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ical demonstration of the transmitter content of the receptor-bearing cell types. Therefore, previous attempts to immunocytochemically characterize radioligand-labeled cells have involved in vivo autoradiography or neighboring tissue sections. To overcome some of the obvious disadvantages connected with these methods, we have developed a technique using primary cell cultures to which radiolabeled peptides are bound while still viable, followed by fixation and immunocytochemical staining of the hormone content.

Materials and Methods

Chemicals. Carrier-free Bolton–Hunteriodinated substance P ([125I]-BH-SP) was purchased from Amersham (Buckinghamshire, UK). Substance P, leupeptin, chymostatin, and bacitracin were obtained from Cambidge Research Biochemicals (Cambidge, UK). Cell culture media were from Gibco (Paisley, UK). All other chemicals used were obtained from Sigma Chemical (St Louis, MO). [125I]-BH-SP was dissolved in 0.1 M aetic acid and stored at −20°C under nitrogen. Under these conditions the tracer was stable for at least 2 months, as evaluated by HPLC analysis, which in addition confirmed that the tracer was carrier free.

Preparation of Dispersed Anterior Pituitary Cell Cultures. Adult male Wistar rats were decapitated, the pituitaries removed, and the neurointermediate lobe separated from the anterior lobe. The anterior lobes were cut into about 10 smaller fragments and isolated pituitary cells were prepared by trypsinizing the organs according to the method of Gillies and Lowry (1978), as modified by Larsen et al. (1989b). The dispersed cell sample was re-suspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 15 ug benzylpenicillin/ml, and 25 ug streptomycin/ml to a final concentration of 1 x 10^6 cells/ml. The dispersed anterior pituitary cells were placed in 8- or 16-well Tissue-Tek Chamber slides (Nunc; Roskilde, Denmark) with 5 x 10^3 to 1 x 10^4 cells per well and kept at 37°C in a CO2/air incubator until the cells were adherent to the well, which usually took place after 3 to 5 days.

Substance P Receptor Binding Study. The binding experiments were performed essentially as described earlier (Larsen et al., 1989b), with minor variations. To abolish internalization of the ligand and minimize the action of enzymatic degradation, pre-incubation and incubation with the radioactive ligand were performed at 0°C. Primary anterior pituitary cell cultures were washed twice with Earl’s balanced salt solution (EBSS) containing 0.2% bovine serum albumin (BSA), followed by pre-incubation for 20 min in 0.5 ml EBSS containing 0.2% BSA and 3 mM MgCl2 per well (Buffer A). Thereafter, the cell cultures were incubated in 0.5 ml of Buffer A to which 40 µg bacitracin, 40 µg chymostatin, 4 µg leupeptin, and aprotonin (100 kallikrein-inactivating units/ml) were added. At time zero, [125I]-BH-SP (spec. act. 2000 Ci/mmol) was added, giving a final concentration of 0.5 nM of the radioactive tracer. Nonspecific binding was estimated from incubations with the presence of 1.5 µM non-iodinated substance P. After 20 min incubation, the wells were washed with chilled EBSS-BSA four times for 30 sec, followed by addition of 0.5 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) fixative for 1 hr.

Immunocytochemistry. After fixation, the wells were washed twice with PBS, pH 7.4. Immunocytochemical staining of fixed primary anterior pituitary cell cultures to which [125I]-BH-SP was bound was performed no longer than 3 days after the binding experiment. The wells were washed twice for 10 min with PBS and then incubated for 30 min in 5% pre-immune swine serum in PBS. Thereafter, the cell cultures were incubated at 4°C for 24 hr in either rabbit anti-prolactin (PRL, 1:1000) antiserum, rabbit anti-luteinizing hormone (LH, 1:1000) antiserum, or a mixture of both dissolved in PBS + 1% BSA. The PRL and LH antisera were supplied by the National Pituitary and Hormone Distribution, NIDDK (Bethesda, MD) and have previously been used successfully in several immunohistochemical studies (Nikitowitch–Winer et al., 1987).

After incubation with the primary antiserum, the cell cultures were washed three times for 10 min in PBS with 0.25% BSA, followed by incubation in biotinylated swine anti-rabbit IgG (Dakopatts; Copenhagen, Denmark) diluted 1:400 in PBS containing 1% BSA at room temperature for 60 min. The cell cultures were then washed three times for 10 min in PBS with 0.25% BSA and finally incubated at room temperature for 60 min in strepavidin–HRP complex (Dakopatts). Thereafter, they were washed in PBS with 0.25% BSA for 10 min and finally incubated in 0.05 M Tris-HCl buffer (pH 7.4) for a further 10 min. The cell cultures were reacted for peroxidase activity by incubation in the latter buffer containing 0.025% 3,3'-diaminobenzidine (DAB) and 0.001% H2O2 for 20 min. After washing twice for 10 min in distilled water, the top section of the Tissue-Tek Chamber slide was removed and the tissue slides were air-dried and covered with Ilford K-2 emulsion diluted 1:1 in distilled water as described by Hösli et al. (1975). The emulsion-coated slides were exposed in the dark for 4–7 days and finally developed with Kodak D19 developer. After developing the emulsion, the slides went through a series of ethanol and were cover-slipped.

