α-MSH potentiates the responsiveness of mammotropes by increasing Ca^{2+} entry

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Nuñez, Lucia, and L. Stephen Frawley. α-MSH potentiates the responsiveness of mammotropes by increasing Ca^{2+} entry. *Am. J. Physiol.* 274 (*Endocrinol. Metab.* 37): E971–E977, 1998.—It is well known that the suckling stimulus renders mammotropes considerably more responsive to prolactin (PRL)-releasing stimuli, and the neurointermediate lobe peptide α-melanocyte-stimulating hormone (α-MSH) has been proposed to play a pivotal role in this priming. The objectives of the present study were to determine whether α-MSH could act directly on pituitary cells to potentiate PRL release in response to two physiologically relevant PRL secretagogues, thyrotropin-releasing hormone (TRH) and ATP, and, if so, to identify the mechanism by which this priming phenomenon is manifested. To this end, we cultured anterior pituitary cells from lactating rats overnight and then subjected them to a reverse hemolytic plaque assay for PRL to evaluate their responses to various test agents. We found that α-MSH, which had no effect on PRL export when tested alone, augmented by more than threefold the secretory responses to TRH and ATP. Next, we utilized digital-imaging fluorescence microscopy of fura 2 to evaluate the role of intracellular Ca^{2+} in this process. We found that PRL export induced by pharmacological activation of L-type voltage-operated calcium channels was also potentiated by α-MSH, as was Ca^{2+} entry induced by TRH. Our results indicate that α-MSH acts as a mammotrope-priming agent on a subset of mammotropes by increasing Ca^{2+} entry induced by PRL secretagogues.

prolactin; calcium; suckling; thyrotropin-releasing hormone; adenosine 5′-triphosphate

IT HAS LONG BEEN ESTABLISHED that suckling is the most potent physiological stimulus for prolactin (PRL) release in mammals (4, 23, 28), but the mechanisms by which its effects are transduced are extremely complex and incompletely understood. A critical piece of the mechanistic puzzle resides in the fact that the suckling stimulus induces a potentiation of the secretory responsiveness of mammotropes to physiologically relevant secretagogues such as thyrotropin-releasing hormone (TRH) (14, 21, 24). This was first demonstrated almost two decades ago by Grosvenor et al. (15), who showed that even a brief nursing episode rendered mammotropes enormously sensitive and responsive to the PRL-releasing effects of TRH administered in vivo. More recently, conceptually similar results have been obtained with ex vivo models (21, 22).

Attempts to identify the chemical agent that imparts responsiveness to mammotropes have led to the proposal that the hypophysial neurointermediate lobe (NIL) plays a pivotal role in this process (10, 20). This view is based on observations that the NIL can communicate directly with the anterior pituitary through the short portal vasculature (1, 27) and that surgical removal of the NIL abolishes the acute discharge of PRL evoked by nursing (20). At present, the most likely candidate molecule to subserve the role of an NIL-derived potentiator of mammotrope responsiveness is α-melanocyte-stimulating hormone (α-MSH). This peptide is released abruptly after the suckling stimulus (2, 9, 30), and its in vivo immunoneutralization leads to a severe attenuation of suckling-induced PRL release (17). Moreover, the peptide can substitute in vitro for the nursing stimulus by making mammotropes more responsive to at least one PRL-releasing agent, low-dose dopamine (18). These observations, coupled with our recent report that a discrete subpopulation of rat mammotropes possesses functional α-MSH receptors (35), comprise a convincing (albeit indirect) case that α-MSH is the mammotrope responsiveness agent. However, the final pieces of direct evidence in this regard are currently lacking, as is information about the intracellular mechanisms that govern the potentiation response. Accordingly, the purpose of the present study was to evaluate whether α-MSH could enhance the export of PRL induced by two physiologically relevant secretagogues, TRH and ATP. The former is a bona fide PRL-releasing factor of hypothalamic origin, whereas the latter is released during PRL exocytosis and acts in an autocrine/paracrine manner on mammotropes to induce additional PRL release, thereby amplifying the secretory response (25). In addition, we attempted in this study to determine whether the priming of PRL release by α-MSH was mediated by the potentiation of Ca^{2+} entry induced by PRL secretagogues.

