somatostatin[14,16], VIP (refs 8, 13, 21, 22), bombesin[13,23,24] and cholecystokinin[25], suggesting that the mammalian form of physalaemin may belong to this diverse group of neuropeptides.

Column chromatography of extracts of guinea pig stomach on Bio-Gel P-4 columns ($n = 15$) gave only one symmetrical peak of PSLI (molecular weight 1,700) regardless of the area of the stomach examined. This was larger than any of the known tachykinins[6] and no other major peak with PSLI eluted from the columns. Cyanogen bromide digestion of the peak material gave an immunoreactive profile with the same elution volume as that of the original extract, suggesting that PSLI could be NH_2-terminally extended. This indeed was found for a mammalian bombesin-containing peptide[11], angiotensin from frogs[5] and somatostatin from porcine intestine[12]. Reverse-phase HPLC was used to resolve whether PSLI resembled physalaemin or uperolein: in acidic or neutral conditions, PSLI co-eluted with physalaemin several minutes earlier than uperolein (Fig. 3*b*). These data suggest that even though PSLI seemed to be larger than physalaemin, its ionic properties are very similar, if not identical, to those of physalaemin.

PSLI differs from substance P on the basis of several criteria: specifity of antiserum PS-1 (Table 1); tissue distribution and cellular localization[21]; substance P has not been localized in Brunner's glands[27] which contain cells immunopositive to the COOH terminus of gastrin[28] and bombesin[23]; and PSLI co-eluted only with physalaemin on HPLC (Fig. 3*b*).

The physiological effects of physalaemin may indicate a possible role for PSLI. Physalaemin affects mucous production *in vitro*[29], and *in situ* stimulates secretion of the lachrymal and salivary glands[3,30] with the secretion of K^+ and reabsorption of Na^+ (ref. 31). These activities may account for the presence of PSLI in tissues with mucous production, Brunner's glands and in mucosal layer of trachea and oesophagus. Physalaemin produces acute hypotension by vasodilation[2,3,6], and a spasmogenic action on extravascular smooth muscle[2,3] which suggests that locally released PSLI could regulate muscle tone; for example, in gastro-oesophageal sphincter[8] or enteric plexi[22] the relaxation induced by VIP may be antagonized by PSLI. The presence of PSLI nerves in the vicinity of acinar cells suggests that it could interact on specific physalaemin membrane receptors on acinar cells[32] leading to cellular secretion[32–34]. Thus the release of an endogenous PSLI may be involved in different physiological functions depending on its neuronal or cellular location.

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Dual ionic controls for the activation of protein synthesis at fertilization

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The general metabolic activation of the sea urchin egg at fertilization is dependent on a release of intracellular stores of calcium and the subsequent transient elevation of intracellular Ca^{2+} (refs 1–3). However, this elevation does not by itself lead to increased macromolecular synthesis and development but initiates steps which result in a long-term elevation of intracellular *p*H (refs 4–6). Among the developmental processes dependent on the elevation of intracellular *p*H is the large acceleration in the rate of protein synthesis at fertilization[6]. Weak penetrating bases such as ammonia can be used to mimic the processes resulting in an increase in intracellular *p*H and so show the corresponding increases in protein synthesis rate[6–9]. Conversely, it is possible to demonstrate a gradual but complete shut down of protein synthesis if the intracellular *p*H is reduced to the unfertilized level with penetrating weak acids[6]. However, the rate of protein synthesis in ammonia-activated eggs lags behind that of fertilized controls even though ammonia activation can result in an intracellular *p*H increase greater than occurs in the fertilized egg[6,8,10]. This result has led to the suggestion that factors other than intracellular *p*H may be regulating protein synthesis following fertilization[6,10]. To investigate the possibility that the Ca^{2+} transient may have such a role, we measured the rate of amino acid incorporation in eggs that were activated in various ionic conditions which enabled the effects of Ca^{2+} and *p*H changes to be studied separately. Our results, reported here, show that if intracellular *p*H is elevated, increases in intracellular Ca^{2+} play an additional part in the activation of protein synthesis at fertilization.

