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# Induction of $Ca^{2+}$ transport in liposomes by insulin

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The requirement of extracellular $Ca^{2+}$ for insulin action has been indicated by past studies. With a view to understand the interaction of insulin with $Ca^{2+}$ in the vicinity of the cell membrane, we have examined the ability of insulin and its constituent polypeptide chains A and B to translocate $Ca^{2+}$ and $Mg^{2+}$ across the lipid bilayer in two sets of synthetic liposomes. The first were unilamellar vesicles made of dimyristoylphosphatidylcholine and contained the $Ca^{2+}$ sensor dye arsenazo III. Peptide-mediated $Ca^{2+}$ and $Mg^{2+}$ transport in these vesicles was monitored at 37°C in a neutral buffer containing $CaCl_2$ or $MgCl_2$ using a difference absorbance method. In the second set, multilamellar vesicles of egg lecithin containing trapped fura-2 were employed and the cation transport was followed at 20°C by fluorescence changes in the dye. Control experiments indicated that the hormonal peptides caused no appreciable perturbation of the vesicles leading to leakage of contents or membrane fusion. In both liposome systems, substantial $Ca^{2+}$ and $Mg^{2+}$ transport was observed with insulin and the B chain; the A chain was less effective as an ionophore. Quantitative analysis of the transport kinetic data on the B chain showed a 1 : 1 peptide-$Ca^{2+}$ complex formed inside the membrane. In light of the available structural data on $Ca^{2+}$ binding by insulin and insulin receptor, our results suggest the possibility of the hormone interacting with the receptor with the bound $Ca^{2+}$.

## Introduction

A variety of biochemical events triggered by the action of insulin are known to be modulated by extracellular $Ca^{2+}$ [1,2]. These include glucose transport [3], protein synthesis [4] and receptor phosphorylation [5]. In addition, both insulin [6] and insulin receptor [7] have been shown to contain $Ca^{2+}$-binding sites and the binding of insulin to the receptor has been shown to be $Ca^{2+}$-dependent [8]. In spite of the importance of extracellular $Ca^{2+}$ in insulin action suggested by these data, the exact role of $Ca^{2+}$ in insulin's signal transduction pathway remains unclear. In the present study, we have examined the ability of insulin and its two peptide constituents, the A and B chains, to interact with $Ca^{2+}$ and $Mg^{2+}$ at the membrane surface. This information was obtained by studying the ability of the hormonal peptides to translocate the above cations from the aqueous to the lipid environment. For this purpose, we have used model bilayer liposomes containing the dyes fura-2 [9] and arsenazo III [10] which alter their fluorescence and absorbance characteristics, respectively, on binding $Ca^{2+}$. We provide here evidence that insulin can transport $Ca^{2+}$ and $Mg^{2+}$ present in the external buffer across the lipid bilayer of the liposomes, indicating that insulin binds to these ions at the membrane/water interface and facilitates their translocation into the lipid bilayer. This activity is retained in the B chain of insulin but is markedly reduced in the A chain. These results suggest that insulin may interact with its cell surface receptor with bound $Ca^{2+}$ and may provide clues to the observed $Ca^{2+}$-dependence of insulin-mediated events.

## Materials and Methods

### *Materials*

The following were purchased: Hepes, Mops, insulin, oxidized forms of insulin A and B chains, valinomycin, melittin, A23187, arsenazo III, safranine O, EDTA and EGTA from Sigma Chemical Company (St. Louis, MO); ionomycin from Calbiochem (La Jolla, CA), CF from Eastman Kodak (Rochester, NY); Triton

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid; Hepes, *N*-(hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); CF, 6-carboxyfluorescein; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid, disodium salt; DPX, *p*-xylene-bispyridinium bromide; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s).

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X-100 from Pierce Chemical Co. (Rockford, IL); egg PC and DMPC from Avanti Polar Lipids (Birmingham, AL); fura-2, ANTS, and DPX from Molecular Probes (Eugene, OR); $CaCl_2$ and $MgCl_2$ from Fisher Scientific (Unionville, Ontario); Chelex 100 from Bio-Rad (Mississauga, Ontario); and Sephadex resins from Pharmacia (Toronto, Ontario). All other reagents were of analytical grade.

