PKA inhibitor, H-89, affects the intracellular transit of regulated secretory proteins in rat lacrimal glands

P. ROBIN, B. ROSSIGNOL, AND M. N. RAYMOND
Laboratoire de Biochimie des Transports Cellulaires, Centre National de la Recherche Scientifique, ERS 0571, Université Paris XI, 91 405 Orsay Cedex, France

Robin, P., B. Rossignol, and M. N. Raymond. PKA inhibitor, H-89, affects the intracellular transit of regulated secretory proteins in rat lacrimal glands. Am. J. Physiol. 274 (Cell Physiol. 43): C262–C271, 1998.—We tested the effect of H-89, a protein kinase A (PKA) inhibitor, on the intracellular transit of the regulated secretory proteins in rat lacrimal glands. We show that H-89, by itself, induces the secretion of newly synthesized proteins trafficking in its presence but not of proteins already stored in the mature secretory granules. This secretion does not depend on the presence of extracellular Ca2+. The proteins released are identical to those secreted after cholinergic stimulation or under the action of the ionophore A23187, but the secretion level is ∼40% lower. The effect of H-89 seems to be due to PKA inhibition because other protein kinase inhibitors (calphostin C, chelerythrine, H-85) do not induce secretion. We further show that H-89 does not modify the rate of glycoprotein galactosylation but induces the secretion of newly galactosylated glycoproteins. Finally, we used a “20°C block” procedure to show that H-89 affects a trans-Golgi network (TGN) or post-TGN step of the secretory pathway. Our results demonstrate that, in lacrimal cells, H-89 affects the intracellular trafficking of secretory proteins, suggesting a role for PKA in this process.

protein kinase A; regulation of intracellular transit; protein sorting; protein secretion

EXTRAORBITAL LACRIMAL GLANDS are exocrine glands specialized in the secretion of ions and proteins, which are constituents of tears. The proteins, synthesized in polarized cells organized in acini, are delivered to the lumen of these acini.

The following two distinct, well characterized, secretory pathways have been described for these proteins: the constitutive pathway and the regulated pathway (6). In the constitutive pathway, proteins are transported to the plasma membrane in small vesicles originating from the trans-Golgi network (TGN) and are immediately released. In contrast, the proteins destined to be secreted via the regulated pathway are packaged into large granules that undergo a maturation process. The mature secretory granules thus formed accumulate at the apex of the cells until their exocytosis is triggered by an external stimulus. The proteins destined for each of these pathways are sorted in the TGN by mechanisms that are still under discussion. The selective aggregation of the regulated secretory proteins is thought to be the main event controlling the sorting toward the secretory granules, but other phenomena, such as binding to a sorting receptor, may be implicated (4, 12, 21).

In some cells, a third kind of secretion has been described, referred to as “constitutive-like” secretion (2, 3). The proteins secreted via that pathway are identical or derived (after proteolysis) from those secreted via the regulated pathway. This pathway seems to be connected to the maturation process of regulated secretory granules; vesicles would be generated from maturing granules and directly fuse with the plasma membrane in an unregulated way.

In the extraorbital lacrimal gland, two main signal transduction pathways can regulate the exocytosis of the proteins stored in the secretory granules (14); in one pathway, the activation of β-adrenergic receptors leads to the generation of adenosine 3′,5′-cyclic monophosphate (cAMP) by adenyl cyclase and the subsequent activation of protein kinase A (PKA). In the other pathway, the activation of cholinergic muscarinic receptors is coupled to the hydrolysis of phosphatidylinositol bisphosphate by phospholipase C, which produces diacylglycerol and inositol trisphosphate. Inositol trisphosphate production then leads to Ca2+ mobilization and diacylglycerol to protein kinase C (PKC) activation.

Although the role of these different signal transduction pathways in the exocytosis of granule contents (that is, the most distal step of the secretory pathway) has been extensively studied, their possible involvement in the control of intracellular protein transit has hardly been investigated. Over the last few years, evidence has accumulated to indicate that anterograde transport along the secretory pathway is regulated by protein kinases. First, membrane trafficking between the endoplasmic reticulum (ER) and the Golgi apparatus was found to be regulated by protein phosphorylation (15, 16). Soon afterward, in Madin-Darby canine kidney (MDCK) cells, Pimplikar and Simons (36) and Cardone et al. (7), respectively, described activation of apical transport of influenza hemagglutinin and stimulation of transcytosis of polymeric immunoglobulin A receptor by phorbol esters. Recently, in cell-free systems or cultured cells, PKC has been shown to play a role in the formation of vesicles from the TGN (5, 39, 43).

