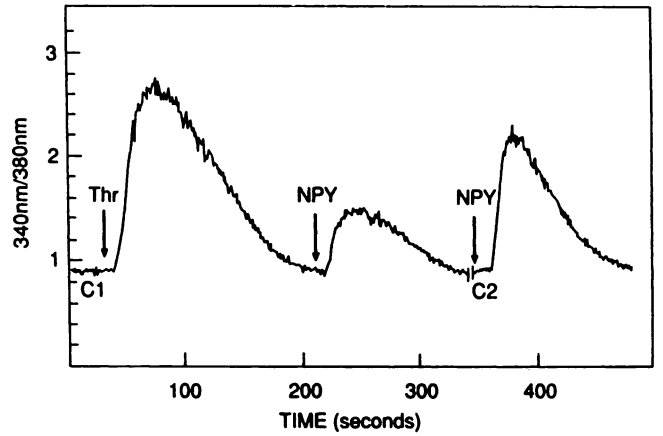

Fig. 3. Heterologous desensitization of the intracellular Ca^{2+} release induced by NPY and α-T. Fura-2-loaded cells were suspended in calcium-free medium containing 6 mM EGTA and were stimulated first with 10 nM α-T and, after return to base line, with 100 nM NPY. Control release induced by 100 nM NPY was followed in a separate cuvette (*C2*).

pool?) for the two agonists or an apparent heterologous desensitization of the NPY receptor.

TG is a tumor-promoting sesquiterpene lactone that has been shown to deplete calcium from the IP_3-sensitive intracellular pool through a direct and selective inhibition of a microsomal Ca^{2+}-ATPase that is responsible for loading Ca^{2+} into the IP_3-sensitive store (20). Because we had shown previously that the NPY-induced release of intracellular Ca^{2+} in HEL cells was associated with the formation of IP_3 (11), we used the calcium-depleting agent in an attempt to characterize and identify the intracellular Ca^{2+} pool activated by NPY and/or α-T and to establish whether a common calcium pool could account for the heterologous desensitization of the response to the aforementioned agonists. In the absence of external calcium, TG induced a slow and transient release of intracellular Ca^{2+} that stabilized at a slightly higher basal level after 8 min. Thereafter, the response to NPY (Fig. 4A), as well as that to α-T (Fig. 4C), was completely abolished. This indicated that NPY and α-T would mobilize calcium from a common, TG-sensitive, intracellular calcium storage site. Moreover, under the same conditions as described above (calcium-free medium), when the release of intracellular Ca^{2+} was first induced by a maximal dose of NPY (Fig. 4B) or α-T (Fig. 4D) the subsequent response to TG was significantly reduced, by 27% and 60%, respectively, in good correlation with the amount of intracellular Ca^{2+} that each agonist was able to deplete.

The possibility that the homologous or apparent heterologous desensitization of the NPY receptor was mediated by activation of PKC, as a consequence of phospholipase C-induced diacylglycerol formation, was examined. Fig. 5 shows that preincubation of HEL cells with the active, but not the inactive, stereoisomer of PMA strongly inhibited the NPY-induced release of intracellular Ca^{2+}. The inhibitory effect of PMA could be partialy prevented by a prior incubation of the cells in the presence of H7, a PKC inhibitor. Partial protection from the PMA-induced inhibition of intracellular Ca^{2+} release by NPY was also obtained by preincubation with 1 μM staurosporine (data not shown), thus strongly suggesting a PKC-dependent mechanism for the inhibition of the NPY response. Nevertheless, preincubation of HEL cells with H7 failed to prevent the homologous desensitization observed after the second addition of NPY (Fig. 6A). Moreover, H7 could not reverse the apparent heterologous desensitization to NPY after the stimulation of cells with α-T in the absence of extracellular calcium (Fig. 6B).

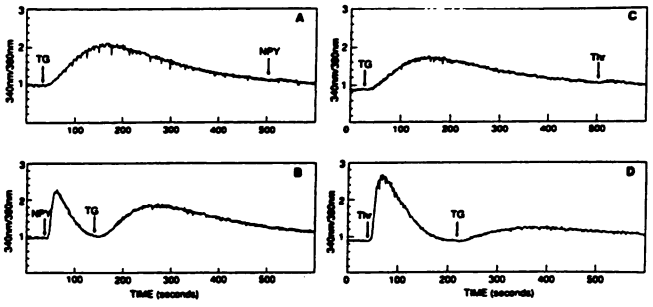

Fig. 4. Effect of TG on the release of intracellular Ca^{2+} induced by NPY or α-T. Fura-2-loaded cells were suspended in calcium-free medium containing 6 mM EGTA. A, Cells were exposed to 50 nM TG and then to 100 nM NPY. B, Cells were exposed to 100 nM NPY and then to 50 nM TG. C, Cells were exposed to 50 nM TG and then to 30 nM α-T. D, Cells were exposed to 30 nM α-T and then to 50 nM TG.

