Rapid effects of insulin on cyclic GMP location in an intact protozoan

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Abstract. We studied rapid changes in location of cyclic GMP in *Tetrahymena pyriformis*. Insulin caused cGMP localization in cilia and near the plasma membrane (0.5–1 min). Later (1–5 min) cGMP localization was diffuse in cytoplasm with perinuclear accentuation. Inactive insulin analogs did not elicit these changes.
Key words. Insulin; cyclic GMP; immunocytology; *Tetrahymena pyriformis*.

Tetrahymena pyriformis is a unicellular eucaryote, that has been used as a model for many biological processes[1–3], including signal transduction. Receptor-mediated activation of guanylate cyclase[4, 5] and adenylate cyclase[6, 7] has been demonstrated in *Tetrahymena*. Modulation of cGMP and cAMP have been associated with several physiologic processes in *Tetrahymena*, including cell cycling[8, 9] and phagocytosis[10]. Changes in cyclic nucleotide concentrations after exposure to various agonists were previously characterized with radioimmunoassays of cyclic nucleotides extracted from whole cells[11]. Such analyses provide quantitation of temporal changes of total cAMP or cGMP but no information on spatial distribution.

Regulation of compartmentalization of cyclic nucleotides has been predicted[12] but not evaluated in intact unicellular organisms. A recently developed method for cyclic nucleotide immunocytology in cultured human cells suggested the possibility of examining localization of cyclic nucleotides after hormone exposure in a single celled organism[13]. In this study our goal was to image the cGMP location in the intact *Tetrahymena* cell and to test for agonist-dependent and time-dependent changes of cGMP location.

Materials and methods

Materials: Pancreatic insulin and its derivatives were used except where noted. Bovine insulin A-chain and porcine insulin were from Sigma (St. Louis, MO); chicken insulin was from Sigma and also from Litron Labs (Rochester, NY); porcine desoctapeptide insulin was from Litron Labs; recombinant human insulin and human proinsulin were from Eli Lilly (Indianapolis, IN). 3-Isobutyl-1-methyl-xanthine (IBMX) and sodium nitroprusside were from Sigma. Bovine serum-albumin was from Miles Diagnostics (Kankakee, IL). Phosphate buffered-saline (PBS) was from Biofluids (Rockville, MD). 8-(Fluoresceinl)thioguanosine 3′,5′-cyclic monophosphate (FITC-cGMP) was from Molecular Probes (Eugene, OR). Immunocytology reagents were from Vector Labs (Burlingame, CA); monoclonal mouse antibody against cyclic-GMP was a gift from Dr Michael A. Kaliner[14].

Cell culture conditions: Tetrahymena pyriformis GL cells were from Dr Joseph Shiloach. Cells were grown at 28 °C in a chemically defined medium containing glucose, amino acids, minerals, vitamins and inorganic salts but free of macromolecules (Gibco, Grand Island, NY, product No. 83/5053). Cells were subcultured at 1:20 dilution every second day. For experiments, cells were separated by centrifugation from the media at the end of the logarithmic phase of growth (typically 48 h after subculturing), resuspended in growth medium at a density of 5×10^4 cells/ml, and plated on 12 precoated spots (8 mm diameter) of adhesion slides (MM Developments, Ottawa), with 500 cells/spot (i.e. 10 µl/spot).

Agonist exposure: Agonists were added 1 min after cells were plated onto adhesion slides. Agonists (sodium nitroprusside or insulin analogs) were in Eagle's minimal essential medium, without phenol red, without sodium bicarbonate, containing 25 mM HEPES, 0.3 g/l bovine serum albumin, 800 units/l aprotinin, 0.5 mM calcium and 0.5 mM magnesium, pH 7.4, at 28 °C. In certain experiments 0.5 mM IBMX was also included in the assay buffer.