*Methods*

All solutions were made with Millipore-filtered deionized water to ensure minimal $Ca^{2+}$ levels and were subsequently passed through a $30 \times 1$ cm Chelex 100 column. The commercial samples of insulin and oxidized insulin A and insulin B chains were checked for their purity by gel-filtration HPLC using a Beckman Ultraspherogel TSK-2000 column. The column was calibrated in the 1000–60000 $M_r$ range with standard peptides and proteins in the buffer system used in the cation transport experiments. From the areas under the respective chromatograms, the insulin samples were judged to be 92–98% pure. Zinc-free insulin was obtained by passing a 1.75 mM solution of commercial insulin in 1% acetic acid through a $30 \times 1$ cm column of Sephadex G-75 equilibrated with the same solvent and lyophilizing the eluted sample overnight. Concentration of insulin was determined by measuring the absorbance at 278 nm (pH 7.3) using an absorption coefficient of 1.05 $lg^{-1} cm^{-1}$ [11].

*Preparation of liposomes*

MLV of egg PC containing trapped fura-2 were made essentially as described previously [12]. Briefly, thin lipid films were formed by removing the solvent from a 2:1 (v/v) chloroform/methanol solution of the lipid by rotary evaporation followed by drying under high vacuum for a minimum of 2 h. The lipid film was then hydrated in 10 mM Hepes, 145 mM KCl, and 100 $\mu$M fura-2 (pH 7.2) at a lipid concentration of 26.3 mM. The suspension was then vortexed and freeze-thawed five times employing liquid nitrogen for freezing to ensure homogeneous distribution of solutes [13]. The MLV were kept at room temperature for one hour, then refrigerated at 4°C and passed through a $30 \times 1$ cm Sephadex G-75 column to remove extraneous fura-2. Complete removal of the dye was verified by the addition of $CaCl_2$ to the eluted MLV and measuring the fluorescence spectrum.

LUV containing trapped arsenazo III were made from DMPC by first treating the dried lipid film (52 mM) with 20 mM Mops, 125 mM KCl (pH 7.3) containing 3.6 mM arsenazo III and subjecting the vortexed vesicles to ten repetitions of freeze-thawing. The resulting MLV were extruded through a 0.1 $\mu$m polycarbonate membrane filter using a commercial extruder (Lipex Biomembranes, Vancouver). The vesicles were then passed through a $30 \times 1$ cm Sephadex G-75 column to remove the excess dye [12]. Lipid concentration was determined from inorganic phosphate measured according to Ames [14]. LUV made from DMPC using the above protocol tended to leak fura-2 significantly and hence were not used in the present studies.

*Cation translocation measurements*

Fluorescence measurements were recorded on a Perkin-Elmer LS-50 fluorescence spectrometer, operating in the ratio mode during the time course experiments and excitation mode for individual scans and for experiments with model ionophores. Slitwidths of 5 and 10 nm were used, respectively, for the excitation and emission modes in the fura-2 experiments. Samples were magnetically stirred to ensure rapid mixing of the $Ca^{2+}$ and hormone or ionophore. When using A23187 the intrinsic fluorescence of the ionophore was corrected from the total signal. Absorbance measurements were recorded on a Perkin-Elmer Lambda 6 UV/Vis spectrophotometer. Temperature was controlled at $20 \pm 1$°C during the fluorescence experiments and $37 \pm 1$°C during the absorbance measurements using a refrigerated liquid circulator.