As far as a regulatory effect of PKA on intracellular traffic is concerned, few data are available. The involvement of PKA in regulating membrane traffic was first suggested when it was found that the regulatory RII subunit of PKA was in part associated with Golgi membranes (18, 19, 34). Recently, Pimplikar and Simons (36) and Hansen and Casanova (22), respectively, showed that PKA activators increase the apical transport of influenza hemagglutinin and the secretion of the endogenous glycoprotein gp80 in MDCK cells. More recently, Jilling and Kirk (27) showed that, in cultured colonic epithelial cells, cAMP stimulated constitutive membrane traffic from the TGN to the apical cell surface, and Muniz et al. (33) demonstrated that, in...
NRK cells, the transport of vesicular stomatitis virus (VSV)-G glycoprotein was blocked at the exit from the Golgi complex by the PKA inhibitor H-89. Moreover, the inhibition of PKA has been shown to inhibit protein transit from the ER to the Golgi (33), and PKA activation was found to facilitate the restoration of the Golgi complex after treatment with brefeldin A (37).

In this work, we tested the effect of H-89 on the transit of regulated secretory proteins. We show that this PKA inhibitor, in the absence of any secretagogue, induces the release of newly synthesized proteins, normally destined for the regulated secretory pathway. This inhibitor provokes, in a Ca\(^{2+}\)-independent way, the release of those proteins that are in the process of trafficking but is ineffective toward proteins already packaged in secretory granules. Furthermore, we propose that H-89 affects a TGN or post-TGN event of the secretory pathway. Taking together all of our data, the H-89 effect can be interpreted as the result of a mis-sorting of proteins from the regulated secretory pathway. This work further suggests that PKA is involved in the regulation of the intracellular transit of proteins in exocrine cells.

**MATERIALS AND METHODS**

Chemicals. \(^{[3]}\)H\)lecine (83 Ci/mmol, 3.07 TBq/mmol - 1 mCi/ml) was purchased from ICN Pharmaceuticals (Orsay, France). \(^{[3]}\)H\)lecine (151 Ci/mmol, 5.59 TBq/mmol - 5 mCi/ml) and \(^{[14]}\)C\)galactose (53 mCi/mmol, 1.96 GBq/mmol - 200 µCi/ml) were obtained from Amersham France (Les Ulis, France). Carbachol, the calcium ionophore A-23187, calphostin C, chelerythrine, and dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) were from Sigma Chemical (Saint Quentin Fallavier, France). H-85 and H-89 were purchased from Seikagaku-Coger (Paris, France).

Preparation of gland lobules. Male Sprague-Dawley rats (5 wk old) were used throughout this study. They were killed by 1 min of CO\(_2\) inhalation. Lacrimal glands were immediately removed and distended by injecting Krebs-Ringer bicarbonate buffer supplemented with 1 g/l glucose (KRBG buffer) into their stroma. Lobules were carefully dissected at room temperature to remove connective tissue and were subsequently rinsed three times with a large volume of KRBG buffer at 37°C.

Secretion of newly synthesized proteins. Protein secretion was measured by quantifying the labeled proteins secreted in the incubation medium after \(^{[3]}\)H\)lecine pulse labeling of gland lobules incubated in KRBG buffer at 37°C under a 95% O\(_2\)-5% CO\(_2\) atmosphere. All experiments were performed using previously described procedures (37); gland lobules were pulse labeled for 10 min with \(^{[3]}\)H\)lecine (5-10 µCi/gland for the kinetic experiments, 100 µCi/gland for the autoradiography analysis of the secretory products) followed by washing three times in KRBG buffer containing 1 mM leucine to stop the labeling. Gland lobules were then resuspended in KRBG buffer containing 1 mM leucine in the presence or absence of one of the following secretagogues (2 µM carbachol, 10 µM calcium ionophore A-23187) or one of the different protein kinase inhibitors (5-50 µM H-89, 50 µM H-85, 4 µM calphostin C, 20 µM chelerythrine). At different times after addition of the drugs, duplicate aliquots of the incubation medium were subjected to two cycles of 20% trichloroacetic acid - 0.1% phosphotungstic acid precipitation, and the insoluble radioactivity was counted in a 1212 Rack-beta liquid scintillation counter (LKB Pharmacia, Saint-Quentin en Yvelines, France). After the last sampling, the lobules were dried on paper, homogenized in water for 20 s with an Ultra Turrax homogenizer, and centrifuged for 20 min at 38,000 g. Two fractions of the supernatants were subjected to the same treatment as the incubation medium samples.