Figure 1 shows the amount of ^{3}H-valine incorporated after activation in these different ionic conditions. Eggs were preloaded with ^{3}H-valine to circumvent problems of differential uptake of label in different experimental conditions. Fertilization typically results in a 10–30-fold stimulation of protein synthesis over the unfertilized rate. The variation between experiments stems from differences in both the basal unfertilized rate and the degree of stimulation resulting from activation.

Fertilization results in an increase of intracellular *p*H of about 0.45 *p*H units[5]. This seems to occur by a mechanism by which protons are released from the egg in the presence of external Na^+ (ref. 4). To determine how Ca^{2+} release in the absence of an

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intracellular *p*H increase would affect the protein synthesis rate, eggs were activated with the Ca^{2+} ionophore A23187 in zero-sodium (0-Na^+) artificial seawater. The ionophore causes the release of intracellular Ca^{2+} and the discharge of the cortical granules, but in the absence of external Na^+ there is no intracellular *p*H increase[11], and the rate of protein synthesis is the same as in the unfertilized controls (Fig. 1*a*). Similarly, if eggs are fertilized in seawater and immediately transferred to 0-Na^+ artificial seawater before the onset of the Na^+-dependent H^+ efflux, there will be a Ca^{2+} release without an increase in intracellular *p*H. Protein synthesis remains at the unfertilized rate until Na^+ is added back and the intracellular *p*H increases (Fig. 2). Thus, a Ca^{2+} release in the absence of a *p*H rise does not stimulate protein synthesis.

Treatment with 10 mM NH_4Cl increases the intracellular *p*H by about 0.80 *p*H units and does so by an entirely different mechanism[5,9]. NH_4Cl in alkaline solutions will generate ammonia, which passively diffuses across the egg plasma membrane raising the intracellular *p*H, bypassing the transient