MATERIALS AND METHODS

Animals and primary pituitary cell culture. Primiparous lactating female (*days 6–10* postpartum) rats (Harlan Sprague Dawley, Madison, WI) were provided with food and water ad libitum and maintained under a standard 12:12-h light-dark cycle. Animal housing and handling were performed in accordance with procedures approved by our university animal care committee. Litter size was standardized to eight pups from the first day postpartum. Before decapitation, the pups were separated from their mothers for 4 h. Pituitaries were removed aseptically and dispersed into single cells as described previously (5). Unless otherwise stated, all cell culture reagents were obtained from GIBCO/BRL (Grand Island, NY), and all treatments were purchased from Sigma Chemical (St. Louis, MO).

Reverse hemolytic plaque assay. PRL release measurements from individual anterior pituitary cells were performed by reverse hemolytic plaque assay (RHPA) in accordance with the protocol described by Boockfor et al. (5). Briefly, monodispersed pituitary cells were plated in DMEM supplemented with 0.1% BSA, 10% fetal bovine serum (FBS), and antibiotics. The next day, cells were resuspended by mild trypsinization, mixed with protein A-coated ovine erythro-

cytes, and infused into Cunningham incubation chambers. After a 1-h attachment period, cells were rinsed with DMEM-0.1% BSA, and the resulting monolayers were then flooded with 5 vol (150 μl) of medium containing PRL antiserum (1:80), with or without treatments, and incubated for specified times. Subsequently, guinea pig complement (1:80, 50 min) was flooded into the chambers to develop the plaques. Cells were then fixed and stained for storage and quantification. Three replicates were performed per treatment in each experiment. Series of chambers were incubated with antibody and/or treatments for 60, 90, or 120 min, and the percentage of PRL secretors was determined in each case. Inasmuch as the 90-min incubation time was sufficient to detect a maximal percentage of plaque formers, we used this time frame for quantification of plaque areas. At least 200 plaque areas were quantified in each treatment group by use of a calibrated ocular reticule to derive mean plaque area and frequency distributions of plaque areas.

Digital-imaging fluorescence microscopy. Monodispersed anterior pituitary cells were cultured overnight on plastic Petri dishes in DMEM-10% FBS and subsequently subjected to measurements of intracellular free calcium concentration ($[Ca^{2+}]_i$) by digital-imaging fluorescence microscopy. This was accomplished essentially as reported elsewhere (35). Briefly, cells were loaded with 2 μM fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR) for 1 h in external medium containing (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH, 7.4) and 10 glucose. Then Petri dishes were mounted under the heated stage (37°C) of an Axiovert 35 inverted microscope (Zeiss, Jena, Germany). Cells were epi-illuminated at 340 and 380 nm. Light emitted above 520 nm was recorded and analyzed using an Attofluor Ratio Vision System (Atto Instruments, Rockville, MD). Four video frames of each wavelength were averaged with an overall resolution time of 4 s. Ratios for consecutive frames obtained at 340- and 380-nm excitation were calculated, and $[Ca^{2+}]_i$ was estimated by comparison with fura 2 standards (16). During experiments, cells were bathed in 2 ml of external medium. Addition of test substances was achieved by removing 1 ml of medium and adding 1 ml of warm medium containing no secretagogue or a twofold concentration of the required secretagogue.

To quantify $[Ca^{2+}]_i$ oscillations, we used the Oscillation Index, a parameter developed elsewhere (32, 33). The Oscillation Index represents the rate of change for $[Ca^{2+}]_i$ during the period of measurement and reflects the frequency and/or amplitude of $[Ca^{2+}]_i$ oscillations. To calculate this parameter, absolute differences in $[Ca^{2+}]_i$ levels between successive measurements (taken at 4-s intervals) were averaged for the length of the entire sampling period.

