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Phosphorylation of elongation factor 2 during Ca^{2+}-mediated secretion from rat parotid acini

Maxwell T. HINCKE*† and Angus C. NAIRN‡

*Department of Anatomy, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5, and ‡Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, U.S.A.

In this paper we report the rapid phosphorylation of a cytosolic 100 kDa protein during stimulation of secretion from dispersed aggregates of parotid acinar cells with Ca^{2+}-mobilizing secretagogues (carbachol, Substance P, ATP and the Ca^{2+} ionophore A23187). Phosphorylation was inhibited by removal of extracellular Ca^{2+}, but was not observed during stimulation with phorbol esters, suggesting that this protein is not a substrate for protein kinase C. Two-dimensional PAGE and immunoprecipitation with a specific antiserum indicated that this protein is elongation factor 2, whose Ca^{2+} calmodulin-dependent phosphorylation has been shown to inhibit protein synthesis [Nairn & Palfrey (1987) J. Biol. Chem. **262**, 17299–17303]. These results suggest that phosphorylation of elongation factor 2 is the molecular mechanism for the inhibition of protein synthesis which has been previously observed in rat parotid cells during stimulation with Ca^{2+}-mobilizing secretagogues.

INTRODUCTION

In the rat parotid gland, stimulus–secretion coupling occurs via at least two pathways [1,2]. Binding of ligand to the β-adrenergic receptor results in cyclic AMP synthesis and is accompanied by strong stimulation of protein exocytosis which is believed to be mediated by protein kinase A (PK-A). Another class of receptors (muscarinic cholinergic, α-adrenergic and Substance P) is coupled to a phospholipase C which specifically degrades PtdInsP_2 to generate polyphosphoinositides and 1,2-diacylglycerol [3,4]. Ins(1,4,5)P_3 releases Ca^{2+} from vesicular stores [5], and its metabolite Ins(1,3,4,5)P_4 is believed to mediate refilling of these Ca^{2+} stores [5,6]. Purinergic (P_{2y}) stimulation leads to Ca^{2+} influx in the absence of phosphoinositide turnover [7]. Protein phosphorylation is stimulated during both cyclic-AMP- and Ca^{2+}-mediated amylase secretion from parotid acinar cells [8–14], and activation of protein kinases is believed to underlie exocrine stimulus–secretion coupling (reviewed in ref. [2]). At present, however, only the ribosomal S-6 subunit (protein I) has been identified in parotid cells as a substrate for protein kinase C (PK-C), PK-A and Ca^{2+}-dependent protein kinases, and it is phosphorylated during stimulation of secretion [12,15]. Other proteins which are observed to be phosphorylated during secretion have not yet been identified, and the functional significance of their phosphorylation is unknown. In the course of our studies on this problem, we observed the phosphorylation of a 100 kDa protein during Ca^{2+}-mediated secretion from parotid acini. Characterization of this protein reveals that it is elongation factor 2 (EF-2).

EXPERIMENTAL

Materials

[^{32}P]P_i and BSA (fraction V) were obtained from ICN. Isoprenaline, substance P, carbachol, A23187, basal medium Eagle (containing 20 mM-Hepes), ATP and phorbol esters were obtained from Sigma. The bicinchoninic acid protein assay reagent and Aquasil were from Pierce. Pansorbin was purchased from Calbiochem. All other chemicals were of the highest available quality. The protein standards for SDS/PAGE were purchased from Bio-Rad laboratories. Zero-phosphate BME was prepared with the following composition: $CaCl_2$, 1.80 mM; $MgSO_4$, 0.81 mM; KCl, 5.36 mM; NaCl, 103 mM; D-glucose, 5.55 mM; Hepes, 26.0 mM; $NaHCO_3$, 26.2 mM; pH 7.4.

Methods

Dispersed aggregates of parotid acini were prepared with tissue from male Wistar rats (125–150 g), and secretagogue-stimulated amylase release was determined exactly as previously described [16]. For phosphorylation studies, cells were incubated with [^{32}P]P_i (1 mCi/ml) in BME (Sigma)/0.2% BSA for 45 min, diluted about 10-fold with BME (Sigma)/0.1% BSA, and then divided into 1 ml batches in silicone-treated glass vials for stimulation with secretagogues [16,17]. The reactions were terminated by centrifugation (10 s at 5000 rev./min; Eppendorf microcentrifuge) and rapid aspiration of the supernatant. The pellet of lightly packed parotid acini was rapidly dispersed in 250 μl of SDS-stop solution [4% SDS, 125 mM-Tris/HCl, pH 6.8, 25% (v/v) glycerol] and heated for 5 min at 100 °C. In some experiments, cells were incubated with [^{32}P]P_i in zero-phosphate BME in order to increase the specific radioactivity of the labelled ATP pool and allow briefer autoradiographic exposure periods. Preliminary experiments in which the cells were incubated with [^{32}P]P_i for 1.5 h yielded identical phosphorylation results upon stimulation.