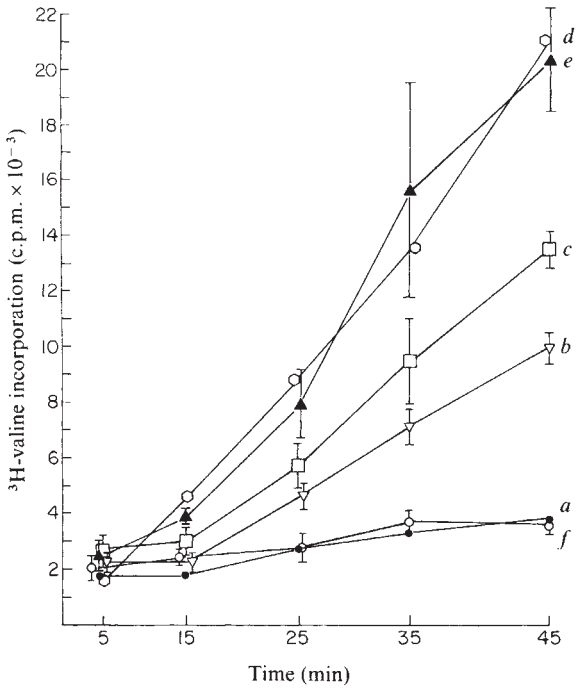

Fig. 1 Total ^{3}H-valine incorporation over a 45-min period starting 5 min after activation in various ionic media. The mean incorporation of label into unfertilized eggs from four experiments was determined for each time point. The ratio of this mean to the unfertilized rate in each of these four experiments was used to normalize the data presented for the different ionic conditions. The plotted data points represent the mean of the normalized incorporation data. The value shown represents the amount of incorporation ±s.e.m. for four repeats of this experiment testing five different conditions plus fertilization. The standard errors for fertilization have been omitted and the time points slightly spread out for clarity. *a*, Eggs activated by 2.5 μM A23187 in 0-Na^+ artificial seawater: intracellular Ca^{2+} release without an intracellular *p*H rise. *b*, Eggs activated by 10 mM NH_4Cl in 0-Ca^{2+} artificial seawater: a rise in intracellular pH without a Ca^{2+} release or influx. *c*, Eggs activated in 10 mM NH_4Cl in 0-Na^+ artificial seawater: an intracellular *p*H rise with some Ca^{2+} influx. *d*, Eggs activated in 10 mM NH_4Cl and 2.5 μM A23187 in 0-Na^+ artificial seawater: both an intracellular *p*H rise and a intracellular Ca^{2+} release. *e*, Eggs fertilized normally in natural seawater for comparison purposes. *f*, Unfertilized eggs in natural seawater. Artificial seawater: 484 mM NaCl, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 11 mM $CaCl_2$, 2.4 mM $NaHCO_3$, *p*H 8.0. 0-Na^+ artificial seawater had choline Cl substituted for NaCl and $KHCO_3$ for $NaHCO_3$. 0-Ca^{2+} artificial seawater had 16 mM NaCl substituted for $CaCl_2$ and 1 mM EGTA added. Eggs of the sea urchin *Lytechinus pictus* were obtained and preloaded as described previously[6]. Briefly, a 0.75-ml volume of packed dejellied eggs was preloaded in 15 ml of seawater containing 5 μCi ml^{-1} [2, 3-^{3}H]valine (Schwarz–Mann, 26 Ci mmol^{-1}) for 45 min. The eggs were then washed twice in seawater and once in the test solution before resuspending to a 0.1% concentration in the test solution. Aliquots (2 ml) were taken at 10-min intervals and processed for scintillation counting as described previously[6].

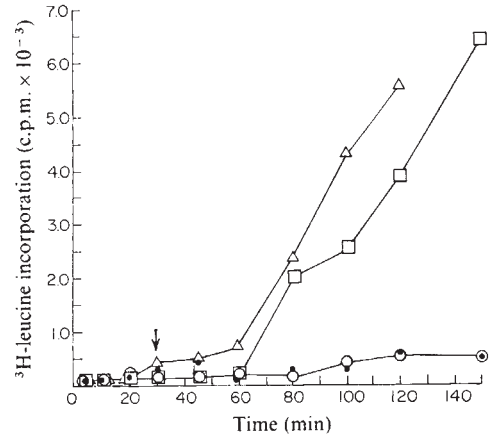

Fig. 2 Total ^{3}H-leucine incorporation over a 150-min period showing the effect of Na^+ on the acceleration of protein synthesis at fertilization. ●, Unfertilized eggs in seawater; ○, Eggs fertilized in normal seawater and completely transferred to 0-Na^+ seawater 50 s after fertilization; △, fertilized eggs in seawater; □, eggs fertilized in normal seawater and completely transferred to 0-Na^+ seawater 50 s after fertilization. At the arrow, 30 min after fertilization, eggs were transferred back to normal seawater. Eggs of the sea urchin *Strongylocentrotus purpuratus* were treated with 5 mM dithiothreitol (DTT), *p*H 8.5, until test aliquots showed that fertilization would not produce a fertilization envelope. Eggs were then preloaded with 5 μCi ml^{-1} [4, 5-^{3}H]leucine for 1 h. Aliquots (2 ml) of a 1% suspension of eggs were put into polylysine-coated Petri dishes, one for each time point. This treatment firmly attaches the eggs to the Petri dish where they will remain even if fertilized because the DTT treatment prevents the elevation of the fertilization envelope. These attached eggs could then be quickly and completely transferred in and out of the 0-Na^+ seawater. The attachment of DTT-treated eggs to polylysine-coated surfaces delays the response of protein synthesis to fertilization by 20 min in both the experimental and the control conditions. At each time point the seawater was removed and 5 ml of 10% trichloroacetic acid added to the Petri dish. At the completion of the experiment the eggs were scraped from the dishes using rubber policemen, collected on glass fibre filters and processed as described previously[6].