Results

Receptor Autoradiography

Binding of [125I]-BH-SP to anterior pituitary cell cultures in primary culture resulted in uptake and breakdown of the ligand at 37°C but...
not at 0°C (Larsen et al., 1989b). After incubation of primary anterior pituitary cultures at 0°C with [125I]-BH-SP, many cells showed binding of this radioligand when subjected to autoradiography. Cells were considered positively labeled when they were overlaid with a grain density five times that of the background. The majority of labeled cells were large and rounded (Figure 1), while a minor population appeared multiform, probably representing flattened cells more extensively attached to the chamber slide. Some cells, however, remained free of [125I]-BH-SP (Figure 1).

Developed grains were unevenly distributed throughout the area of the culture chamber. Close to the periphery of the chamber a moderate number of grains were associated with cell bodies, while the highest number of grains was observed within the center of the chamber. As shown in Figure 2, it was impossible to evaluate the immunocytochemical staining properties of such heavily labeled cells after 7 days of exposure. Therefore, the evaluation of combined radioligand labeling and immunohistochemical staining was performed on cells after 4 days of exposure. The radioligand labeling was clearly associated with the surface of cultured cells, although it was impossible at the light microscopic level to exclude intracellularly localized radioligand. Most heavily labeled was the central part of cells, but the cell outgrowths characteristic of adherent cultured cells were also labeled (Figure 6). The binding of [125I]-BH-SP was reversible, as demonstrated in cultures in which 1.5 μM non-radioactive SP was present during the incubation (Figures 4, 7D, and 7E).

Immunocytochemistry

Among the cells of primary anterior pituitary cell cultures, a high number of PRL-IR cells was present, whereas the number of LH-IR cells was low. In a separate series of experiments the PRL and LH antisera were mixed, and using this procedure a very high number of immunoreactive cells was revealed. Despite the nature of the immunocytochemical reaction, a number of non-immunoreactive cells were always present, probably representing other cell types in the heterogeneous anterior pituitary cell population. The immunoreactive material was granular, and usually the nuclear area was seen as non-immunoreactive centrally located ovoid spot (Figures 3, 6, and 7E). In the combined experiments, this phenomenon served as a useful guideline to determine whether a cell possessing [125I]-BH-SP binding sites was at the same time an immunopositive cell. Cell outgrowths, when present, were less immunoreactive than the central part of the cells. Control cell cultures were incubated with pre-immune serum or primary antisera pre-absorbed with the peptides against which they had been raised. Pre-immune serum was unable to visualize immunoreactive material, and only peptides against which the antisera were raised could abolish the immunohistochemical staining.

Combined Results

When immunocytochemistry was combined with binding of [125I]-BH-SP to anterior pituitary cell cultures, it was revealed that PRL-IR as well as LH-IR cells were labeled with the radioligand (Figures 7A–7C). The immunoreactive material was clearly distinguishable in most labeled cells but, as mentioned above, the density of grains was, in some cases exposed for 7 days, so high that evaluation of the immunocytochemical reaction was impossible. However, a careful examination of cell cultures exposed for 4 days revealed that all PRL-IR and LH-IR cells possessed SP receptors. In some cases non-immunoreactive cells were labeled with the radioligand (Figure 7F). When the intensity of the immunocytochemical staining was compared to cultures in which [125I]-BH-SP was not bound, no apparent difference in staining intensity could be observed.

Discussion

The present study demonstrates that binding sites for SP are localized on a large proportion of cultured anterior pituitary cells. Binding of [125I]-BH-SP appeared to be specific, because it was inhibited to a great extent by addition of 1.5 μM unlabeled SP to the incubation medium. The presence of SP binding sites on anterior pituitary cells is consistent with previously performed biochemical receptor binding experiments (Larsen et al., 1989a,b; Kerdelhué et al., 1985). The combination with immunocytochemistry has made it possible to confine the binding of a radioligand to biochemically defined cells. The number of SP binding sites within the anterior pituitary gland is fairly low, and the receptor ligand complex is internalized and recycled (Larsen et al., 1989b). Therefore, it is likely that the synthesis rate of SP receptors in this organ is very low, and this could explain why a recent attempt to demonstrate mRNA coding for the SP receptor in anterior pituitary tissue extracts found specific mRNA content to be below the detection limit (Hershey et al., 1991).