Fixation and immunocytology: Cells were fixed as described previously[13] with modifications including the use of adhesion slides, increase in fixation time, and postfixation treatment of cells. Cells were fixed on adhesion slides without a covering lid. Optimal fixation time was determined by monitoring the temperature of the cells during fixation (Sensortek TS-4 Controller) and by microscopically monitoring cell morphology after various fixation conditions. Optimal fixation time for *Tetrahymena pyriformis* in an Amana 700 W oven was 30 s. The associated temperature of the cells for optimal fixation was 48–50 °C. Immediately after microwave exposure, slides were placed into a water bath at 26 °C, and 80 µl PBS/spot was added to the cells. Immunocytology was as described earlier[13]. Briefly, we added normal horse serum (4% diluted in PBS) for 30 min, then primary mouse monoclonal anti-cyclic GMP antibody (1:2000) for 1 h, then R-phycoerythrin labeled horse anti-mouse IgG as second antibody for 30 min. Incubations were in a humidified dark chamber at room temperature with continual rocking. Each incubation step was followed by 5 washes for 5 min with 3 × PBS. Slides were mounted with aqueous mounting solution from Zymed Lab Inc. (San Francisco, CA).

Cell morphology after microwave fixation was compared with cell morphology after three other fixation methods: 4% formaldehyde in PBS[15], 15% picric acid + 4% formaldehyde in PBS[16], or 75% ethanol[17]. After each fixation method, cell morphology was evaluated with phase contrast microscopy. Cells were also stained with standard methods: hematoxylin and eosin[18], toluidine blue[19], or fast green[20].

In pilot experiments we examined the effect of microwave fixation on the pattern of fluorescent cGMP in a manner analogous to prior studies[13]. *Tetrahymena* were incubated for varying times with FITC-cGMP. Up to 10 min FITC-cGMP accumulated progressively, near the plasma membrane and in the cytoplasm; the fluorescence intensity and distribution remained the same after microwave fixation and after microwave fixation followed by 5 10-min washes.

Image processing and analysis: Images were photographed directly from a Zeiss Photomicroscope III with a built-in camera. Paired control and experimental fluorescent images were photographed with similar illumination and camera settings. Alternately fluorescent images were captured with a Dage/MTI CCD-72 solid state camera equipped with an Opelco intensifier KS3-CD. Color images were captured with a Sony XC-007 precision tricolor camera. Digital images were analyzed with a computer system from G. W. Hannaway and Associates (Boulder, CO).

Results

Microwave fixation of Tetrahymena. Cellular structures, including nucleus, vacuoles, mucocysts, peritrich cilia, and oral field were well preserved with microwave fixation (fig. 1). Cell structures appeared similar after microwave fixation and after the other three conventional fixation methods (not shown).

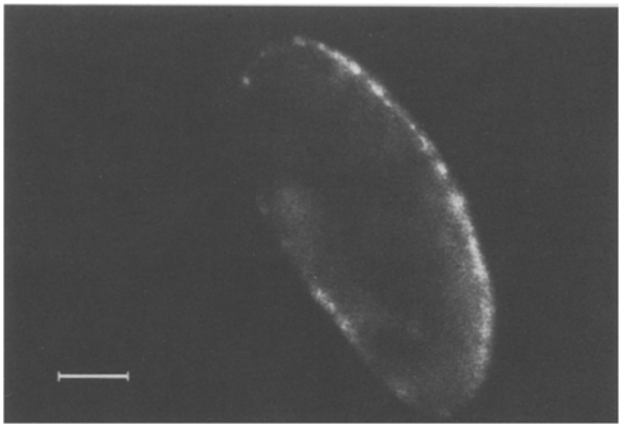
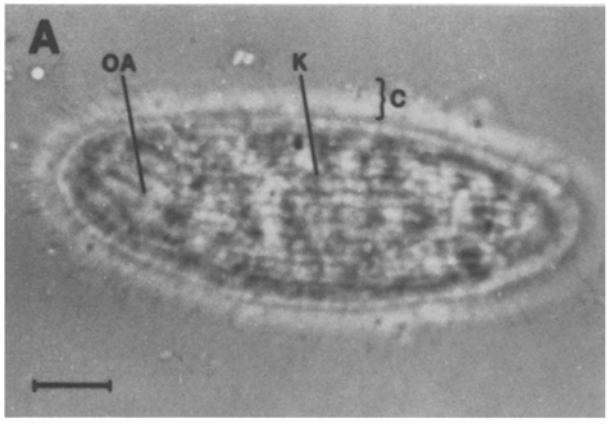


Figure 2. Cyclic GMP localization pattern in *Tetrahymena* without any cGMP agonist added. Cyclic GMP localization is diffuse in cilia. Bars indicate 10 microns.

Basal cyclic GMP distribution in Tetrahymena. Under control conditions (incubation of cells up to 5 min with assay buffer only) the cyclic GMP signal was weak. The distribution was diffuse along the plasma membrane and within the cilia (resembling a halo outside the plasma membrane) (fig. 2).