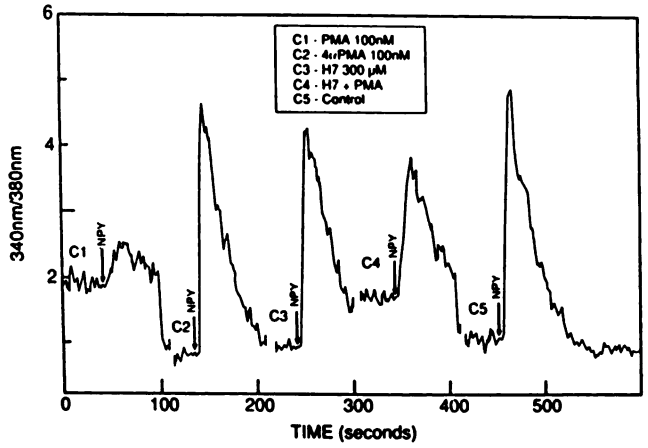

Fig. 5. Effect of activation of PKC by phorbol esters on the NPY-induced release of intracellular Ca^{2+}. Cells were incubated with or without PMA or 4α-PMA for 30 min at 37°. When present, H7 was added 5 min before the phorbol esters. The results represent a typical experiment repeated at least four times with similar results. *C1–C5*, different cuvettes.

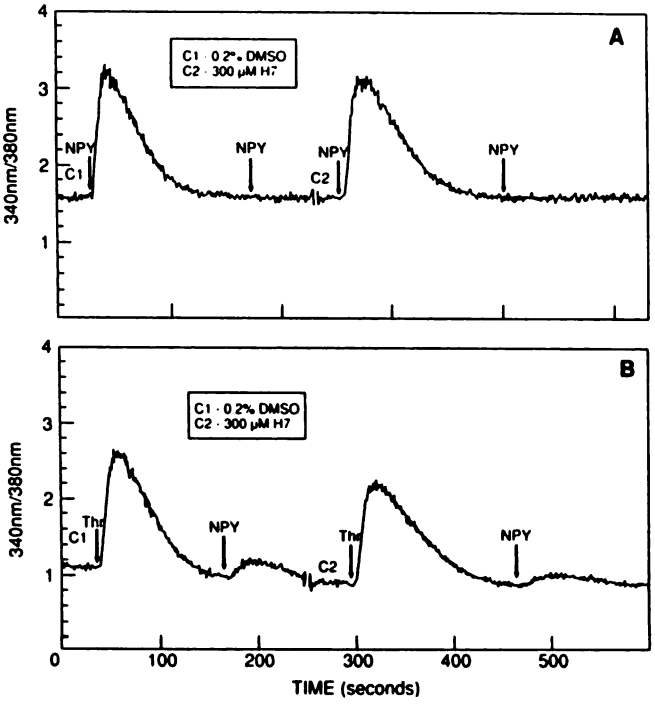

Fig. 6. Effect of the PKC inhibitor H7 on homologous and heterologous desensitization of the NPY-induced release of intracellular Ca^{2+}. Fura-2-loaded cells were incubated for 30 min in the presence of 0.2% dimethyl sulfoxide (*C1*) or 300 μM H7 (*C2*) and were stimulated in normal medium with 100 nM NPY (A) or in calcium-free medium (6 mM EGTA) first with 30 nM α-T and then with 100 nM NPY (B).

Discussion

Physiological (17) as well as biochemical (18, 19) evidence has prompted general acceptance of the classification of NPY receptors into at least two different types (Y_1 and Y_2). The Y_1-type receptor has been found, in most tissues and species, to be the predominant postsynaptic receptor accounting for the vasoconstrictor effect of NPY.

The NPY-induced mobilization of intracellular calcium in HEL cells has been previously reported (10, 11); however, the receptor type was not characterized. The fact that in the present study we have found a 4-fold lower ED_{50} for the Y_1-specific

agonist (Leu-31,Pro-34)-NPY and a much higher ED_{50} for the carboxyl-terminal fragment NPY13–36, with respect to NPY, indicates that HEL cells express the Y_1-type receptor and that, through its activation, NPY induces the release of intracellular calcium. We have previously provided preliminary evidence that this effect of NPY is dependent on the formation of inositol phosphates (11). Thus, the HEL cells may constitute a good model to study the signal transduction pathway for the action of NPY through Y_1 receptors and, by extrapolation, provide information on the mechanisms that lead to the enhancement of contraction of the vascular smooth muscle. Interestingly, endothelin, another highly potent vasoconstrictor peptide, does not induce the release of intracellular Ca^{2+} in HEL cells,[1] although it markedly increases the intracellular Ca^{2+} concentration in cultured smooth muscle cells (21, 22).