During the course of this work, we noticed that extended heating of cell samples in SDS decreased the phosphate content of high-molecular-mass proteins and increased the autoradiographic background in the low-molecular-mass region. No effect on the appearance of the Coomassie-Blue-stained protein bands was observed. The origin of this phenomenon is unknown, but it could be avoided by dissolving the cells in a SDS solution containing proteinase and phosphatase inhibitors at room temperature. However, even after centrifugation, the unheated supernatants exhibited extremely high viscosities, and quantitative pipetting was difficult. Preliminary results indicated that

Abbreviations used: BME (Sigma), basal medium Eagle, containing 20 mM-Hepes; EF-2, elongation factor 2; PK-A, cyclic-AMP-dependent protein kinase; PK-C, protein kinase C; PMA, phorbol myristate acetate.

† To whom correspondence should be addressed.

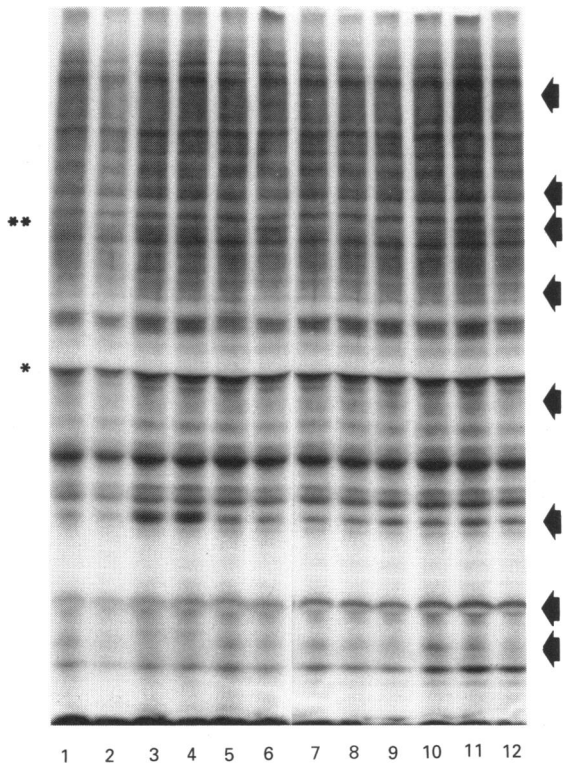


Fig. 1. Phosphorylation of parotid proteins after stimulation with isoprenaline, A23187, PMA or carbachol

Dispersed aggregates of parotid acini were incubated with [^{32}P]P_i (1 mCi/ml) in BME (Sigma)/0.2% BSA for 45 min, and then stimulated with secretagogues for 10 min. Autoradiogram of SDS/PAGE (5–15% acrylamide) of total parotid protein: lane 1, control; lanes 2–4, isoprenaline (1 nM, 100 nM, 10 μM); lane 5, dimethyl sulphoxide control (0.5%); lane 6, A23187 (1 μM); lanes 7–9, PMA (300 pM, 30 nM, 3 μM); lanes 10–12, carbachol (10 nM, 1 μM, 100 μM). Dimethyl sulphoxide was the vehicle for A23187 and PMA. **indicates the 100 kDa band whose intensity was dependent on agonist stimulation. *indicates the 50 kDa band used for normalization of the densitometric data, Positions of the molecular-mass markers (kDa) are indicated by right-hand arrows (top to bottom): myosin (200), β-galactosidase (116), phosphorylase *b* (97.4), BSA (66), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), lysozyme (14.4).