Protein secretion was expressed as the amount of \(^{3}\)H\)-labeled proteins released in the incubation medium as a percentage of the total \(^{3}\)H\)-labeled soluble proteins.

To obtain net protein secretion, the basal secretion from control samples without drug was subtracted from the protein secretion values obtained with the different drugs tested.

In some experiments, kinetics were not followed, but protein secretion was determined 3 or 5 h after the addition of the drugs. Finally, in some experiments performed with H-89, the drug was added at different times after the pulse, or a preincubation with the drug was carried out before the pulse.

In the case of experiments performed at two temperatures, lobules were incubated just after the pulse, in the absence of drug, either at 20°C or at 37°C; 150 min later, H-89 or the ionophore A-23187 was added to the medium, and the lobules were further incubated at the same temperature or transferred from 20 to 37°C or from 37 to 20°C.

In the case of the experiments performed in the absence of Ca\(^{2+}\), pulsed lobules were washed and incubated in a modified KRBG buffer in which CaCl\(_2\) was omitted and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid was added.

When the effect of H-89 was tested on regulated exocytosis, lobules were submitted to a chase period of 150 min after the end of the pulse. The duration of this period is sufficient to allow the radiolabeled proteins to accumulate in secretory granules (37). H-89 was then added and, 30 min later, exocytosis was triggered by adding 1 mM DBcAMP to the medium. Secretion was measured 2 h later, as described above.

Secretion of newly galactosylated proteins. Lacrimal gland lobules were labeled for 30 min with \(^{14}\)C\)galactose (5 µCi/gland) in KRBG buffer at 37°C in the presence of either 2 µM carbachol or 50 µM H-89 (H-89 was added to the medium 30 min before the pulse). At the end of the labeling period, lobules were washed three times in KRBG buffer in the presence of 1 mM galactose; the incubation was then continued in the presence of carbachol or H-89. The subsequent experimental procedure was identical to that performed in the case of \(^{3}\)H\)-labeled proteins.

Glycoprotein secretion was expressed as the amount of \(^{14}\)C\)-labeled proteins released in the incubation medium as a percentage of the total \(^{14}\)C\)-labeled soluble proteins.

Leucine and galactose incorporation. Lobules were preincubated with 50 µM H-89 for 30 min and pulse labeled (with \(^{3}\)H\)lecine or \(^{14}\)C\)galactose) as for secretion assays, but in the presence of 50 µM H-89. At the end of the pulse, lobules were washed three times in KRBG buffer, dried on paper, and homogenized in water. The homogenates were processed for radioactivity determination as described above for secretion assays. Control lobules were treated similarly but in the absence of H-89.

Assay of peroxidase activity. The amount of peroxidase released into the incubation medium after addition of carbachol or H-89 to gland lobules was determined using the method described by De (17); the initial velocity of formation of I\(_2\) was measured at 365 nm. One unit of peroxidase was defined as a change of absorbance at 365 nm of one in 1 min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and fluorography of secreted proteins. The radiolabeled proteins secreted in the incubation media were solubi-
lized in a minimal volume of buffer, pH 7, after trichloroacetic acid precipitation; 5x denaturating sample buffer was added to each sample followed by heating to 100°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 17% acrylamide gels. After fixation and staining with Coomassie blue, the gels were soaked in Amplify (Amersham), dried, and exposed to Amersham Hyperfilm-MP. Films were then quantified by laser scanning densitometry with a densitometer SI and the ImageQuaNT software (Molecular Dynamics, Evry, France).