Statistical analysis. All data are reported as the result of at least three completely independent experiments. A two-way ANOVA was employed to analyze the data. Differences in mean plaque areas and Oscillation Index values were compared by use of Bonferroni's multiple comparisons test. Differences were considered significant at $P < 0.05$.

RESULTS

α-MSH potentiates the secretory response of a discrete mammotrope subpopulation to TRH and ATP. Our initial studies were aimed at determining whether α-MSH could modulate the amount of PRL released by a consensus PRL-releasing factor, such as TRH, or by a more recently established autocrine/paracrine stimulator of PRL export, ATP. The strategy was to treat pituitary cells obtained from lactating rats with these secretagogues and then to quantify PRL release from individual living cells by RHPA. Plaque area, a reliable index of the relative amount of hormone released (11, 19), was used as the biological end point for these studies. We found, as we had found previously (25), that TRH and ATP (both at maximal doses) could each evoke a modest increase of PRL release, whereas α-MSH alone was ineffective in this regard (Fig. 1). It is noteworthy that TRH and ATP induced comparable increments (25 and 26% above control value, respec-

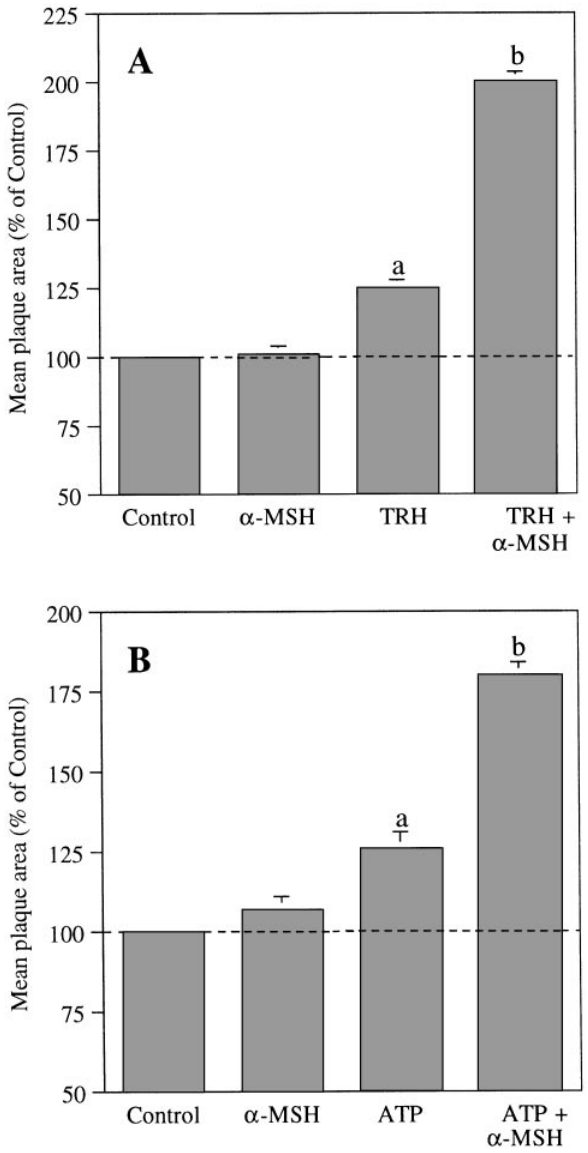

Fig. 1. α-MSH potentiates effects of thyrotropin-releasing hormone (TRH) or ATP on prolactin (PRL) secretion. Anterior pituitary cells derived from lactating rats deprived of their pups for 4 h were cultured overnight and then subjected to reverse hemolytic plaque assays (RHPA) for determination of PRL release. Cells were stimulated for 90 min with TRH (100 nM, *A*) or ATP (10 μM, *B*) in the presence or the absence of α-melanocyte-stimulating hormone (α-MSH, 100 nM). Mean plaque areas reflective of cumulative PRL release are expressed here as a percentage of control. Values are means ± SE of 3 completely independent experiments in which 3 replicates were measured per sample. Values having different letters are significantly different ($P < 0.05$) from each other or from control group.

tively) of PRL release. Interestingly, coexposure to α-MSH augmented the amount of PRL released by either TRH or ATP (Fig. 1, *A* and *B*). More specifically, the secretory response induced by the combination of α-MSH and TRH was fourfold higher than that elicited by TRH alone. Likewise, the combination of α-MSH and ATP led to a response 3.2 times that evoked by ATP alone. Thus it is clear that the secretory responses of mammotropes to PRL secretagogues are potentiated by concurrent exposure to α-MSH.