$Ca^{2+}$ translocation across the fura-2-loaded MLV was measured in a 1.0 ml volume (650 nmol lipid). Experiments were initiated by adding an appropriate concentration of A23187, ionomycin or the insulin peptides, followed by the addition of 2 mM $CaCl_2$. The rate of $Ca^{2+}$ influx was monitored by following the change in the fluorescence ratio of fura-2 at excitation wavelengths of 340 and 380 nm (with emission wavelength set at 510 nm) as a function of time. (Switching between these wavelengths was performed at 1500 nm/min using the fura-2 software program in the instrument). Ratio measurements were corrected for background fluorescence and scattering by preparing MLV under identical conditions in the absence of fura-2. The concentration of lipid was adjusted to that of the fura-2 vesicles by measuring the absorbance at 700 nm. A fluorescence scan was taken of these vesicles and the spectrum was subtracted from all experimental scans before calculations were performed. The ratio was then converted to the internal $Ca^{2+}$ concentration as described elsewhere [9]. From this the actual amount of $Ca^{2+}$ translocated could be determined by estimating the intravesicular volume. The latter was obtained by comparing the observed fluorescence intensity of the vesicle solution with that expected for the trapped concentration (100 $\mu$M) of fura-2 from a standard curve for fura-2 fluorescence. The fluorescence spectra for $Ca^{2+}$-bound fura-2 ($R_{max}$) and $Ca^{2+}$-free fura-2 ($R_{min}$) were measured by permeabilizing the vesicles with 20 $\mu$M ionomycin and adding either excess $Ca^{2+}$ or EGTA, respectively [15].

$Ca^{2+}$ translocation across DMPC LUV was measured by monitoring the change in absorbance at 650–700 nm with time [10,16,17]. The sample and reference cuvettes initially contained 700 $\mu$l buffer, 150 $\mu$l of vesicles (3.5 $\mu$mol lipid) and 30 $\mu$l of 100 mM $CaCl_2$ (final concentration: 3.5 mM). Cation transport was initiated by adding the required concentration of the hormone or A23187 to the sample cuvette and the same volume of solvent to the reference. Difference spectra were recorded between 400 and 700 nm at specific time intervals. (This was more convenient than recording absorption spectra of the sample and eliminated scattering artifacts [10]). EDTA was added at the end of the experiment at a final concentration of 5 mM to determine the amount of dye which had leaked out. The difference in absorbance change ($\Delta A$) was converted to internal $Ca^{2+}$ concentration using the procedure of Sokolove and Kester [16]. This could then be converted to moles of $Ca^{2+}$ by estimating the intravesicular volume by the procedure analogous to that described earlier for fura-2 experiments. In $Mg^{2+}$ transport experiments, an appropriate volume of 100 mM $MgCl_2$ replaced the $Ca^{2+}$ solution in the above method. A titration experiment was carried out between $Mg^{2+}$ and the dye under our conditions using the latter's absorption at 617 nm as a monitor. A value of $K_d = 960$ $\mu$M was obtained, as compared to 20.3 $\mu$M obtained for the arsenazo III-$Ca^{2+}$ complex.

*Liposome stability*

Membrane permeabilty was measured by using the CF method [18]. LUV made with DMPC were loaded with 20 mM CF in 20 mM Mops, 125 mM KCl (pH 7.3) and the fluoresence emission of the dye was monitored as a function of time at 515 nm with excitation at 470 nm. Dye leak induced by either of the peptide hormones or A23187 would cause an increase in the fluorescence intensity caused by decreased extent of concentration-quenching experienced by the dye. LUV (3.5 $\mu$mol lipid) were added to a 850 $\mu$l volume of buffer (20 mM Mops, 125 mM KCl (pH 7.3)) in a cuvette, followed by addition of either 30 $\mu$M insulin, insulin B, or 20 nM A23187. In all experiments this was followed by the addition of $CaCl_2$ stock solution to obtain a final concentration of 3.5 mM. The fluorescence increase caused by the addition of 10 $\mu$l 10% (v/v) Triton X-100 was taken to represent 100% leakage and the hormone-induced leak was measured as the difference between the rate of change of fluorescence before and after hormone addition.