RESULTS

H-89 inhibits the PKA-dependent exocytosis of proteins. To determine the sensibility of lacrimal glands to H-89, we measured the inhibitory effect of this drug on the PKA-dependent exocytosis of proteins stored in secretory granules. For this purpose, lacrimal lobules were pulse labeled with [3H]leucine and chased to allow the radiolabeled proteins to accumulate in secretory granules. H-89 and the secretagogue DBcAMP were added to the medium 150 and 180 min after the end of the pulse, respectively. Two control experiments were performed in parallel; in the first experiment, nothing was added to the chase medium, and, in the second experiment, H-89 was omitted. We tested different concentrations of H-89 with the range of the usual concentrations used to inhibit PKA in cells (1, 9, 22, 24, 33). When H-89 was used at a concentration of 50 µM, net secretion, measured 2 h after the addition of DBcAMP, was inhibited by 64 ± 5% (n = 3); a lower concentration of H-89 (5 µM) did not significantly inhibit net secretion (4 ± 7% of inhibition, n = 3).

These results indicate that, in lacrimal glands, H-89 inhibits regulated exocytosis involving PKA activity but probably poorly penetrates into the cells, as high concentrations are required to give a significant inhibitory effect.

H-89 triggers protein release. To investigate a possible role of PKA in the regulation of intracellular transit, we tested the effect of H-89 in other experimental conditions. Lacrimal lobules were pulse labeled with [3H]leucine, and H-89 was immediately added in the chase medium so that the intracellular transit and the maturation of the proteins occurred in its presence. Two control experiments were performed in parallel; in one experiment, lobules were not treated during the chase to determine the level of basal secretion, and, in the other experiment, lobules were treated with carbachol to determine the maximal level of secretion. The results of these experiments, presented in Fig. 1A, clearly show that H-89 induces a secretion of radiolabeled proteins that is about two times the basal secretion but less than the carbachol-triggered secretion. Five hours after the end of the pulse, the net secretion triggered by H-89 represents ~23 ± 2% (n = 10), whereas net secretion induced by carbachol is 38 ± 2% (n = 8). The lag time of ~45–60 min observed in the secretion kinetics triggered by carbachol and H-89 corresponds to the minimal duration of the intracellular transit in the lacrimal gland. When H-89 was added to the medium 30 min before the pulse, during the pulse, and during the 5 h after the pulse, the results obtained were identical (data not shown).

To check if the H-89-induced secretion could be due to an enhanced rate of unlabeled protein synthesis occurring after the pulse, we tested the effect of H-89 on [3H]leucine incorporation. When lobules were pulsed in the presence of H-89, [3H]leucine incorporation was inhibited by 30 ± 8% (n = 6), indicating that the
H-89-triggered secretion cannot be due to an enhanced rate of protein synthesis. Neither can the secretion triggered by H-89 be explained by a lysis of the cells, as the proportion of lactate dehydrogenase found in the media during the experiments is not increased by H-89 (data not shown).

When we used 10 and 30 µM H-89 instead of 50 µM, the net secretions represented 50% and 80%, respectively, of the response obtained with 50 µM. In further experiments, we therefore used 50 µM H-89 to obtain the highest protein secretion.

When secretion was triggered by the ionophore A-23187 (10 µM), another secretagogue that increases the intracellular Ca2+ concentration, the kinetics of protein release were identical to those observed with carbachol. We used this secretagogue to determine whether the fraction of the radiolabeled proteins that are not secreted by H-89 treatment is still secretable via classical regulated exocytosis. Lobules were pulse labeled as in the previous experiment and incubated in the presence of H-89 for 5 h; the medium was then removed, and the lobules were incubated for a further 4 h in the presence of the ionophore A-23187 (10 µM). For the two control experiments performed with ionophore A-23187 or H-89 for the whole 9 h, the medium was also changed after the first 5 h of incubation but kept the same composition. Figure 1B shows that, for the two control experiments, a plateau was obtained after 5 h for H-89 and ionophore A-23187. In contrast, in the lobules treated first with H-89 and then with the calcium ionophore, the secretion also reached a plateau at 5 h but continued to increase and tended to be the same value as that obtained with the ionophore A-23187 alone for the whole 9 h. This indicates that some proteins escape the H-89 action and behave as normal regulated secretory proteins.