We established recently that only a subpopulation of mammotropes, accounting for about one-fifth of the total, exhibits functional α-MSH receptors (35). Accordingly, we tested whether the potentiating effects of α-MSH could be localized to such a subpopulation. We found, as we had previously, that addition of either TRH or ATP induced a unimodal shift to the right in the frequency distribution of plaque areas, indicating that most if not all mammotropes released PRL in response to these agents (data not shown). However, when each of these secretagogues was applied in combination with α-MSH, the frequency distribution of plaque areas shifted from unimodal to bimodal (Fig. 2, *A* and *B*). The first of these modes corresponded precisely to that found with TRH- or ATP-treated cells, whereas the second mode (representing cells that released considerably more hormone) was found only when α-MSH was present. These data demonstrate that only a subpopulation of mammotropes was rendered much more responsive to PRL secretagogues by α-MSH.

α-MSH potentiates PRL release induced by Ca^{2+} entry through L-type voltage-operated Ca^{2+} channels. Because it is well established that TRH and ATP induce PRL release, at least in part, through calcium-dependent mechanisms (6, 13), we asked whether α-MSH was able to potentiate Ca^{2+}-mediated PRL export. To this end, we stimulated pituitary cells with each of two Ca^{2+} agonists, either alone or in combination with α-MSH. One of these was BAY K 8644 (BK), a dihydropyridine derivative that induces Ca^{2+} entry into the cells through L-type voltage-operated Ca^{2+} channels (VOCC) (12). The other was ionomycin, an ionophore that induces a $[Ca^{2+}]_i$ increase primarily by release from intracellular Ca^{2+} stores and does not influence L-type VOCC (3, 32). In preliminary experiments, we established the doses of BK (1 nM) and ionomycin (200 nM) that could stimulate PRL release to a degree indistinguishable from that of ATP, and this is illustrated in Fig. 3. Interestingly, when α-MSH was coadministered with BK, it augmented PRL release just as it had potentiated that of ATP. In striking contrast, cotreatment with α-MSH had no effect on the amount of PRL released by the Ca^{2+} agonist ionomycin (Fig. 3). These data indicate clearly that α-MSH augments the release of PRL induced by Ca^{2+} entry through L-type Ca^{2+} channels but not that evoked by mobilization of intracellular Ca^{2+} stores.

α-MSH is able to potentiate Ca^{2+} entry induced by a PRL secretagogue. Having established that α-MSH potentiates PRL release induced by Ca^{2+} entry through L-type VOCC, we asked whether the peptide interacts