Test for the leak of either DPX or ANTS was made by measuring the fluorescence enhancement in a batch of DMPC LUV containing both the fluorophore ANTS and the quencher DPX [19]. LUV were made containing 12.5 mM ANTS, 45 mM DPX, 10 mM Mops, 68 mM NaCl (pH 7.4). LUV (3.5 $\mu$mol lipid) were added to the external buffer (10 mM Mops, 0.1 mM EDTA, 150 mM NaCl (pH 7.4)) (final volume: 850 $\mu$l) and fluorescence monitored at 545 nm (384 nm excitation). Fluorescence was monitored until the signal was steady before the addition of either A23187 (20 nM) or the hormone (30 $\mu$M). $Ca^{2+}$ was subsequently added at a final concentration of 3.5 mM. Addition of Triton X-100 (10 $\mu$l of a 10% (v/v) solution) caused disruption and the resulting fluorescence signal was used as the 100% reference value for quantitative estimation of membrane leakage caused by peptides.

Perturbation of the lipid bilayer of the vesicles by the hormonal peptides was assessed by the use of the dye safranine O whose fluorescence increases when a negative membrane potential is present [20]. Relatively low concentrations of the dye (about 1 $\mu$M) were used so as to avoid possible quenching artifacts. DMPC LUV were made as described above in the presence of 20 mM Mops, 125 mM KCl (pH 7.3). Vesicles were then passed through a 30 × 1 cm Sephadex G-75 column equilibrated with 20 mM Mops, 149 mM NaCl and 1 mM KCl (pH 7.3), thus creating a large negative $K^+$ gradient across the membrane. In one experiment, safranine O was added at a final concentration of 1.4 $\mu$M to 850 $\mu$l vesicles (3.5 $\mu$mol lipid) and its basal fluorescence monitored at 581 nm with 522 nm excitation [20]. Valinomycin was then added to a final concentration of 8.5 $\mu$M (using a 1 mg/ml stock solution in dimethylsulphoxide), and the increase in the dye fluorescence caused by the creation of a membrane potential due to valinomycin-mediated transport of $K^+$ was measured as a function of time. The experiment was then repeated with either 30 $\mu$M insulin, insulin B or 0.69 $\mu$M melittin added prior to the addition of valinomycin. Any leak of $K^+$ due to membrane perturbation caused by the hormone's presence would be revealed as a decrease in the extent of the safranine O fluorescence enhancement upon valinomycin addition.

Membrane fusion was tested using the procedure employed by Ellens et al. [19]. Two sets of DMPC LUV were prepared, one containing the fluorophore ANTS (25 mM ANTS, 10 mM Mops, 89 mM NaCl (pH 7.4)) and the other containing the quencher DPX (90 mM DPX, 10 mM Mops, 47 mM NaCl (pH 7.4)). Equal amounts (1.75 $\mu$mol) of these two LUV were mixed in 850 $\mu$l of external buffer (10 mM Mops, 0.1 mM EDTA, 150 mM NaCl (pH 7.4)). Fluorescence was monitored as described in the ANTS/DPX leakage assay before the addition of either A23187 (20 nM) or the hormone (30 $\mu$M). $Ca^{2+}$ was subsequently added at a final concentration of 3.5 mM. The quenching of fluorescence at 545 nm (384 nm excitation) due to the intermixing of internal aqueous contents of the DPX and ANTS vesicles was taken as a measure of membrane fusion. Melittin, which induces membrane fusion was used as the positive control.

In all of the above tests for membrane integrity except in the safranine O experiments, the conventional $Ca^{2+}$ ionophore A23187 (about 20 nM) was employed as an effective model ionophore.

## Results

*Integrity of the vesicles in the presence of the hormonal peptides*

Several methods were employed to assess the structural integrity of the vesicles during the time course of the experiments in the presence of insulin and the A and B chains. The procedure used routinely in all our experiments is the one employed by Weissman and his colleagues [10] as modified recently by Smaal et al. [17] and by Sokolove and Kester [16]. It consisted of the addition of sufficient EDTA or EGTA to the test solution in the sample cell at the completion of the ionophore/hormone-mediated transport experiment so as to chelate all extravesicular $Ca^{2+}$. Any decrease in fluorescence of fura-2 in the egg PC MLV or absorbance of arsenazo III in the DMPC LUV (caused by the leaking of the trapped dye and formation of the dye-$Ca^{2+}$ complex *outside* the vesicles) was subtracted from the total spectral change and the net increment due to the hormone-mediated cation influx was used in the quantitative treatment of the transport data (see data shown later in Figs. 2 and 5). In all experiments, the decrease in signal due to dye leak was found to make only a rather small contribution (0–15%) to the total value.