H-89-induced secretion is Ca2+ independent. We tested comparatively the Ca2+ dependence of the carbachol-induced secretion and the H-89-induced secretion. The results presented in Fig. 2 show that the carbachol-induced secretion was strongly inhibited in the absence of Ca2+ (67% inhibition). This result is consistent with previous investigations showing that, in lacrimal glands, regulated secretion is Ca2+ dependent (31). On the other hand, the secretion triggered by H-89 was not inhibited in the absence of extracellular Ca2+, indicating that H-89 does not trigger secretion in a classical regulated way.

H-89 triggers the release of the regulated secretory proteins. The [3H]leucine-labeled proteins released after the action of either H-89 or ionophore A-23187 were analyzed by SDS-PAGE and fluorography. Figure 3A shows the autoradiographic patterns of the secreted proteins. In the case of basal secretion, seven major radiolabeled polypeptides with molecular masses ranging from 50 to 10 kDa (lane A) were detected. It is worth noting that most of these polypeptides are found in rat tears (lane D), showing that the proteins found in the tears are mainly synthesized by the extraorbital lacrimal glands. After addition of the ionophore A-23187, the same polypeptides were released into the medium (lane B); finally, we observed an identical autoradiographic pattern for the medium obtained after incubation of the lobules with H-89 (lane C). The intensity of each band was quantified by laser densitometry, and Fig. 3B shows the relative amounts of the seven major radioactive polypeptides secreted under the three experimental conditions tested. The statistical analysis of these results using the Student’s t-test allowed us to conclude that H-89 did not significantly affect the respective ratios of the different polypeptides; thus we can conclude that H-89 triggered the release of the proteins usually stored in the regulated secretory granules.

The proteins released in the media during the experiments shown in Fig. 1B were also analyzed by fluorography. No difference was observed in the autoradiograms corresponding to the medium obtained after 5 h in the presence of H-89 and the medium obtained after the four additional hours in the presence of the ionophore A-23187 (data not shown).

In the case of protein release triggered by carbachol, the polypeptides detected after autoradiographic analysis of the medium were identical and in the same proportions as those observed when the secretion was triggered by the ionophore A-23187 (data not shown).

H-89 specificity. H-89 was described as a specific PKA inhibitor, but it is known that in vitro, and at high
doses, it can also inhibit other kinases. Keeping in mind that PKC has been involved in some trafficking mechanisms (5, 36, 39, 43), we verified that the H-89 effect was not due to PKC inhibition. With that aim, we tested the effect of different protein kinase inhibitors on the protein release. The two inhibitors of PKC, calphostin C and chelerythrine, were used at 4 and 20 µM, concentrations, respectively, 80 and 30 times higher than their half-maximal inhibitory concentration determined in vitro (23, 28). H-85, which resembles H-89 in its chemical structure, was used as a negative control for H-89 (9) at a concentration of 50 µM, as for H-89. These three inhibitors were added just after the pulse. As shown in Fig. 4, none of these inhibitors was able to trigger the release of radiolabeled proteins.

These results eliminate the possibility that PKC is the site of H-89 action. This result, taken together with the fact that H-89 inhibits the PKA-regulated secretion, strengthens the idea that the H-89 effects may be due to PKA inhibition.

H-89 does not induce the release of the proteins stored in the secretory granules. To investigate the action of H-89 further, we added the drug at different times after the pulse. The sooner the drug was introduced, the higher the protein secretion was; when H-89 was added just after the pulse, net secretion determined 3 h later was 15 ± 6.1% (n = 8), whereas, when the drug was added 2 h after the end of the pulse, the net secretion decreased to 3.5 ± 3% (n = 4). As we had previously shown that, 2 h after the pulse, most of the newly synthesized proteins are stored in the secretory granules (37), it can be concluded that H-89 is unable to trigger the release of the secretory granule content.