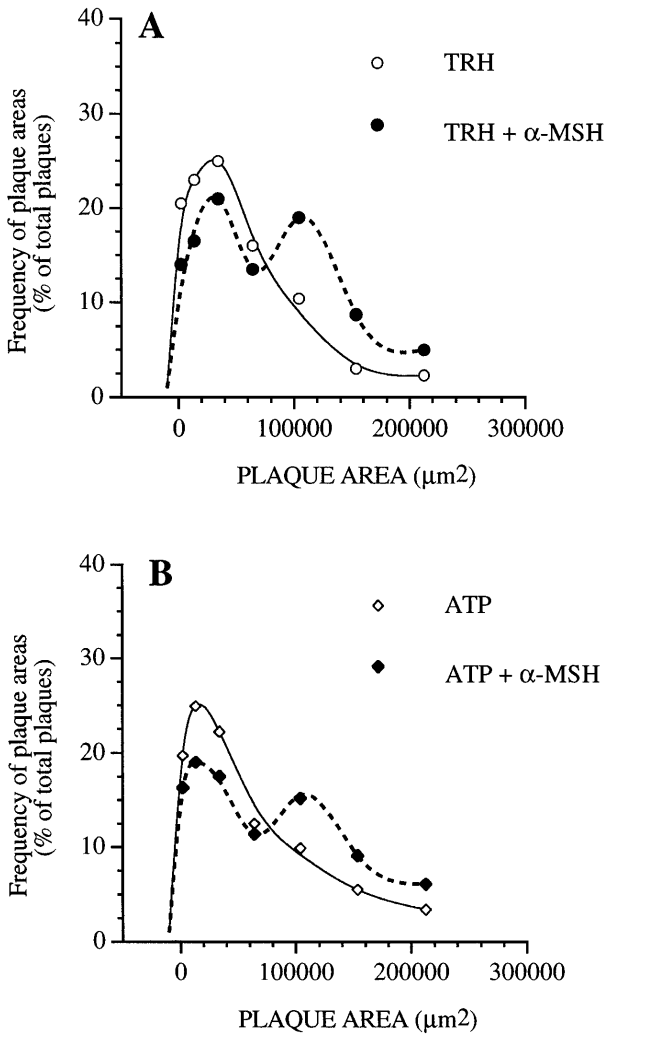

Fig. 2. Effects of α-MSH on TRH- or ATP-induced PRL secretion are only observed in a subpopulation of mammotropes. Frequency distributions of PRL plaque areas after stimulation with TRH (○) or ATP (◇) are shown. Cotreatment with α-MSH (solid symbols) evoked a shift from a unimodal frequency distribution to a bimodal one (conditions as in Fig. 1). Distributions were generated by plotting areas of 3 replicates in 7 different groups. Values are the average of 3 independent experiments.

with a bona fide PRL secretagogue via a similar mechanism. Accordingly, we made $[Ca^{2+}]_i$ measurements during the exposure of pituitary cells to TRH (or BK), administered alone or after priming with α-MSH. Figure 4 shows the averaged responses of representative pituitary cells stimulated with TRH or BK at the same concentrations employed in our measurements of PRL release by RHPA. Consistent with our previous results (35), we found that a subpopulation of pituitary cells (17%) exhibited a moderate increase of $[Ca^{2+}]_i$ secondary to activation of α-MSH binding [we had established elsewhere (35) that such binding was restricted to mammotropes]. However, this increase of $[Ca^{2+}]_i$ was transient and returned to values indistinguishable from basal levels within 5–10 min (Ref. 35 and unpublished observations). To determine whether α-MSH could modify Ca^{2+} responses to TRH and BK, we averaged the $[Ca^{2+}]_i$ profiles for those cells exhibiting a

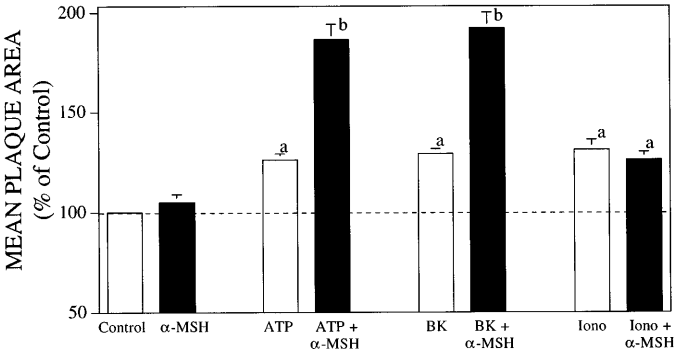

Fig. 3. α-MSH potentiates secretory response to an L-type calcium channel agonist but not to a calcium ionophore. PRL secretion was measured by RHPA in response to 90-min incubations with an L-type calcium channel agonist, BAY K 8644 (BK, 1 nM), or to a calcium ionophore, ionomycin (Iono, 200 nM), in the presence or the absence of α-MSH (100 nM). Values are means ± SE of 3 completely independent experiments in which 3 replicates were measured per sample. Values having different letters are significantly different ($P < 0.05$) from each other or from control group.