The data obtained with CF-loaded vesicles indicate that addition of either insulin or insulin B had no significant effect on the rate of leakage from the vesicles (Fig. 1, panel A). This leak in LUV at 37°C was found to be less than 3.0% of the total encapsulated dye during a 15 min duration used for initial rate measurements. The corresponding value for the well-

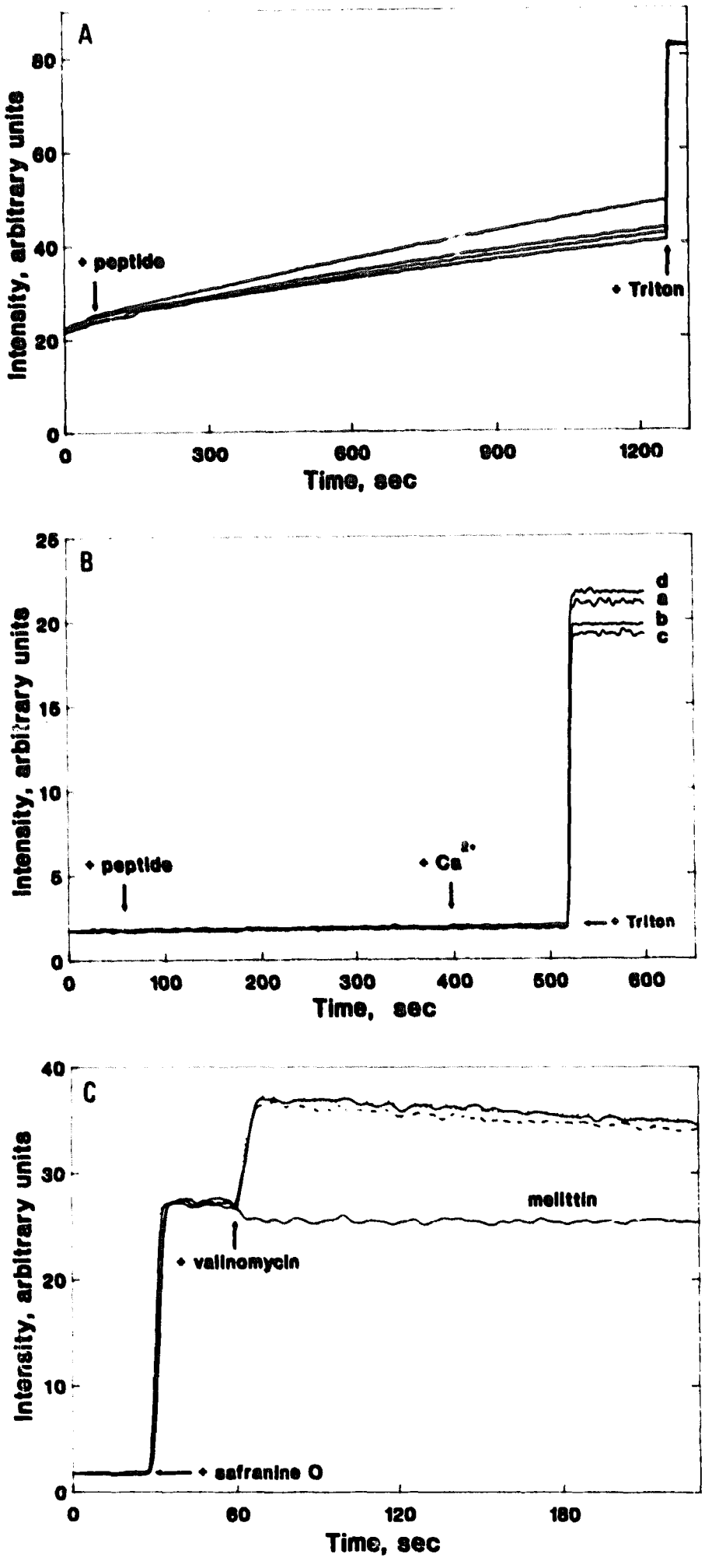


Fig. 1. Tests for leakage of solutes through LUV made of DMPC (lipid content: 3.5 $\mu$mol). (A) CF method: Vesicles contained CF (20 mM) in 20 mM Mops (pH 7.3) and 125 mM KCl. Fluorescence excitation: 470 nm, emission: 515 nm. 850 $\mu$l of LUV in the cuvette contained 3.5 mM $CaCl_2$ and, from bottom curve to the top: 30 $\mu$M of insulin A chain, insulin, insulin B chain or 20 nM of A23187. Peptide (or A23187) and Triton X-100 were added at the times indicated by the arrows. The control curve containing no peptide or ionophore but having 3.5 mM $Ca^{2+}$ is superimposable on that of oxidized insulin A. Fluorescence increase caused by adding 10 $\mu$l of 10% (v/v) Triton X-100 was taken to represent 100% leakage. (B) ANTS/DPX method: Vesicles contained 12.5 mM ANTS and 45 mM DPX in 10 mM Mops (pH 7.4) and 68 mM NaCl. Excitation: 384 nm and emission: 545 nm. Hormonal peptides, $Ca^{2+}$, and Triton X-100 were added at the times shown by the arrows: (a) insulin; (b) insulin B; (c) insulin A; and (d) A23187. The percent leak was computed as in (A). (C) Safranine O method: Vesicles containing 20 mM Mops (pH 7.3) and 125 mM KCl were made with a $K^+$ concentration gradient as described in Methods. LUV were pre-incubated without peptides (control (top solid curve)) or with 30 $\mu$M insulin (· · · · · ·), 30 $\mu$M insulin B (– – –) or 0.69 $\mu$M melittin (lower solid curve) for 10 min before the addition of safranine O (final concentration: 1.4 $\mu$M) whose fluorescence emission was monitored at 581 nm (excitation: 522 nm). Valinomycin (final concentration: 8.5 $\mu$M) was added to develop the membrane potential. Fluorescence signals in all cases were normalized to the initial level of dye fluorescence. Times of addition of compounds are indicated by the arrows.