The observed variation in grain density between the center and the outer perimeter of the culture chamber is likely to be caused by variations in thickness of the emulsion layer covering the cultures. First, the surface of a tissue culture is uneven, raising the possibility that the emulsion layer is thinner immediately above the cell body. Second, the tissue chamber slides employed have their removable top glued to the plastic slide with a silicone membrane. This membrane is highly hydrophobic, leaving the emulsion most likely to attach to the central area of the culture chamber. Because of the previously mentioned variability of radioligand labeling in a single culture chamber, a quantitative estimate of the receptor density was not given. After 7 days of exposure, a high grain density was obtained in the central area of the culture chamber, obscuring the immunocytochemical staining. Therefore, it was not possible to assess with certainty the proportion of immunoreactive cells labeled with [125I]-BH-SP. However, after only 4 days of exposure the grain density was sufficiently low to enable evaluation of most immunocytochemically stained cells labeled with the radioligand, revealing that all PRL- and LH-containing cells possess SP receptors.

The use of primary cell cultures instead of post-mortem tissue sections makes it possible to bind radioligands to living cells with presumably unaffected surface receptors, although it is still possible that the dispersion and culturing procedures may have affected the number of binding sites as well as the biochemical properties of the receptor in question. Our earlier binding experiments with [125I]-BH-SP clearly show that the kinetic data obtained from anterior pituitary cell membranes closely match those obtained from cultured anterior pituitary cells (Larsen et al., 1989a,b). The pres-
Figure 7. (A) Medium-power photomicrograph showing $^{125I}$-BH-SP-labeled anterior pituitary cells immunoreactive for either PRL or LH. The grains of the photographic emulsion are closely associated with the immunocytochemically stained cells. In a number of immunoreactive cells the nucleus is clearly designated as a clear spot, helping to determine the specificity of the staining. (B,C) Photomicrographs showing $^{125I}$-BH-SP labeled anterior pituitary cells immunoreactive for either PRL (B) or LH (C). When $^{125I}$-BH-SP was bound in the presence of 1.5 μM unlabeled substance P, the number of grains associated with PRL-immunoreactive (D) or LH-immunoreactive (E) cells was very low, thus representing nonspecific labeling. (F) Two anterior pituitary cells immunoreactive neither for PRL nor for LH and possessing $^{125I}$-BH-SP binding sites are seen. (G) PRL/LH-immunoreactive cells concomitantly labeled with $^{125I}$-BH-SP (open arrow) are seen close to a non-immunoreactive cell possessing SP binding sites (solid arrow). Bars: A = 100 μm; B = 50 μm; C-G = 25 μm.
corticotropin from anterior pituitaries was inhibited by addition of SP to the incubation medium (Nicholson et al., 1989). Moreover, the number of SP binding sites within the anterior pituitary lobe is inversely correlated with the number of LHRH binding sites during the estrous cycle (Kerdelhut et al., 1988). However, it is still speculative whether corticotrophs are in fact influenced by SP.

Concomitant visualization of radioligand binding sites and intracellular neurotransmitters has been done previously using techniques different from the present (Szegethy and Beaudet, 1989; Akesson and Micevych, 1988; Morell and Pfaff, 1983). The most successful report of combined receptor autoradiography and immunocytochemistry involved in vivo binding of radiolabeled steroids (Morell and Pfaff, 1983). Not all cells labeled with $[^{125}]$-BH-SP were immunocytochemically stained, and this was true even when the PRL and LH antisera were mixed. The anterior pituitary gland consists of a heterogeneous population of cells identifiable according to their content of pituitary hormones produced (Herlant, 1964). Therefore, it is likely that cell types other than the two presently investigated may possess SP receptors.

From several reports, SP has been shown to stimulate the release of PRL from anterior pituitary cells in vivo as well as in vitro (Eckstein et al., 1980; Chihara et al., 1978; Vijayan and McCormick, 1978; Kato et al., 1976). The presence of $[^{125}]$-BH-SP binding sites on PRL-containing cells provides evidence that SP exerts its prolactin-releasing action directly on mammatrophs. In addition to this, SP may influence LH-containing gonadotrophs, which is in accordance with the finding that SP inhibits LHRH-stimulated LH release from anterior pituitary cell cultures (Kerdelhut et al., 1979). Furthermore, the number of SP binding sites within the anterior pituitary lobe is inversely correlated with the number of LHRH binding sites during the estrous cycle (Kerdelhut et al., 1985). However, the influence of SP on the release of other anterior pituitary hormones is more complex. It has been reported that corticotropin-releasing hormone and arginine-vasopressin-stimulated release of corticotropin from anterior pituitaries was inhibited by addition of SP to the incubation medium (Nicholson et al., 1984). Recently, another study could not confirm any effect of SP on corticotropin-releasing hormone-stimulated release of corticotropin from dispersed anterior pituitary cell cultures (Chowdrey et al., 1990). Therefore, it is still speculative whether corticotrophs are in fact influenced by SP.

We have previously shown that SP induces receptor-mediated hydrolysis of polyphosphoinositides in the rat anterior pituitary lobe (Mau et al., 1990). Consistent with the findings of the present study, it seems likely that SP induces its effects on mammatrophs and gonadotrophs via the polyphosphoinositide pathway. Since many cells in cultures of anterior pituitary glands, not merely mammatrophs, can be labeled by incubation with $[^{125}]$-BH-SP, their mixed cultures cannot be used for experiments concerned with second messenger pathways leading only to PRL secretion.

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