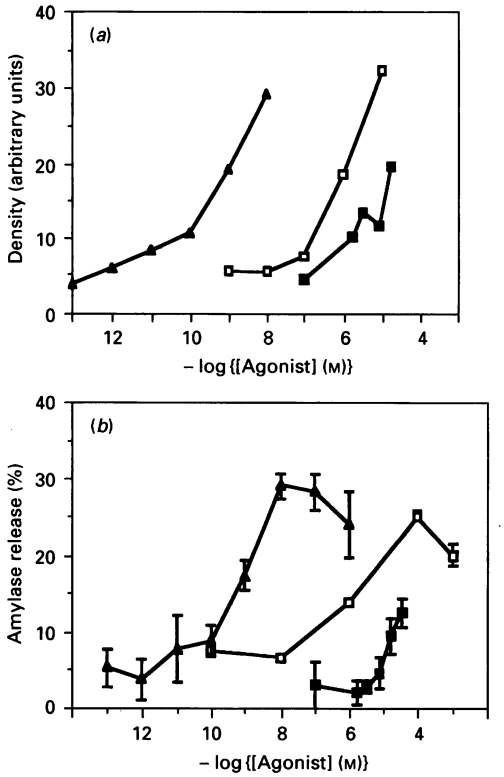


Fig. 2. (*a*) Densitometry of the 100 kDa band after stimulation with different concentrations of Ca^{2+} mobilizing agonists and (*b*) dose-dependent stimulation of amylase secretion by different agonists

(*a*) Cells were stimulated with different concentrations of carbachol (□), Substance P (▲) and ATP (■) for 5 min. These results are representative of three experiments. The ATP concentration plotted here is that calculated for ATP^{4-} at 1.8 mM-Ca^{2+} and 0.81 mM-Mg^{2+} with the following expression [19]:

$$[ATP^{4-}] = [\text{total ATP}]/(10^{3.94}\,[Ca^{2+}] + 10^{4.23}\,[Mg^{2+}])$$

(*b*) Release of amylase during 20 min [Substance P (▲) and carbachol (□)] or 10 min [ATP (■)] was determined.

the 100 kDa phosphoprotein was soluble. Therefore, in some experiments, pelleted cells were lysed in a buffer containing proteinase and phosphatase inhibitors (50 mM-NaF, 20 mM-$Na_4P_2O_7$, 5 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride, 40 μg of pepstatin/ml, 20 μg of leupeptin/ml, 0.3% Triton X-100, pH 6.8) and rapidly frozen in liquid nitrogen. The suspensions were thawed and centrifuged at 14000 rev./min for 15 min (Eppendorf 5415 microcentrifuge). The supernatant was diluted with an equal volume of SDS-stop solution, and protein concentration was measured before SDS/PAGE or immunoprecipitation.

Protein concentration of all samples was determined with the bicinchoninic acid reagent (micro-plate modification) before addition of dithiothreitol (to 50 mM) and Bromophenol Blue (to 0.0125%), with BSA as a standard. SDS/PAGE was performed on 5–10% or 5–15% gradient gels with sample loadings of 100 μg of protein per lane (total cell) or 1.7 μg per lane (soluble cell extracts) [18]. Gels were dried and submitted to autoradiography for 1–5 days at room temperature on Kodak MIN R film.

The immunoprecipitation procedure was slightly modified from previously [19]. All samples were adjusted to the same protein concentration by adding a solution of 1% SDS/10 mM-Na-Hepes (pH 7.4)/1% 2-mercaptoethanol, such that their final volumes were all 100 μl. Next, 900 μl of a 1.1-times-concentrated wash buffer was added to all samples (wash buffer = 25 mM-Tris/HCl, pH 7.5, 200 mM-NaCl, 5 mM-EDTA, 1% Nonidet P-40, 100 mM-NaF, 20 mM-$Na_4P_2O_7$, 20 μg of leupeptin/ml, 40 μg of pepstatin A/ml, 0.1 mM-phenylmethanesulphonyl fluoride). To pre-clear, 50 μl of Pansorbin (a 10% suspension of cells) was added to each tube. This was incubated for 15 min at room temperature, and centrifuged for 10 min at 14000 rev./min. To the supernatants was added 10 or 20 μl of anti-EF-2 (thawed serum), and the solutions were refrigerated and incubated for 90 min, 16 h or 40 h. Each incubation time yielded equivalent results. Pansorbin (100 μl) was then added, and after 30 min the suspensions were centrifuged (1 min, 5000 rev./min). The pellets were washed four times in wash buffer with centrifugation at the same speed, and finally dissolved in 85 μl of SDS-stop and heated for 2 min (100 °C). After addition of Bromophenol Blue (5 μl) and dithiothreitol (10 μl), 40 or 50 μl of each sample was electrophoresed on 5–15% gels. The dried gels were submitted to autoradiography with XOMAT-AR film, at −80 °C, with an intensifying screen for 4–6 days.

Two-dimensional PAGE was performed by a modification [20] of the method of O'Farrell [21]. Densitometry of X-ray films was performed with an LKB Ultroscan XL laser densitometer.