known ionophore A23187 (Fig. 1, panel A) was 7%. In contrast, addition of compounds like lidoflazine caused significant leak (over 15%) of the trapped CF, possibly due to the highly hydrophobic character of the drug. The absence of significant leakage of vesicle contents was also verified by means of the ANTS/DPX assay (Fig. 1, panel B). Using the fluorescence signal caused by Triton X-100-induced membrane disruption as 100% leak it was found that insulin and its two chains caused a negligible increase in the steady-state leakage at 37°C (< 3%). This was also the case for A23187.

Experiments were also performed to determine if the hormone or its individual polypeptide chains perturbed or disrupted the lipid bilayer of the vesicles sufficiently to allow the non-specific movement of solutes, particularly small ones such as $Ca^{2+}$, down their concentration gradients. Our experiments on the effect of insulin and insulin B on the membrane potential, monitored by the use of the potential-sensitive fluorophore safranine O, show that these peptides do not significantly enhance the membrane's permeability to $K^+$ which has an ionic radius comparable to $Ca^{2+}$ (Fig. 1, panel C).

Membrane fusion was also found to be an improbable event as addition of either of the hormones or the conventional ionophore had little effect on membrane fusion as measured by the ANTS/DPX assay; no quenching of the fluorescence of ANTS by DPX was observed on addition of these compounds with or without $Ca^{2+}$ (data not shown). Membrane fusion is generally observed in the case of unsaturated diacyl phosphatidylethanolamines and acidic phospholipids such as cardiolipin which are capable of undergoing transitions from bilayer to non-bilayer phases rather than in neutral lipids such as DMPC [21].