intracellular Ca^{2+} release and not causing the discharge of the cortical granules. However, NH_4Cl in seawater containing Ca^{2+} does provoke a transient Ca^{2+} influx[12]. NH_4Cl activation in seawater or zero 0-Na^+ artificial seawater (both of which have the normal amount of Ca^{2+}) will result in similar stimulations of protein synthesis to levels less than those achieved by fertilization. If extracellular Ca^{2+} is present during NH_4Cl activation there will be a measurable influx of Ca^{2+}, which although insufficient to cause discharge of the cortical granules, does affect the level of protein synthesis. If protein synthesis is compared in eggs activated in 0-Na^+ seawater where Ca^{2+} influx can occur (Fig. 1*c*) and in 0-Ca^{2+} artificial seawater where there is no measurable Ca^{2+} influx, there is a significantly lower level of protein synthesis in the absence of the Ca^{2+} influx (Fig. 1*b*).

If eggs are treated with A23187 and 10 mM NH_4Cl in 0-Na^+ artificial seawater, there will be both an intracellular Ca^{2+} release caused by the ionophore and an intracellular *p*H increase caused by the NH_4Cl. The rate of protein synthesis approaches that of normal fertilized eggs (Fig. 1*d*). Thus, Ca^{2+} release and an increase in intracellular *p*H, although induced by mechanisms other than the normal ones used by the egg at fertilization, will lead to the fertilized level of accelerated protein synthesis. The higher levels of amino acid incorporation with both a Ca^{2+} and *p*H stimulation are unlikely to be due to a change in amino acid pools because pulse labelling experiments give an identical ordering of incorporation rates (unpublished results).

Thus, Ca^{2+} release in the absence of an intracellular *p*H increase does not stimulate protein synthesis. A *p*H increase in the absence of a Ca^{2+} release will yield a partial stimulation of protein synthesis. A small Ca^{2+} influx in the presence of a *p*H increase will yield higher levels of protein synthesis. Only in the presence of both an intracellular Ca^{2+} release and a *p*H increase will protein synthesis be maximally stimulated.

Brandis and Raff[10] have provided evidence for a increase in the peptide elongation rate associated with fertilization that is not seen with NH_4Cl stimulations. It is possible that the

increased elongation rate is dependent on the Ca^{2+} release at fertilization. However, it is difficult to be sure of such an interpretation of the differences between NH_4Cl activation and fertilization because (1) the rate of protein synthesis is changing very rapidly, (2) it is affected by the cell cycle changes, and (3) extracellular Ca^{2+} enters during NH_4Cl activation in seawater. In our experiments, however, the magnitude of the response to NH_4Cl stimulation with varying amounts of Ca^{2+} entry or release clearly show that if the intracellular *p*H is elevated, Ca^{2+} plays an additional role in accelerating the rate of protein synthesis at fertilization.

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Mutagenic deamination of cytosine residues in DNA

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Spontaneous deamination converts cytosine to uracil, which is excised from DNA by the enzyme uracil-DNA glycosylase, leading to error-free repair. 5-Methylcytosine residues are deaminated to thymine, which cannot be excised and repaired by this system. As a result, 5-methylcytosine residues are hotspots for spontaneous transitions, as demonstrated in the *lacI* gene of *Escherichia coli*. We show here that in bacteria which lack uracil-DNA glycosylase (Ung^-) and cannot excise uracil residues from DNA, the rate of spontaneous transition at cytosine residues is raised to the hotspot rate at 5-methylcytosine residues. These studies provide direct evidence that the deamination of cytosine is a significant source of spontaneous mutations.