RESULTS

Stimulation of parotid acini with carbachol or A23187 caused marked phosphorylation of a protein of 100000 ± 1800 Da, which could be observed in samples of total cell protein (Fig. 1). Densitometry indicated that the increase in phosphorylation resulting from stimulation with carbachol and Ca^{2+} ionophore was of a similar magnitude. Phosphorylation of this protein was not observed during treatment with phorbol esters such as phorbol myristate acetate (PMA) (up to 3 μM; Fig. 1) or up to 100 μM-phorbol dibutyrate (results not shown). A small stimulation of phosphorylation in the 100 kDa region was observed with the β-adrenergic agent isoprenaline, although it was not a single intense band as with carbachol and A23187 (Fig. 1). Stimulation of phosphorylation of lower-molecular-mass proteins (31.6, 18.8 kDa) in response to isoprenaline was apparent, in addition to the concomitant dephosphorylation of a 14.8 kDa protein (Fig. 1, lanes 3 and 4).

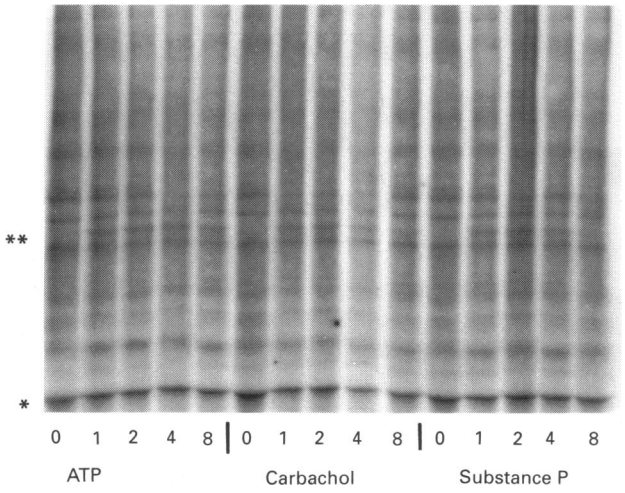


Fig. 3. Time course of phosphorylation of 100 kDa protein after stimulation with different Ca^{2+}-mobilizing agonists

Autoradiogram of SDS/PAGE (5–15% gel) of parotid cell aggregates stimulated with ATP (0.5 mM = 16 μM-ATP^{4-}), carbachol (1 μM) or Substance P (10 nM) for the indicated periods of time (min). This result is representative of two independent experiments. Asterisks are explained in Fig. 1 legend.

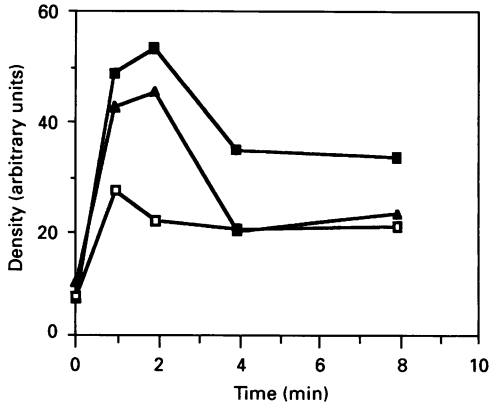


Fig. 4. Densitometry of the time course of phosphorylation of the 100 kDa protein

Cells were stimulated for different times with carbachol (1 μM) (□), Substance P (10 nM) (▲) and ATP (16 μM) (■).

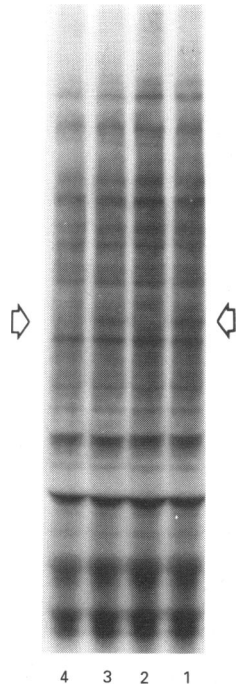


Fig. 5. Extracellular EGTA inhibits phosphorylation of the 200 kDa band

Autoradiogram of SDS/PAGE (5–10% gel) of parotid-cell aggregates stimulated with the following agonists for 10 min: lanes 1 and 2, carbachol (1 μM); lanes 3 and 4, Substance P (10 nM). EGTA (2 mM final concn.) was added to the cells in lanes 2 and 4 immediately before adding secretagogue, in order to chelate extracellular Ca^{2+} (1.8 mM). The 100 kDa band is indicated by the arrows.