*Cation transport studies*

The fura-2 and arsenazo III-based methods were employed for studying the transport of $Ca^{2+}$ by insulin and its A and B chains. In both cases, the initial rates of cation transport were estimated by measuring the slopes of the initial linear portions of the spectral change versus time curves [12]. The stoichiometry of the $Ca^{2+}$ complex was obtained in each case from the slope of the plot of log (initial rate) versus log (ionophore concentration) [12,22]. The data on the calcium transport by A23187 yielded a value of 1.8:1 (ionophore/$Ca^{2+}$) for the stoichiometry of the calcium complex of the ionophore using fura-2 loaded vesicles, and a value of 1.95:1 using the arsenazo-loaded vesicles (data not shown). Calcium transport across the PC MLV by ionomycin was also studied using the fura-2 method, yielding a 1.1:1 (ionophore/$Ca^{2+}$) stoichiometry. These are close to those reported by others [12,23] thus indicating the suitability of the lipid systems chosen for ionophore-mediated cation transport studies.

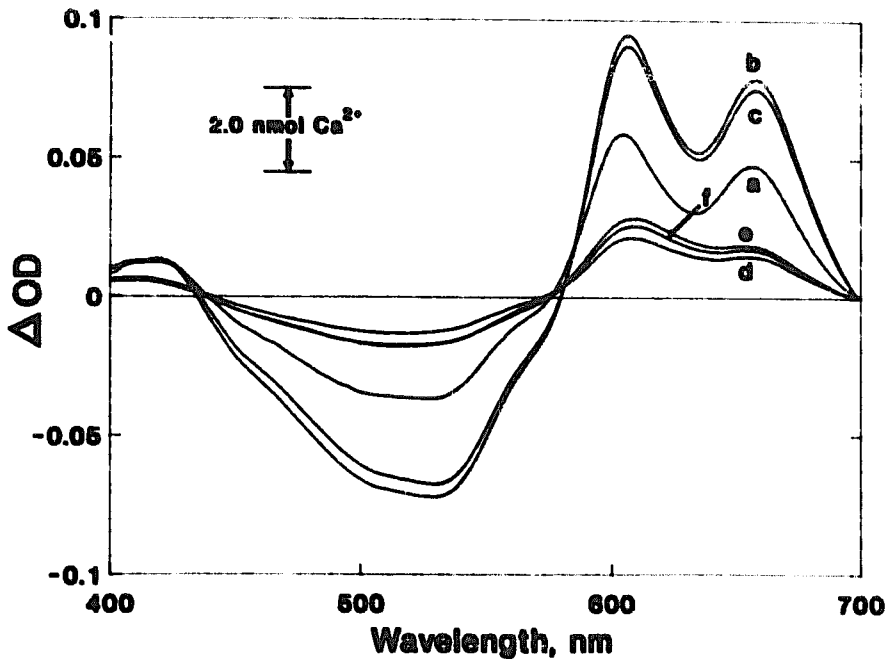


Fig. 2. $Ca^{2+}$ transport measured by the arsenazo III method: Sample cuvette had DMPC LUV (3.5 $\mu$mol lipid) containing 3.6 mM arsenazo III, 20 mM Mops (pH 7.3) and 125 mM KCl in the presence of 3.5 mM external $CaCl_2$, and, 60 $\mu$M insulin, or insulin B. Reference cuvette had the same solution but without the peptide. Temperature: 37 °C. Difference spectra shown are: (a) insulin B, 8 min; (b) insulin B, 15 min; (c) insulin B + EDTA (final concentration: 5 mM), 15 min; (d) insulin, 8 min; (e) insulin, 15 min; and (f) insulin + EDTA, 15 min. Conversion of absorbance to $Ca^{2+}_f$ is described under Methods.