The following controls, included to test the specificity of cGMP immunocytology, gave negative results: 1) substitution of primary antibody with nonimmune ascites fluid; 2) substitution of primary antibody with the same primary antibody preincubated overnight with cyclic GMP (1 mM) at 4 °C.

Sodium nitroprusside and cyclic GMP location. Incubation of cells with sodium nitroprusside for 1 min caused a striking increase in the intensity of the cGMP signal. Cyclic GMP was located diffusely in the cilia, plasma membrane, and cytoplasm (compare fig. 2 versus fig. 3 A). The half-maximal effect of sodium nitroprus-



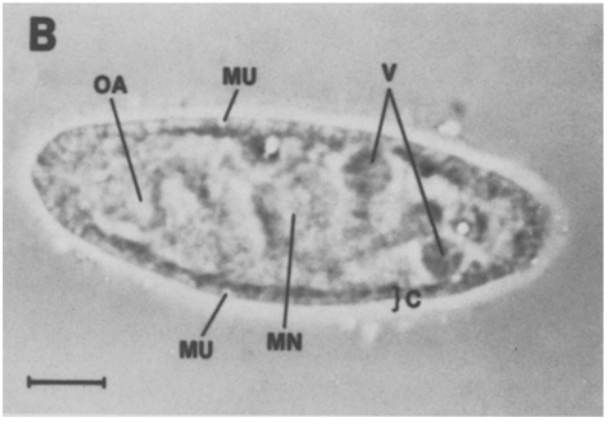


Figure 1. Phase contrast images of *Tetrahymena pyriformis* fixed by microwave radiation. *A* Focal plane near the surface of the same cell; *B* focal plane within a cell. MN = macronucleus, V = food vacuole, C = ciliary zone, K = kinetosome zone (the primary meridians are long dark lines of ciliary attachment; these alternate with light parallel rows of secondary meridians), Mu = mucocyst, OA = oral area. Bars indicate 10 microns.

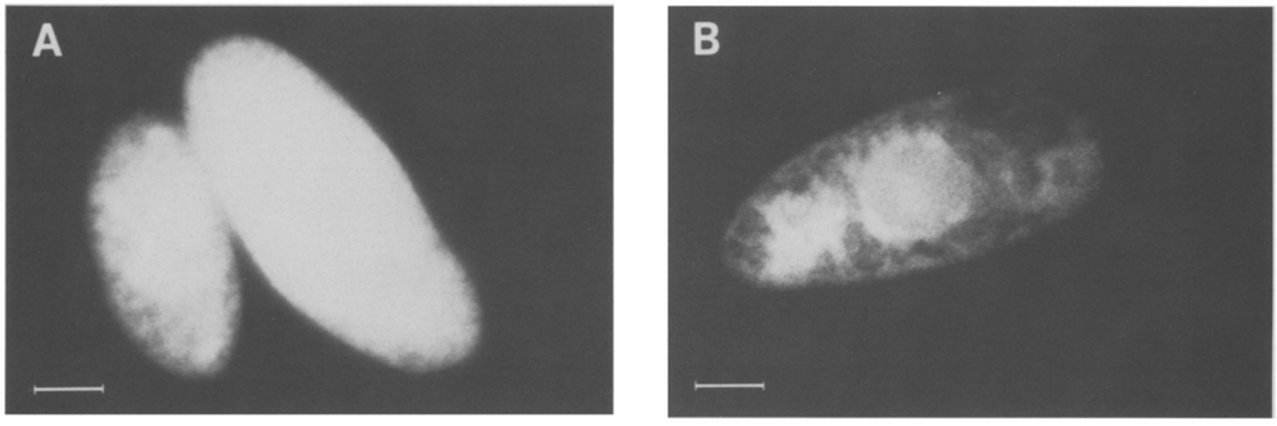


Figure 3. Cyclic GMP localization pattern in *Tetrahymena* after incubation with sodium nitroprusside (10^{-5} M) *A* for 1 min; *B* for 5 min. Bars indicate 10 microns.

side was at 5×10^{-6} M; the maximal effect (approximately 2.5-fold over baseline) was observed at 10^{-4} M. The effects of nitroprusside were rapid; 10^{-5} M sodium nitroprusside increased cGMP signal intensity with 15 s (the earliest time tested), and a decline in the signal was evident after 5 min.