The dose-dependent phosphorylation of the 100 kDa protein was investigated with agonists that mobilize Ca^{2+} in parotid cells (Fig. 2*a*). Carbachol, Substance P and ATP each stimulate the phosphorylation of this protein. These agonists are also secretagogues and stimulate the secretion of amylase, although ATP was less potent than either carbachol or Substance P (Fig. 2*b*). Release of amylase in response to stimulation was linear throughout the indicated time period. From these results, the K_d values for stimulation of amylase secretion and phosphorylation were estimated, and are similar for each agent (K_d for secretion and phosphorylation: carbachol, 2.5 μM and 1 μM; Substance P, 1 nM and 1 nM; ATP, 20 μM and 10 μM).

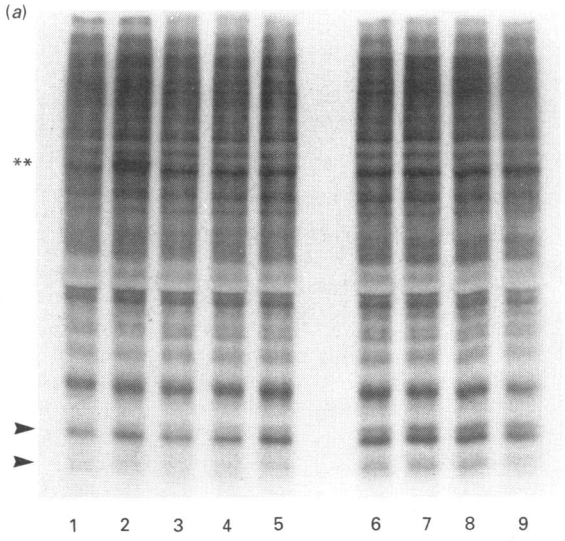
The time course of the phosphorylation reaction after stimulation with different Ca^{2+}-mobilizing agonists was studied, and an autoradiogram from such an experiment is shown in Fig. 3. Phosphorylation of the 100 kDa protein is maximal several minutes after stimulation. This was also seen with analysis by densitometry, which additionally revealed that the phosphate content declines after several minutes to a level which remained elevated over basal during continuous stimulation (Fig. 4). The effect of carbachol (1 μM) was completely antagonized when added together with 100 μM-atropine (results not shown). The Ca^{2+}-dependence of phosphorylation was confirmed by adding EGTA to the incubation medium just before stimulation with carbachol and Substance P. Sufficient EGTA (2 mM) was added to chelate the 1.8 mM-$CaCl_2$ present in the medium, and completely inhibited secretagogue-stimulated phosphorylation of the 100 kDa band (Fig. 5).

Since the dose–responses for phosphorylation and secretion were similar, the possibility that phosphorylation of this protein played a role in stimulus–secretion coupling was further investigated by evaluating the effect of acute EGTA addition upon

Table 1. Effect of Ca^{2+} chelation on agonist-stimulated amylase release

Amylase release was measured at 5, 10 and 15 min, by the microplate assay [16]. Rates of release from three independent experiments were averaged, and analysed with the unpaired *t* test.

Secretagogue	Amylase release (%/min) 2 mM-Hepes	2 mM-EGTA	Level of significance
Control	−0.074±0.08	−0.112±0.022	$P > 0.555$
Carbachol (10 μM)	0.252±0.055	−0.014±0.131	$P < 0.057$
Substance P (1 μM)	0.519±0.073	0.187±0.089	$P < 0.015$
Isoprenaline (1 μM)	1.30±0.26	1.31±0.15	$P > 0.958$