The observation by Lindahl that potentially mutagenic cytosine deamination occurs at observable rates in *in vitro* physiological conditions led to the discovery of uracil-DNA glycosylase[1]. This enzyme initiates the repair of uracil from DNA by a 'base-excision' repair pathway[2,3]. *E. coli* uracil-DNA-glycosylase-deficient mutant strains[4] have been shown to have a weak G:C→A:T specific mutator phenotype[5]. Presumably, a G:U mismatched base pair resulting from cytosine deamination generates an A:U base pair after replication. Thus, one of the physiological roles of uracil-DNA glycosylase may be to repair such pre-mutational lesions resulting from deamination.

A more general description of the possible significance of cytosine deamination as an important mutagenic process comes from studies of the *E. coli lacI* gene[6–8]. Large numbers of independent *lacI* mutations have been characterized by genetic and biochemical methods. Of spontaneous base substitutions leading to nonsense mutations, approximately 75% arise by G:C→A:T transitions[6–8]. Two highly mutable 'hotspot' sites are conspicuous among 14 G:C→A:T amber mutation sites in the *lacI* gene; half of the observed amber transition mutations occur at these two sites. DNA sequence studies have demonstrated the presence of 5-methylcytosine residues at the hotspot sites[6]. It is clear that cytosine methylation increases the mutagenic susceptibility of a cytosine residue, presumably because of the inability of the cell to repair the deamination product, thymine.

We have studied the effect of the *ung* mutation, which results in the loss of uracil-DNA glycosylase activity, on the distribution of I^- nonsense mutations. The *ung-1* allele was inserted by P1 transduction into the *E. coli* GM1 used in previous studies of the *I* gene[6–8]. Table 1 shows that the frequency of amber mutations increased almost fourfold in the presence of the ung^- mutation. Thus, a deficiency of uracil-DNA glycosylase led to an increase in the frequency of mutations due to base substitutions. The I^- amber mutations were assigned to one of the known amber sites by deletion mapping and by examining the response to different amber suppressors, as described previously[7,8].

G:C→A:T transitions generate amber mutations at 14 different sites in the *lacI* gene. This represents 38% of the 37 characterized amber mutation sites in *lacI* which can be derived by a single base change. As Table 2 shows, 93% of the amber mutations detected in the Ung^- background arose by way of a G:C→A:T transition. In Ung^+ cells (GM1), 75% of all the amber mutations were G:T→A:T transitions, principally due to the 5-methylcytosine hotspots *A6* and *A15* (and to a lesser extent *A34*).

Table 1 Frequency of spontaneous I^- mutants ($\times 10^6$)

Strain	Approx. I^- mutant frequency	% Amber mutants
GM1	2.5	1.0
BD1322	2.5	1.3
BD1323 (Ung^-)	2.5	4.6

I^- mutants were selected and screened against different suppressors to detect amber mutations, as described previously[7,8]. BD1322 is an Ung^+ derivative of the Ung^- strain BD1323. It is isogenic to GM1.

Table 2 Distribution of amber mutations in Ung^+ and Ung^- strains

Base substitution	Site	No. of occurrences: Ung^+ GM1	Ung^- BD1323	Ung^- BD1323 (2AP)
G:C→A:T	*A5*	0	2	0
	*A6**	51	4	39
	A9	8	18	4
	*A15**	37	0	29
	A16	9	1	2
	A19	11	2	1
	A21	8	2	0
	A23	8	7	2
	A24	10	1	5
	A26	6	2	2
	A31	1	2	3
	A33	5	4	1
	*A34**	12	3	11
	A35	1	2	0
Total G:C→A:T mutations		167	50	99
Transversions		55	4	0
Total amber mutations detected		222	54	99

Distribution of amber mutations in Ung^+ and Ung^- strains, showing the distribution found in GM1 and in BD1323 with and without 2-aminopurine (2AP). The number of occurrences refers to the number of mutations detected at a given locus in the collections of 222, 55 and 99 *lacI* mutations, respectively. The characterization of the GM1 amber mutations has been reported previously[6–8]. The I^- collection from BD1323 was analysed in the same manner.

* Presence of 5-methylcytosine at the mutational site[6].

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