Between 3 and 5 min, cGMP localization was greatest within the macronucleus (fig. 3B).

Effect of insulin on cyclic GMP localization. Incubation of cells with human insulin (10^{-12}–10^{-6} M) caused cyclic GMP accumulation. The concentration of human insulin that yielded the minimal detectable effect on cGMP was 10^{-12} M, and the concentration for maximal effect was 10^{-10} M.

We observed sequential changes in cGMP localization patterns when cells were exposed to human insulin at 10^{-10} M. At 15 s (the earliest time tested), cGMP was occasionally visible in individual cilia extending from the cell margin (not shown) or overlaying the cell (fig. 4A, left). At 30 s cGMP localization was diffuse in the zones of the cilia and plasma membrane (fig. 4B, left). The cGMP had a speckled pattern near the plasma membrane at 30–60 s (fig. 4C, left). This pattern was identical to that of the ciliary attachment sites, i.e. kinetosomes. This suggested cGMP localization near the base of the cilia. Between 1 and 5 min the cGMP localization pattern became diffuse near the plasma membrane, and cGMP was also apparent in the cytoplasm.

Between 1 and 5 min 40–60% of the cells showed a perinuclear location of cGMP (fig. 4, right panels). Cyclic GMP seemed to remain outside the nucleus at all observed times after insulin addition.

Analog specificity of insulin effect on cyclic GMP. To quantify dose-dependency, we measured the integrated intensity of cGMP signal within single cells (in 20 cells per experimental point) after 60 s incubation with graded concentrations of insulin analogs. The maximal cGMP signal was approximately 400% of the basal signal. We found similar effects with human proinsulin and insulin from human, pig, or chicken (table). Insulin A chain and des-(B23–B30) insulin (10^{-12} to 10^{-6} M) did not induce cyclic GMP accumulation with incubations up to 5 min. The time-dependent changes (between 15 s and 5 min) in cGMP localization were similar with porcine, chicken, or human insulin or human proinsulin (not shown).

Effect of isobutyl-methylxanthine. Addition of the phosphodiesterase inhibitor IBMX in the assay buffer did not prevent the insulin effect on cGMP within cilia at early times or around the nucleus at later times.

Discussion

Insulin rapidly increases cGMP in fat[21,22], liver[23], and intestinal mucosa cells[24]. Insulin for 1 h has also been found to stimulate guanylate cyclase in *Tetrahymena*[4]. We have now established that the rapid cGMP accumulation after insulin addition to *Tetrahymena* is localized and that the localization changes rapidly with time.

Effects of insulin analogs for 60 s on cGMP in *Tetrahymena pyriformis*. Data are average response integrated over the entire cell and based on 20 cells per experiment. Each experiment was done three times. Data are mean ± 1 standard deviation.

Insulin analog	Analog concentration for half-maximal effect (pM)
Human proinsulin	40 ± 20
Human insulin	50 ± 12
Chicken insulin	40 ± 10
Porcine insulin	50 ± 9
A-chain human insulin	indet.*
Des-(B23–B30) human insulin	indet.

* indet. = indeterminate. No detectable cGMP stimulation by this analog at concentrations up to 1 μM; thus half maximal effect of the analog was indeterminate.