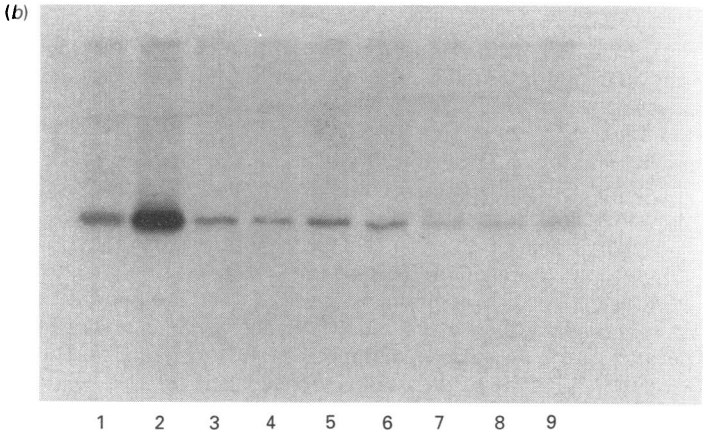


Fig. 6. Autoradiography of immunoprecipitated EF-2

(*a*) Autoradiography of SDS/PAGE of soluble parotid proteins before immunoprecipitation, after stimulation (10 min) with (lanes 1–9): vehicle, 10 μM-carbachol, 1 nM-, 3 nM-, 10 nM-, 30 nM-, 100 nM-, 1 μM-, 10 μM-isoprenaline. The double asterisk marks the position of the 100 kDa phosphoprotein, best seen in lane 2. The arrows indicate the position of the 25.6 and 23.2 kDa phosphoproteins that are further analysed in Fig. 8. (*b*) Autoradiography of SDS/PAGE of soluble parotid protein samples, as in (*a*), after immunoprecipitation with antiserum raised to EF-2. Lanes 1–9 are as (*a*).

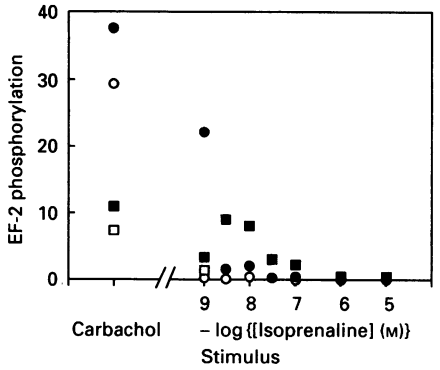


Fig. 7. Phosphorylation of EF-2 in response to carbachol and isoprenaline

Summary of densitometry results for four immunoprecipitation experiments like that presented in Fig. 6(*b*), and for 10 μM-carbachol. The intensities were normalized to the control for each experiment (basal phosphorylation was arbitrarily given a value of 1.0 in each case), and are presented as fold increase in EF-2 phosphorylation. The symbols (●, ○, □, ■) indicate different experiments.

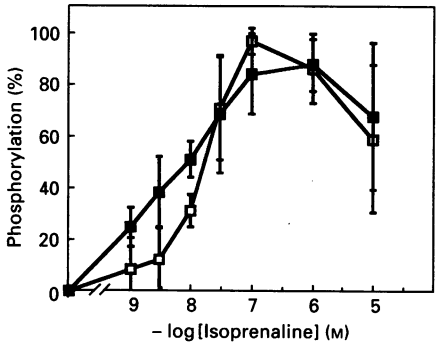


Fig. 8. Phosphorylation of low-molecular-mass substrates in response to isoprenaline

The isoprenaline-stimulated increase in phosphorylation of the soluble 23.2 kDa (□) and 25.6 kDa (■) proteins is presented as an average of four experiments (±S.D.). Before averaging, the densitometric intensities were normalized by setting the unstimulated intensity to 0, and the highest to 100%.

amylase release. As seen in Table 1, both carbachol- and Substance P-stimulated releases are inhibited by this treatment. However, the ability of isoprenaline, a more potent secretagogue which acts via a cyclic AMP-dependent mechanism, to elicit secretion was completely unaltered in the presence of EGTA.

Preliminary studies demonstrated that the 100 kDa protein was soluble and appeared in the supernatant after lysing the cells in a buffer containing protein phosphatase inhibitors (results not shown). Prominent phosphorylation of a protein of this molecular mass during stimulation with carbachol was readily detected by autoradiography after two-dimensional gel electrophoresis of these samples, appearing as several spots at this molecular mass (results not shown). The pI range of the 100 kDa substrate was estimated to be 6.7±0.2 ($n = 4$). At this stage, it became evident that the molecular mass and pI of the parotid phosphoprotein were similar to those of EF-2, which is the substrate of a specific Ca^{2+} calmodulin-dependent protein kinase (Cam kinase III) in a number of tissues. Antiserum raised against EF-2 was used to investigate its phosphorylation in parotid acini during stimulation with secretagogues. A single 100 kDa phosphoprotein was immunoprecipitated from total parotid cell extracts (results not shown), and cell supernatants (Fig. 6), after stimulation with carbachol. The degree of phosphorylation of immunoprecipitated EF-2 closely mirrored that of the 100 kDa

phosphoprotein observed before immunoprecipitation (Fig. 6). Densitometry indicated that the carbachol-stimulated phosphorylation of this protein ranged from 10- to 40-fold over the basal phosphorylation status of EF-2 (four experiments).