$[Ca^{2+}]_i$ increase after α-MSH stimulation and compared them with the profiles obtained in nontreated cells or in those cells that were not responsive to the α-MSH challenge. As shown in Fig. 4, the transient increase of $[Ca^{2+}]_i$ that occurred a few seconds after TRH administration (which is traditionally attributed to Ca^{2+} release from intracellular stores) was similar for α-MSH-responsive cells, nontreated cells, and cells not responsive to α-MSH. However, the second phase of TRH action (widely attributed to enhanced $[Ca^{2+}]_i$ oscillations and Ca^{2+} entry through VOCC) was quite apparent in α-MSH-responsive cells, as opposed to nontreated or nonresponsive cells. A similar output was obtained when the cells were stimulated with BK instead of TRH. Specifically, the increase of $[Ca^{2+}]_i$ induced by BK in α-MSH-responsive cells was larger than the rather modest rise obtained in nontreated cells or those not responsive to α-MSH. These data clearly indicate that Ca^{2+} entry induced by TRH or BK is enhanced in a subpopulation of mammotropes bearing functional α-MSH receptors. To quantify the potentiating effects of α-MSH on Ca^{2+} entry in this subset of mammotropes, we computed the Oscillation Index, a parameter that reflects the frequency and/or amplitude of $[Ca^{2+}]_i$ oscillations (32, 33). Because $[Ca^{2+}]_i$ oscillations in mammotropes are driven by electrical activity and Ca^{2+} influx through VOCC, the magnitude of the Oscillation Index provides an indirect measure of the rate of Ca^{2+} influx through these channels. As shown in Fig. 5, the Oscillation Index after treatment with TRH or BK was enhanced in α-MSH-responsive cells compared with nontreated (Control) cells or cells that were not sensitive to α-MSH stimulation. These data clearly indicate that α-MSH enhanced Ca^{2+} entry induced by TRH or BK in a subpopulation of mammotropes bearing functional α-MSH receptors.

DISCUSSION

The results of the present study demonstrate unequivocally that α-MSH acts on a discrete subset of mammotropes to augment their responsiveness to two physiologically important PRL-releasing agents, TRH and ATP. How do these findings contribute to and extend our current understanding about the mechanisms governing suckling-induced PRL release? It is well established that the suckling stimulus decreases and increases, respectively, the amount of dopamine and TRH secreted by the hypothalamus into the hypophysial portal blood that bathes mammotropes within the adenohypophysis (7, 8, 26). However, the magnitudes of these changes (when viewed either alone or collectively) cannot begin to account for the massive discharge of PRL that occurs in response to nursing. A suckling-induced change of mammotrope responsiveness to these agents has been invoked as a possible explanation for this dilemma, and, as indicated earlier, there is considerable evidence derived from studies conducted in vivo and in vitro to support the idea that α-MSH is the mammotrope responsiveness agent (10). With respect to direct effects on the pituitary gland, α-MSH has been shown to act on mammotropes to override inhibition of PRL release imposed by high concentrations of dopamine (18, 21, 22) and to impart mammotrope responsiveness to the PRL-releasing ac-