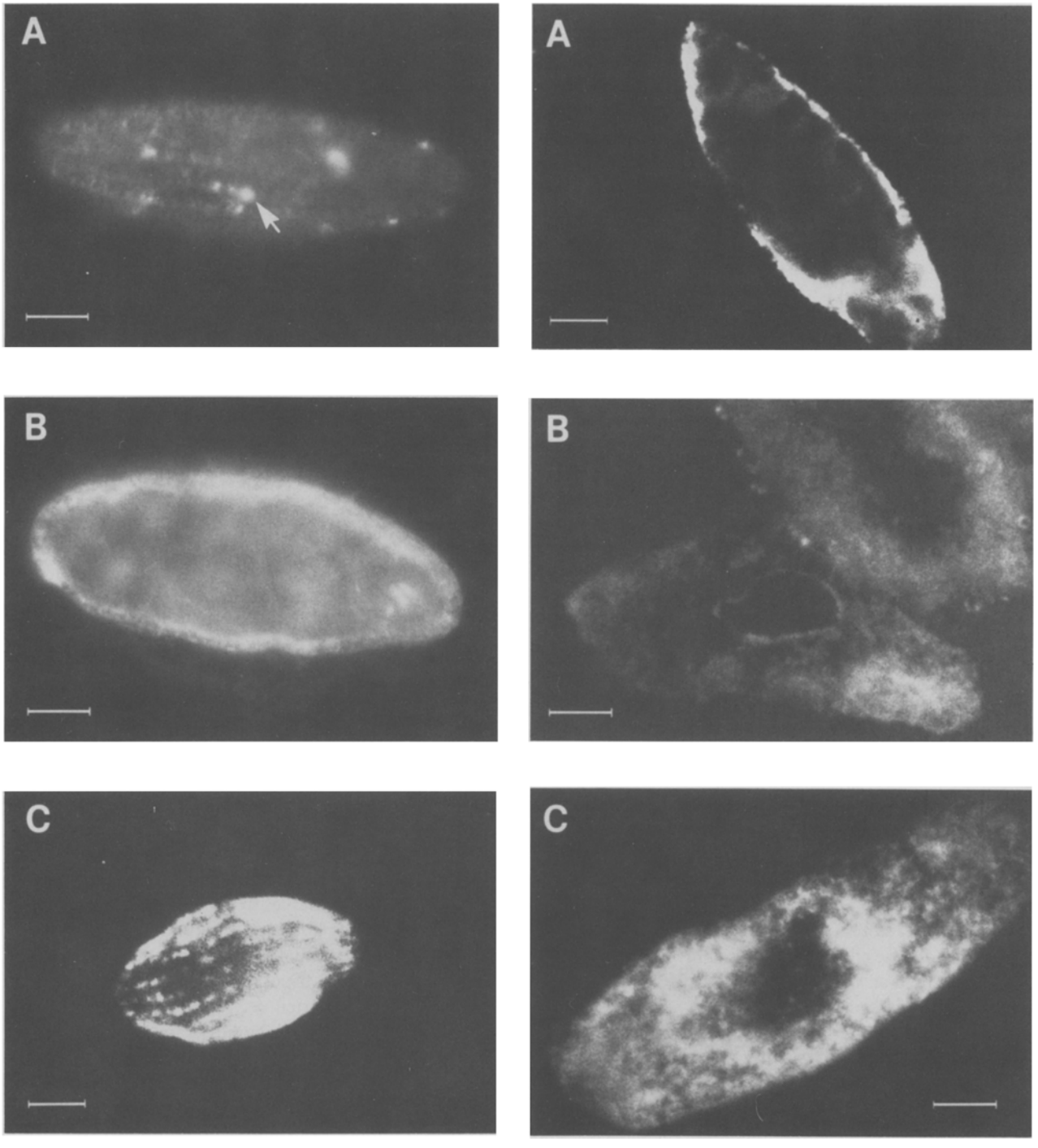


Figure 4. Left panel. Early cyclic GMP localization patterns in *Tetrahymena* after incubation with human insulin (10^{-10} M). *A* At 15 s cGMP localization in individual cilia appears as small striations overlaying the cell (arrow); focal plane near cell surface. *B* At 30 s the ciliary zone and plasma membrane show cGMP localization. Focal plane is within the cell; the cGMP overlaying the cytoplasm is mostly external to the cytoplasm. *C* At 45 s cGMP localization is in parallel foci with distribution of the ciliary attachment sites; focal plane near cell surface. Bars indicate 10 microns. Right panel. Late cyclic GMP localization patterns within *Tetrahymena* after incubation with human insulin (10^{-10} M). *A* At 1 min cGMP is in the cytoplasm mainly near the plasma membrane with slight localization about the macronucleus. *B* At 3 min there is accentuated cGMP localization near the nuclear envelope. *C* At 5 min cGMP localization is diffuse in the perinuclear region of the cytoplasm. Bars indicate 10 microns.

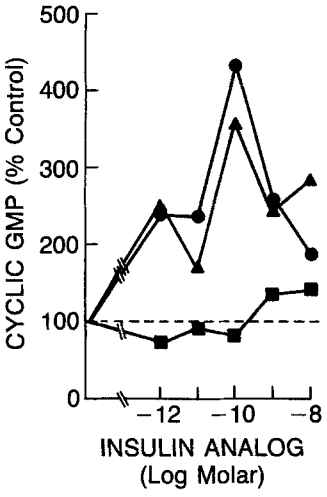


Figure 5. Analog specificity of insulin effect on cyclic GMP in *Tetrahymena*. Each analog was incubated with cells for 60 s prior to fixation. Each point is a mean from 20 whole cells from an experiment that was repeated two additional times (table). (Circles) human proinsulin, (triangles) human insulin, (squares) human insulin A-chain.