To confirm this result, we followed in parallel, in the same lobules, the release of the [3H]leucine-labeled proteins and peroxidase, an enzyme stored in the secretory granules and secreted, in a regulated way, by lacrimal glands (25). Figure 5 shows that, when carbachol was added just after the pulse, the radiolabeled proteins were released after a lag time of ~60 min. The time course of peroxidase release was different and showed the following three phases: an immediate release was observed within the first 30 min; the amount of peroxidase then leveled off for ~60 min and finally increased again over the next 150 min; and the slope of this third part of the curve was superimposable to that

---

**Fig. 3.** Analysis of the radiolabeled proteins secreted by control, calcium ionophore, and H-89-treated lobules. Lacrimal gland lobules were pulse labeled with [3H]leucine and then treated for 5 h in the absence or presence of 2 µM ionophore A-23187 or 50 µM H-89. Incubation media were then processed for SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography. A: fluorographic pattern of the radiolabeled proteins secreted in the absence (lane A) or presence of either ionophore A-23187 (lane B) or H-89 (lane C). For each sample, equal amounts of radioactivity were loaded on the gel. SDS-PAGE pattern (Coomassie blue staining) of rat tears (lane D) and molecular mass standards (lane E) are given for comparison. B: 7 major bands present on the fluorograms were quantified by laser scanning densitometry and expressed as percent of the total radioactivity present in each lane. Values are means ± SE (n = 4). Unpaired Student’s t-test gave P values > 0.1, except for band 2 in which P = 0.04, when we compared the control and the carbachol values.
of radiolabeled protein release and may correspond to the release of newly synthesized peroxidase. When H-89 was added to the lobules just after the pulse, the kinetics of radiolabeled proteins and peroxidase releases were very similar. This experiment confirms that H-89 is unable to discharge the proteins packaged in the secretory granules. Thus we clearly show that H-89 acts on an intracellular transit event and not on the exocytosis of the secretory granules.

H-89 does not affect the early steps of the intracellular transit. To define which step of the secretory pathway was affected by H-89, we checked the effect of the drug on the galactosylation of proteins, which occurs in the trans cisternae of the Golgi apparatus and on the secretion of these glycoproteins. When lacrimal lobules were preincubated and submitted to pulse labeling with [14C]galactose, in the presence of 50 µM H-89, no difference in the amount of galactose incorporated was detected compared with the control performed in the absence of H-89 (99 ± 3% of the control, n = 3), indicating that the transit from the ER to the Golgi was not affected by this drug. Figure 6 shows the glycoprotein secretion of control lobules and of lobules treated with either carbachol or H-89. These kinetic profiles clearly show that H-89 induced the secretion of radiolabeled glycoproteins; the level of secretion was the same proportion of carbachol-induced secretion as for the secretion of [3H]leucine-labeled proteins. It can be concluded from these experiments that H-89 affects a step of the transit occurring after galactosylation.

H-89 affects a TGN or post-TGN event of the secretory pathway. A shift of temperature from 37 to 20°C is known to block protein export from the TGN (20, 26). We used this “20°C block” to define more precisely the step during transit at which H-89 acts. When the lobules are chased at 20°C, the radiolabeled secretory proteins are blocked in the TGN, whereas they are stored in the mature secretory granules when the chase...
is performed at 37°C. In the following experiments, lacrimal lobules were pulsed with [3H]leucine at 37°C and submitted to a 150-min chase at either 37 or 20°C. The lobules were then transferred to either 20 or 37°C and treated with either 50 µM H-89 or 10 µM ionophore A-23187. Figure 7 shows the results of these experiments; when lobules were maintained at 37°C all along the experiment or when they were transferred to 20°C after a chase performed at 37°C, the ionophore A-23187 triggered an immediate release of radiolabeled proteins, whereas H-89 had no effect on protein release (Fig. 7, A and B). In those experiments, after the 150-min chase at 37°C, the radiolabeled proteins were stored in the secretory granules, so H-89 was unable, as previously observed, to trigger their release. When lobules were transferred to 37°C after a chase performed at 20°C (Fig. 7C), both the ionophore A-23187 and H-89 triggered protein release, after a lag time of ~1 h. In this experiment, the radiolabeled proteins were blocked in the TGN at the end of the chase period; when the temperature was increased, protein transit resumed from the TGN to the plasma membrane, and H-89 had a secretory effect. Thus these experiments clearly demonstrate that H-89 affects a TGN or post-TGN step of the secretory pathway.