Since EF-2 is phosphorylated by a specific Ca^{2+}-dependent protein kinase, and the role of Ca^{2+} during cyclic-AMP-mediated secretion remains controversial (see the Discussion section), it was of interest to evaluate the phosphorylation status of EF-2 during stimulation with isoprenaline. In contrast with the consistent and marked phosphorylation of EF-2 seen during stimulation with carbachol, phosphorylation in response to isoprenaline was rarely detected, and when present exhibited an erratic dose-dependence (Fig. 7). In fact, the observed tendency was for decreased EF-2 phosphorylation at higher concentrations of isoprenaline (0.01 μM-isoprenaline: average phosphate content = 3.7-fold over basal, range = 0.6–8.2; 0.1 μM: average = 1.4-fold, range = 0.43–3.3; 1 μM: average = 0.4-fold, range = 0.2–0.4). However, in the same experiments, isoprenaline stimulated the consistent phosphorylation of several low-molecular-mass cytosolic substrates (25.6 ± 0.2 and 23.2 ± 0.2 kDa; K_d = 10–20 nM), as seen in Fig. 8.

DISCUSSION

The phosphorylation of proteins in rat parotid acinar cells during stimulation with the muscarinic cholinergic agonist carbachol has been reported previously [9–12,14]. However, to our knowledge the present study is the first to detect the Ca^{2+}-dependent phosphorylation of a 100 kDa protein, leading to its further characterization and identification as EF-2. Most other workers have utilized a parotid-slice system, where dissolution of cells and inactivation of protein phosphatases with SDS might not occur so rapidly. In addition, electrophoresis conditions and autoradiographic exposures have been generally optimized to monitor the phosphorylation of lower-molecular-mass proteins (i.e. S-6). However, in spite of these differences, our results with the β-adrenergic agonist isoprenaline were similar to those reported in the literature (reviewed in refs. [2,8–11]). We consistently observed the prominent phosphorylation of the ribosomal subunit S-6 (31.6 kDa) and protein III (18.8 kDa) in total cell samples during stimulation with this secretagogue, in addition to the dephosphorylation of a 14.8 kDa protein. Protein III is an intrinsic component of the endoplasmic-reticulum membrane, and therefore cannot correspond to the soluble phosphoproteins which we detected during stimulation with both isoprenaline and carbachol (23.2 and 25.6 kDa). Phosphorylation of these low-molecular-mass proteins and amylase secretion were well correlated and deserves further study. In contrast, phosphorylation of higher-molecular-mass proteins during stimulation of parotid secretion is not readily observed. Isoprenaline, but not carbachol, is reported to stimulate phosphorylation of a 92.5 kDa protein which is associated with purified secretion-granule membranes [14]. One potential problem which is not often recognized is that heating samples in SDS for extended periods, in order to decrease the viscosity of cell samples due to DNA [22], can affect the phosphate content of certain proteins [23]. We observed in this study that improved autoradiographic patterns were obtained, particularly in the high-molecular-mass region, if heating of cell-extract samples was avoided.

Two-dimensional electrophoresis and immunoprecipitation with a specific antiserum indicate that the 100 kDa phosphoprotein is EF-2, which is reported to be the specific substrate of a calmodulin-dependent protein kinase. The two-dimensional pattern of several spots of pI 6.7–6.8 is consistent with that reported for EF-2 in other tissues, and may reflect some post-translational modification [24]. Only one radiolabelled band on one-dimensional SDS/PAGE was ever detected in our experiments after immunoprecipitation with antiserum raised against authentic rat pancreatic EF-2. The molecular mass of this band (100 kDa) was the same as that calculated for the phosphoprotein in one-dimensional SDS/PAGE of total cellular protein or total soluble protein, and for the EF-2 spots in autoradiograms of two-dimensional gels. Since the stimulated phosphorylation of immunoprecipitated EF-2 always mirrored the pattern of relative intensity of the 100 kDa phosphoprotein observed in autoradiograms from total cell or soluble cell samples, we are confident that the same 100 kDa protein was immunoprecipitated.