We established here that microwave fixation can preserve cGMP distribution patterns in *Tetrahymena* analogous to our earlier findings in cultured mammalian cells[13]. We introduced changes from our prior methods to allow fixation of cGMP in a free swimming ciliate. We used adhesion slides to stabilize the cells as a monolayer. Optimal time of microwave radiation was found to be 30 s, as compared to 10 s for human fibroblasts. The requirement for a longer fixation time is probably explained by the difference in size and geometry of the cells or by the special cortical structure of the *Tetrahymena* plasma membrane.

Under control conditions the cyclic GMP signal was weak. The diffuse ciliary and plasma membrane patterns of cGMP are in accord with prior data about cGMP and guanylate cyclase activity in ciliates[8,12,25,26].

Sodium nitroprusside is an agonist for soluble forms of guanylate cyclase[27]. The diffuse and intense cGMP signal after incubation with sodium nitroprusside established that our method can detect cGMP in several portions of the *Tetrahymena* cell, including the nucleus. And the differences in cGMP localization after nitroprusside versus after insulin support biochemical studies in adipocytes, indicating that these two agonists stimulate cGMP synthesis through differing pathways[28].

Studies of interactions with fluorescent labeled insulin[29] and physiologic responses to insulin[4,30–32] previously suggested that *Tetrahymena* bears insulin receptors. Herein we report further evidence for functional insulin receptors in *Tetrahymena*.

The rapid changes in cyclic GMP localization in response to insulin appeared as two phases. The 'early' cGMP localization (from 20 s up to 1 min) was in ciliary and plasma membrane regions. The later cGMP localization (1–5 min) was in the cytoplasm. The perinuclear cGMP pattern in the late period suggests a rapid directional flow of information through this pathway.

The early localization of cGMP in cilia and then at their bases may be related to other observations that insulin receptors are in greatest number along the cilia and that they rapidly accumulate near the ciliary bases after insulin addition[33]. This may be analogous to observations in mammalian cells with insulin receptor accumulation at coated pits on the plasma membrane before receptor internalization[34]. The later (1–5 min) cGMP localization in the perinuclear region could reflect movement of guanylate cyclase inducing elements (such as insulin receptor or another post receptor effector) or movement of cGMP (bound or free).

The sensitivity to low concentrations of insulin in *Tetrahymena* is similar to that in some of the most insulin-sensitive mammalian cells (e.g. insulin-mediated inhibition of lipolysis by adipocytes from rats[35]). The ability of this system to distinguish between insulin-related peptides that are bioactive (such as insulin and proinsulin) and inert (A-chain and des-octapeptide) agrees with results in vertebrates. However, chicken insulin which is typically 2–3 times more potent and porcine proinsulin which is typically 10–100 times less potent than human and porcine insulin, were similar in potency in this system. This evolutionarily remote organism appears to have an insulin receptor which shares with its vertebrate homolog a very high affinity for insulin and an ability to distinguish insulin from noninsulin. Like the insulin receptor homolog in *Drosophila*[36], it shows some differences from the vertebrate receptor in its relative affinity for insulin analogs.

We have not determined the mechanisms through which the insulin receptor couples to cGMP accumulation. The *Drosophila* receptor homolog, like that in vertebrates, is linked to a tyrosine kinase which could couple to some insulin actions[36]; this aspect of the *Tetrahymena* receptor has not yet been reported.

The addition of the phosphodiesterase inhibitor IBMX did not prevent insulin from stimulating the accumulation of cGMP within the cilia at early times or about the nucleus at later times. This suggested that these insulin effects were induced through stimulation of cGMP synthesis and not through inhibition of cGMP degradation. *Tetrahymena* contains several potential mechanisms for stimulating cGMP synthesis, such as rapid modulation of intracellular calcium[12,37]. Further studies will be required to establish the mechanisms of the localized accumulation of cGMP, including whether it is mediated by membrane-bound or soluble forms of guanylate cyclase. Our observations of insulin receptors, cGMP localization, and rapid reorganization of cGMP locations in *Tetrahymena* indicate that these signal transduction phenomena could have retained broad biological importance during evolution.

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