**DISCUSSION**

In this work, we studied the regulation of the intracellular trafficking of proteins in the rat lacrimal gland, an exocrine gland producing the main components of the tears. This study is mainly based on the use of H-89, a specific PKA inhibitor. It must be pointed out that we studied here the effect of H-89 on the trafficking of proteins and not on the last step of the secretory process, exocytosis, which has been known to be regulated by protein kinases for a long time (14). Our results indicate that, in lacrimal lobules incubated in the presence of H-89, a part of the newly synthesized proteins follow an unusual pathway; they are not stored in regulated secretory granules but are discharged in the medium without the requirement for any external stimuli. We have located the site of action of H-89 to a TGN or post-TGN event in the pathway involved in the formation of mature secretory granules.

H-89 was described by Chijiwa et al. (9) and has been shown, in vitro, to be a specific inhibitor of PKA acting in a competitive fashion against ATP and, in vivo, to antagonize the effect of forskolin. Since 1990, this drug has been widely used to demonstrate the implication of PKA in various physiological processes (1, 13, 22, 24, 33). Several of our results favored the hypothesis that,

**Fig. 7.** Effect of 20°C block on H-89-induced secretion. Lacrimal gland lobules were pulse labeled with [3H]leucine at 37°C and either maintained at 37°C all along the experiment (A), chased for 150 min at 37°C and transferred to 20°C (B), or chased for 150 min at 20°C and transferred to 37°C (C). Immediately after the temperature shift (150 min after the end of the pulse), lobules were treated or not treated with 2 µM carbachol or 50 µM H-89. Time 0 corresponds to the time of the temperature shift. Values are results from 1 of 2 typical experiments. Bars on the last point of each curve represent the ranges between the 2 experiments.
in lacrimal glands, the effect of H-89 was due to PKA inhibition and not to inhibition of another protein kinase, as we showed that different protein kinase inhibitors, not specific for PKA, were unable to induce protein secretion in the same way as H-89. We also verified that H-89 was able to inhibit, in gland lobules, the PKA-dependent exocytosis and, in vitro, the cAMP-dependent phosphorylation of proteins present in a lacrimal postnuclear supernatant (data not shown). To further test the specificity of H-89 action, it would have been interesting to antagonize its effect by raising the cAMP level. Unfortunately, an increase of the cAMP level would immediately induce the exocytosis of the pool of radiolabeled proteins stored in the secretory granules. This effect would thus mask any inhibition of the H-89-induced protein secretion.

Because, in our experiments, the cAMP level was not increased by stimulation of adenylyl cyclase and if we assume that the H-89 effect is due to PKA inhibition, our results point out that a physiological process may depend on a basal activity of PKA. A role for a basal PKA activity has been demonstrated in various cell types. For example, in adrenal cells, the constitutive expression of various genes requires PKA basal activity (10). Moreover, in T lymphocytes, the inhibition of this basal activity provokes an enhancement of T cell receptor (TCR)-triggered granule exocytosis (41) and an inhibition of TCR-triggered interleukin-2 secretion (40). The role of such basal PKA activity in protein transit is also suggested by the results of Muniz et al. (33) on unstimulated cells; when studying constitutive transport in VSV-infected NRK cells, they showed that the basal transport of the VSV-G glycoprotein from the TGN to the cell surface was inhibited by H-89. These results suggest that PKA may regulate this process in resting cells.

Our results show that H-89 induces the release of 60% of the newly synthesized proteins usually discharged in the medium after the action of a secretagogue, either carbachol or ionophore A-23187. Nevertheless, after 5 h of incubation with H-89, the remaining radiolabeled proteins can be secreted in a classical regulated way on addition of a secretagogue. Thus it seems that the newly synthesized proteins can follow two different secretory pathways. To determine if the proteins secreted via each of those pathways were different or differently processed, we analyzed the secretory products of lacrimal glands by fluorography. We first showed that the radiolabeled polypeptides discharged into the medium by control lobules were identical and in the same proportions as those released from the secretory granules under the action of either carbachol or the ionophore A-23187. This indicates that the basal secretion is not a constitutive secretion (6) but is probably a combination of both constitutive-like secretion and basal exocytosis of secretory granules, as is the case in parotid glands (42). We showed that the proteins released in the medium under the action of H-89 were also identical and in the same proportions as those released after treatment with a secretagogue. These proteins also seem to be processed in the same way as those contained in secretory granules, suggesting that H-89 acts on a rather late event of the secretory pathway.