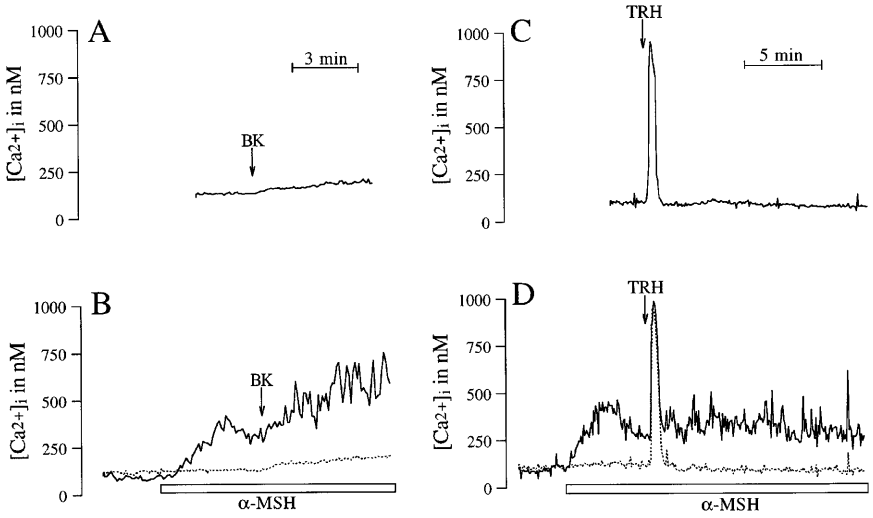

Fig. 4. α-MSH potentiates Ca^{2+} influx induced by TRH and BK. Anterior pituitary cells were cultured overnight, loaded with Ca^{2+}-sensitive probe fura 2, and subjected to measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by digital-imaging fluorescence microscopy. Cells were stimulated with BK (1 nM) or TRH (100 nM). Traces shown are average responses to BK (*A*, $n = 50$) or to TRH (*C*, $n = 42$) in the absence of α-MSH. In *C*, only TRH-sensitive cells were taken into account. Stimulation of cells with α-MSH (100 nM) enhanced $[Ca^{2+}]_i$ oscillations elicited by BK (*B*) or TRH (*D*) only in those cells exhibiting an increase of $[Ca^{2+}]_i$ induced by α-MSH (solid traces, $n = 12$ for *B*, $n = 11$ for *D*) but not in cells not responsive to α-MSH (dotted traces, $n = 50$ for *B*, $n = 31$ for *D*). This experiment is representative of 6 (BK) or 3 (TRH) others.

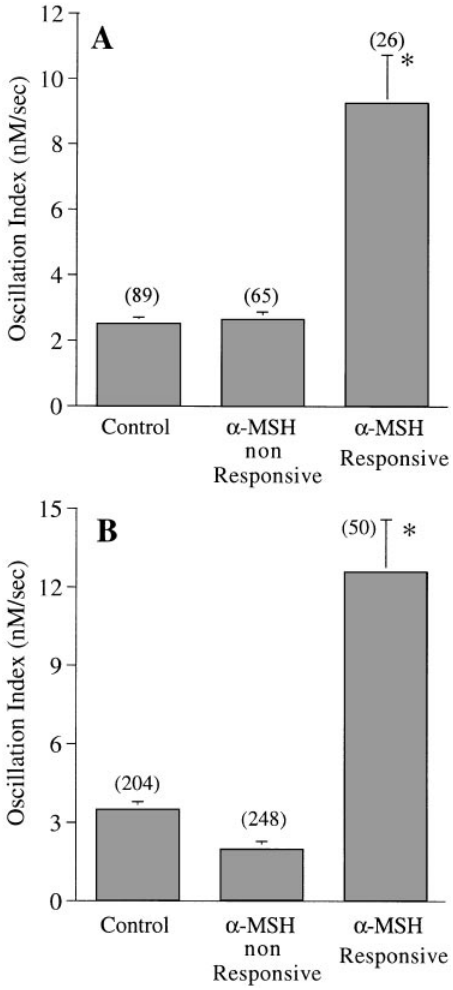

Fig. 5. α-MSH potentiates oscillatory activity induced by TRH or BK. Effects of TRH or BK on $[Ca^{2+}]_i$ oscillations (Fig. 4) were analyzed further here by use of a parameter previously developed for this purpose, the Oscillation Index. *A*: Oscillation Index for 2nd phase of TRH-induced $[Ca^{2+}]_i$ response (100 s after addition of secretagogue). Only this phase was selected for analysis because it corresponded to Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels, which are α-MSH responsive. *B*: results obtained with companion cultures treated with BK. For *A* and *B*, no. of observations is within parentheses above each bar. Values are means ± SE of 3 (TRH) or 6 (BK) independent experiments. $*P < 0.05$ vs. other treatments. Note that TRH and BK increased Oscillation Index only in cells responsive to α-MSH.