The present results indicate that the NPY receptor undergoes homologous desensitization, as indicated by the fact that cells made totally refractory to NPY still respond fully to other agonists. Preincubation of HEL cells with phorbol esters (e.g., PMA), which substitute for diacylglycerol and activate PKC directly, strongly inhibits the NPY-induced release of intracellular Ca^{2+}. However, the homologous desensitization of the NPY response cannot be accounted for by a PKC-dependent protein phosphorylation mechanism, because inhibition of PKC by H7 could not restore the capacity of the HEL cells to release calcium in response to a second addition of NPY. A more likely explanation would be that the NPY receptor either is internalized or undergoes inactivation upon binding of the agonist. Preliminary experiments in our laboratory showed that the loss of responsiveness to NPY persisted even after washing of the cells by centrifugation and resuspension (data not shown). Homologous desensitization was also observed for the response to α-T in HEL cells. While this manuscript was in preparation, Brass *et al.* (23) reported that the mechanism of homologous desensitization to α-T in HEL cells involves proteolytic degradation of the receptor and requires protein synthesis for recovery.

The inability of H7 to prevent the loss of responsivness to NPY after stimulation of the cells with α-T in the absence of extracellular calcium (Fig. 6B) indicates that the mechanism of heterologous desensitization is not through the activation of PKC. The apparent heterologous desensitization of NPY and α-T can, rather, be accounted for by both agonists inducing the release of calcium from the same intracellular pool. The lack of response to NPY and α-T after exposure of the cells to TG, in the absence of external calcium, clearly indicates that both agonists are releasing calcium from a common pool depleted by TG; however, NPY appears to mobilize less intracellular Ca^{2+} than does α-T. This could be accounted for by a smaller accumulation of IP_3 induced by NPY than by α-T. In fact, we have observed that exposure of HEL cells for 30 sec to a maximal dose of NPY (100 nM) generates only 60% of the IP_3 accumulated by a maximal dose (50 nM) of α-T.[1] Alternatively, the NPY receptor could desensitize at a faster rate than the α-T receptor and, thus, limit the efficacy of NPY. Interestingly, maximal stimulation of intracellular Ca^{2+} release by NPY or α-T (in calcium-free medium) does not completely inhibit the subsequent response to TG, indicating that the agent may release calcium from other intracellular pools, in addition to the IP_3-dependent pool. Thus, when intracellular Ca^{2+} release is induced by NPY, α-T, or TG, in the absence of external calcium, the releasable pool cannot be replenished and a second response depends on the degree of depletion of this calcium pool after the first stimulation. The apparent depleting efficacy of the three agonists is TG > α-T > NPY. It has been postulated that the mechanism by which this occurs is by emptying of an IP_3-sensitive pool, which would then function as a signal for calcium entry through the plasma membrane to restore the pool (20, 24).

Although activation of PKC does not account for the desensitization of the HEL cells to NPY, it does inhibit the release of intracellular Ca^{2+} induced by NPY. On the basis of this finding, it is hypothesized that PKC activation may have a role in the termination of the agonist-induced signal transduction in HEL cells. One may speculate that this effect could be due to an inhibition of the formation of IP_3 by inhibition of phospholipase C activation, as suggested in other systems (25, 26), and/or by activation of an IP_3 phosphatase, as has been shown in blood platelets exposed to phorbol esters (27). It should be taken into account, however, that phorbol esters may activate PKC to an extent that would not normally occur in the presence of a physiological agonist. On the other hand, it has been shown that the isolated and purified IP_3 receptor can be phosphorylated and inactivated by a cAMP-dependent protein kinase (28). However, large increases in the HEL cell cAMP content (produced by forskolin, dibutyryl-cAMP, etc.) do not inhibit the release of calcium induced by NPY (10),[1] suggesting that this mechanism would not operate in HEL cells. Alternatively, the IP_3 receptor could be inactivated by PKC-dependent phosphorylation.

In summary, we have shown that NPY releases intracellular Ca^{2+} in HEL cells through the activation of a Y_1-type receptor, from a TG-sensitive pool (IP_3-sensitive pool) common also to α-T. Thus, we have now confirmed our previous finding that NPY activates phospholipase C and releases calcium through an IP_3-dependent mechanism. The latter signal not only releases calcium but also induces the entry of extracellular calcium to maintain the releasable pool.

[1] A. J. Daniels, J. E. Matthews, and E. R. Lazarowski. unpublished observations.

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