To define the level of the secretory pathway at which H-89 acts, we performed two kinds of experiments yielding indirect evidence. We first studied the galactosylation of glycoproteins and their secretion. Our results indicate that H-89 acts after the trans compartment of the Golgi apparatus, the compartment in which the galactosyltransferase is active (29). In the second series of experiments, we took advantage of the findings of Saraste and Kuismanen (38) and Griffiths et al. (20) who reported that incubation of cells at 20°C provokes retention of the secretory products in the TGN. Many authors have used this 20°C block to study the post-Golgi steps of the intracellular traffic and of the posttranslational modifications of proteins (30, 32, 43). In our case, we showed that the proteins blocked at 20°C for 150 min can be discharged by the action of H-89 when the temperature is shifted up to 37°C, whereas the proteins stored in the secretory granules after an incubation of the same length at 37°C cannot. These results indicate that H-89 acts at a step of the secretory pathway occurring between the TGN and the mature secretory granules. Nevertheless, we cannot yet conclude whether the inhibitor acts at the level of the vesicles budding from the TGN or slightly later, when the immature secretory granules form and mature.

A role of PKA at this level of the regulated pathway is not unlikely, since different investigators have shown the involvement of cAMP or PKA in the regulation of constitutive protein transport. They observed that forskolin stimulated the constitutive transport from the TGN to the apical cell surface (27, 33, 36). In the present work, we focused on regulated protein secretion and obtained opposite results to those concerning constitutive secretion; we showed that H-89 induces the secretion of regulated secretory products. As we showed that 1) H-89 does not induce the release of proteins already stored in the regulated secretory granules and 2) the H-89-triggered secretion, unlike regulated secretion (27, 31), is not dependent on extracellular Ca2+, we conclude that the proteins secreted under H-89 treatment do not follow the classical regulated secretory pathway.

One possibility would be that the formation of immature secretory granules from the TGN is impaired by H-89. The secretory proteins thus accumulating in the TGN may then escape from this compartment by constitutive transport vesicles. This inhibition of granule formation could be due to a disturbance of either the budding process or the sorting mechanisms for the regulated secretory proteins. This hypothesis is supported by various observations. Ohashi and Huttner (35) have shown that, in PC-12 cells, the formation of immature secretory granules was dependent on phosphorylation reactions and that cAMP stimulated their formation. It is thus conceivable that the inhibition of PKA by H-89 decreases the rate of formation of immature secretory granules. Another observation that would
support our hypothesis is that the sorting of regulated secretory proteins is closely related to their aggregation in the TGN; this aggregation is dependent on the internal pH and Ca\(^{2+}\) concentration of the TGN lumen (21). It has also been shown that an alteration of the aggregative capacity of regulated secretory proteins causes their missorting to the constitutive secretory pathway (8, 11), and it has been proposed that cAMP could regulate the internal pH of the TGN in epithelial cells (27). Thus one could imagine that the inhibition of PKA by H-89 may modify the internal pH of the TGN, producing a disturbance in the aggregation of the regulated secretory proteins, and cause their missorting to the constitutive pathway.

Another possibility, rather similar in principle to the first, would be that the maturation of the secretory granules is inhibited by H-89, favoring the escape of secretory proteins from the immature secretory granules via the constitutive-like pathway.

The last possibility would be that mature secretory granules are formed normally in the presence of H-89 but lack some signal that causes them to be retained within the cell. The nature of such a signal is as yet totally unknown; it could be a phosphorylated membrane protein or a cytosolic factor in which association with the granule membrane is dependent on its phosphorylated/dephosphorylated state. These last two hypotheses are purely speculative at the moment.

In conclusion, our results clearly demonstrate that, in rat lacrimal glands, H-89 alters at least one step of the intracellular trafficking of regulated secretory proteins, strongly suggesting a role for PKA in this process. The role of this kinase in the control of constitutive transport has been recently described, but our work indicates for the first time that the transit of regulated secretory proteins in exocrine cells could also be controlled by PKA.

We thank Dr. Gillian Barratt for critical reading of the manuscript. Address for reprint requests: M. N. Raymond, Laboratoire de Biochimie des Transports Cellulaires, CNRS, ERS 0571, bâtiment 432, Université Paris XI, 91 405 Orsay Cedex, France.

Received 17 July 1997; accepted in final form 9 October 1997.

REFERENCES