tions of the lower concentrations of dopamine that reach the pituitary gland immediately after nursing (18). Our present efforts expand the scope of this responsiveness phenomenon by demonstrating for the first time that α-MSH administered in vitro can substitute for the suckling stimulus in vivo by rendering a subgroup of mammotropes considerably more responsive to the PRL-releasing effects of TRH. Thus it appears that suckling evokes the concurrent release of hypophysiotropic agents (TRH and low-dose dopamine) that stimulate PRL export and a mammotrope responsiveness factor, which optimizes and embellishes this response. The net effect, then, is an increase in the rate of PRL release. How does ATP fit into this picture? We have previously shown that ATP is coreleased with PRL during exocytosis, that its rate of export is modulated by TRH and the dopamine agonist bromocryptine in a predictable manner (stimulation and inhibition, respectively), that addition of ATP stimulates PRL release, and that removal of the purine from medium bathing pituitary cells causes a diminution of basal and regulated PRL secretion (25). On the basis of these observations, we concluded that ATP acts as an autocrine/paracrine regulator of PRL release that serves to initiate an autoamplification cascade that prolongs the secretory response elicited by a given amount of hypophysiotropic signal. Our present findings add a new dimension to this process in that the PRL-releasing effects of the amplifying agent (ATP) were also found to be augmented greatly in the presence of α-MSH. Taken together, it seems reasonable to conclude that α-MSH, which by itself has no influence on PRL export, potentiates the acute release of PRL by modulating mammotrope responsiveness to at least a trio of regulatory agents.

Having identified many of the major extracellular players involved in elaboration of the responsiveness phenomenon, we next turned our attention to the intracellular processes that might transduce the response. Accordingly, we explored the possibility that changes in $[Ca^{2+}]_i$ contribute to this phenomenon for reasons outlined earlier. Our strategy here was to evaluate the potentiating effects of α-MSH with agents that elevated $[Ca^{2+}]_i$ by different mechanisms. Interestingly, we found that α-MSH greatly augmented the amount of PRL released by BK, which elevates $[Ca^{2+}]_i$ by facilitating Ca^{2+} passage across the cell membrane through L-type VOCC (12). In contrast, α-MSH had no effect on the amount of PRL secreted in response to ionomycin, a Ca^{2+} agonist that liberates the ion from sequestered intracellular stores (3, 32). In light of these findings, we proposed that α-MSH exerted its responsiveness effects by potentiating, at least in part, Ca^{2+} entry induced by physiologically relevant secretagogues. Such a possibility seemed viable, given reports that both TRH and ATP activate Ca^{2+} entry through L-type VOCC (29, 31, 34). In an attempt to test this idea experimentally, we monitored $[Ca^{2+}]_i$ during exposure of pituitary cells to TRH administered alone or after priming with α-MSH. Our results demonstrate that α-MSH did indeed augment TRH-induced $[Ca^{2+}]_i$ dynamics within mammotropes, and that it did so with a profile (enhanced plateau phase of the $[Ca^{2+}]_i$ trace) consistent with a primary effect on Ca^{2+} entry through VOCC. Thus the mammotrope responsiveness changes attributable to α-MSH appear to be mediated by potentiation of extracellular Ca^{2+} entry induced by other secretagogues such as TRH.

In summary, we have provided compelling evidence that α-MSH acts as a mammotrope priming agent by rendering these cells much more responsive to physiologically relevant PRL secretagogues. This effect is attributable to a direct action of α-MSH on a discrete subpopulation of mammotropes bearing the corresponding receptor. In addition, we have shown that the peptide's augmentation of secretory responsiveness is

mediated by potentiation of Ca^{2+} entry through L-type VOCC induced by at least one physiological secretagogue, TRH. It is noteworthy that a number of laboratories, including our own, have pursued for almost two decades the elusive prolactin-releasing factor (PRF) that mediates suckling-induced PRL release. Although there is no shortage of candidate molecules to subserve such a role, none of these is sufficiently potent to account for the massive surge of PRL release that occurs after suckling. Perhaps our search has been complicated by the fact that the "real" PRF is actually the combination of a priming factor (e.g., α-MSH) and other secretagogues that, in the absence of priming, elicit a rather modest increase of PRL secretion. The present results are entirely consistent with this possibility.

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