Our results demonstrate that EF-2 in rat parotid acini is subject to rapid Ca^{2+}-dependent phosphorylation during stimulation by Ca^{2+}-mobilizing secretagogues (ATP, carbachol, substance P and A23187). This conclusion is supported by the observation that chelation of extracellular Ca^{2+} inhibited the reaction. *In vitro*, purified EF-2 is phosphorylated by a specific calmodulin-dependent protein kinase (Cam-kinase III) solely on threonine residue(s), and is not a substrate for other calmodulin-dependent protein kinases, or PK-C or PK-A [25–27]. These properties are consistent with our observation that phorbol esters, which activate PK-C, do not stimulate phosphorylation of this protein in parotid acinar cells. The rapid increase in phosphate content which we observed suggests that phosphorylation of this protein closely parallels the agonist-stimulated increases in intracellular Ca^{2+} reported by others using Ca^{2+}-sensitive fluorescent dyes [3,28,29]. However, differences in the duration of the intracellular Ca^{2+} signal (ATP > carbachol > substance P), reflecting rapid desensitization of substance P receptors, for example, were not apparent in our study. Therefore it is possible that the reversal of phosphorylation observed after several minutes' stimulation is due to the activation of Ca^{2+}-dependent phosphatases. The sustained intermediate level of EF-2 phosphorylation which is maintained for longer periods would then reflect an equilibrium between activated kinase and phosphatase. *In vitro*, EF-2 is not dephosphorylated by calcineurin, but is a good substrate for protein phosphatase 2A (A. Nairn, unpublished work). Apparent differences in the relative ability of ATP to stimulate EF-2 phosphorylation (compare Figs. 2*a* and 4) are likely to be due to the ecto-ATPase activity which parotid acinar cells possess, since hydrolysis of ATP would be proportional to cell density during stimulation [28].

Our studies of EF-2 phosphorylation with isoprenaline were not clear-cut. In these experiments, increased phosphorylation of EF-2 was detected inconsistently, but only at low isoprenaline concentrations. Again, the phosphorylation levels of immunoprecipitated EF-2 were always consistent with that of the 100 kDa phosphoprotein detected after one-dimensional SDS/PAGE of total soluble protein, suggesting that these results were not due to some artifact of the immunoprecipitation procedure. In one case where isoprenaline-stimulated phosphorylation (1 nM) was almost as great as that due to stimulation with carbachol, phospho-amino acid analysis of the immunoprecipitants indicated that only phosphothreonine content was increased with each secretagogue (results not shown), suggesting that Cam-kinase III was activated in each case. The question of whether Ca^{2+}-dependent EF-2 phosphorylation occurs in response to isoprenaline is important because of the long-standing premise that Ca^{2+} is the final mediator of parotid secretion [30]. Although high isoprenaline concentrations (K_a = 10 μM) have been reported to stimulate PtdInsP_2 hydrolysis and mobilize Ca^{2+} in isolated parotid cells [31,32], it has recently become clear that this effect is mediated by the α-adrenergic receptor [33,34] and that increases in basal Ca^{2+} levels are not detected with Ca^{2+}-sensitive fluorescent dyes during isoprenaline-stimulated amylase secretion (K_a = 10 nM [3]). In agreement with these reports, our infrequent

observation of isoprenaline-stimulated EF-2 phosphorylation suggests that β-adrenergic-stimulated increase in intracellular Ca^{2+} is not a common property of parotid acinar cells. One possibility is that it is a consequence of minor and random contamination of the acinar preparations with another cell type, such as parotid-gland ducts, which constitute about 15% of the intact gland by volume [35]. Low levels of isoprenaline (1 nM) depolarize the cells of the main duct of the rat submandibular gland and cause inhibition of net K^+ reabsorption and stimulation of net Na^+ reabsorption [36]. The second messengers responsible for this effect are not yet known; a possible explanation is that salivary-ductal cells possess Ca^{2+} channels which are directly coupled to the β-adrenergic receptor by $G_s\alpha$, as is found in muscle [37]. More work is necessary in order to test this hypothesis. However, since isoprenaline rarely stimulated EF-2 phosphorylation, in contrast with its consistent potency as a secretagogue, these two phenomena are clearly dissociated.

EF-2 is an essential protein in the process of protein synthesis, and catalyses the GTP-dependent translocation of the peptidyl-tRNA from the A site to the P site on the ribosome. Phosphorylation of EF-2 by Cam-kinase III inhibits protein synthesis in the rabbit reticulocyte assay *in vitro* [25,38], and its phosphorylation during mitosis correlates with a decrease in protein synthesis [39]. Although this is not the only mechanism by which protein synthesis is down-regulated [40], it remains one of the best characterized. In rat parotid acinar cells, amylase release stimulated by adrenaline (α_1-adrenoceptor) or carbachol is accompanied by inhibition of [^{3}H]leucine and [^{3}H]phenylalanine incorporation into protein by a Ca^{2+}-dependent mechanism [41–44]. This effect is additional to the Ca^{2+}-dependent inhibition of amino acid uptake, which also occurs during stimulation [45]. Our observation that EF-2 is phosphorylated in parotid acinar cells during stimulation of secretion by Ca^{2+}-mobilizing secretagogues provides one molecular mechanism for this inhibition of protein synthesis.

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