*$Ca^{2+}$ and $Mg^{2+}$ translocation in DMPC vesicles*

Fig. 2 shows a typical experiment with insulin and insulin B in arsenazo III-loaded vesicles at 37°C. In these vesicles, insulin A did not cause an appreciable increase in arsenazo III absorbance indicating negligible transport of $Ca^{2+}$ from the external buffer medium to the intravesicular region (data not shown). Using insulin and its B chain, on the other hand, substantial changes in dye absorbance in the 650–700 nm range took place. The extent of leakage of the dye to the external buffer during the course of the $Ca^{2+}$ transport experiment was found to be relatively small as shown by the data obtained in the presence of EDTA in the external buffer medium (Fig. 2, curves c and f). The spectral change corrected for dye leak and corresponding to the movement of $Ca^{2+}$ across the lipid membrane was linear with time for 5–15 min. A plot of the log of $Ca^{2+}$ transport versus the log of insulin B concentration yielded a slope of 1.0, indicating a first-order interaction between $Ca^{2+}$ and insulin B (Fig. 3). Such an analysis of the insulin-mediated transport data was rendered difficult by the saturating effect found at higher concentrations of insulin. Similar transport rates were seen with Zn-free insulin as with normal insulin (results not shown). Experiments with insulin and insulin B were also conducted at 10°C to assess the effect of temperature on $Ca^{2+}$ transport. There was no observable transport at this temperature.

Experiments were also carried out on arsenazo III-loaded vesicles to measure the ability of insulin and its B chain to transport $Mg^{2+}$ across the lipid membrane.

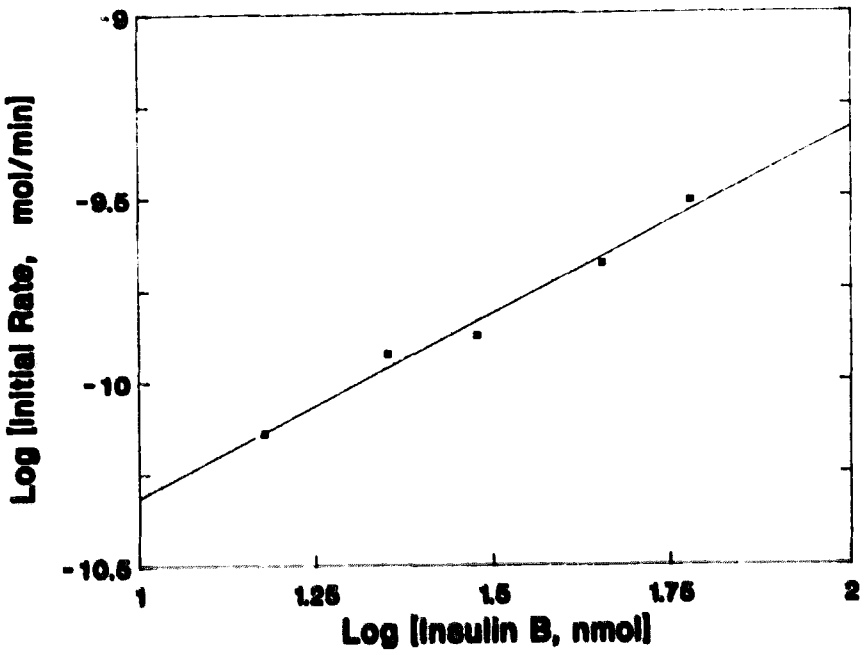


Fig. 3. Log (initial rate) plotted against log (insulin B concentration) for insulin B-mediated $Ca^{2+}$ influx into arsenazo III-loaded DMPC LUV. The rate of $Ca^{2+}$ is expressed in mol/min and insulin B concentration in nmol. The stoichiometry of the transporting species was calculated from the slope to be 1:1 $Ca^{2+}$/peptide.

Transport experiments with $Mg^{2+}$ under identical conditions to the $Ca^{2+}$ experiments revealed that insulin B transported $Mg^{2+}$ at a similar rate to $Ca^{2+}$ (300 pmol/min). On the other hand, insulin transported $Mg^{2+}$ at a higher rate (300 pmol/min) than $Ca^{2+}$ (110 pmol/min). The $Mg^{2+}$ transport data for insulin and insulin B are shown in Fig. 4.

*$Ca^{2+}$ transport in egg PC vesicles*

Fig. 5 shows a typical set of data obtained for $Ca^{2+}$ transport at 37°C in the presence of either 30 $\mu$M insulin or 20 nM A23187 as monitored by fluorescence

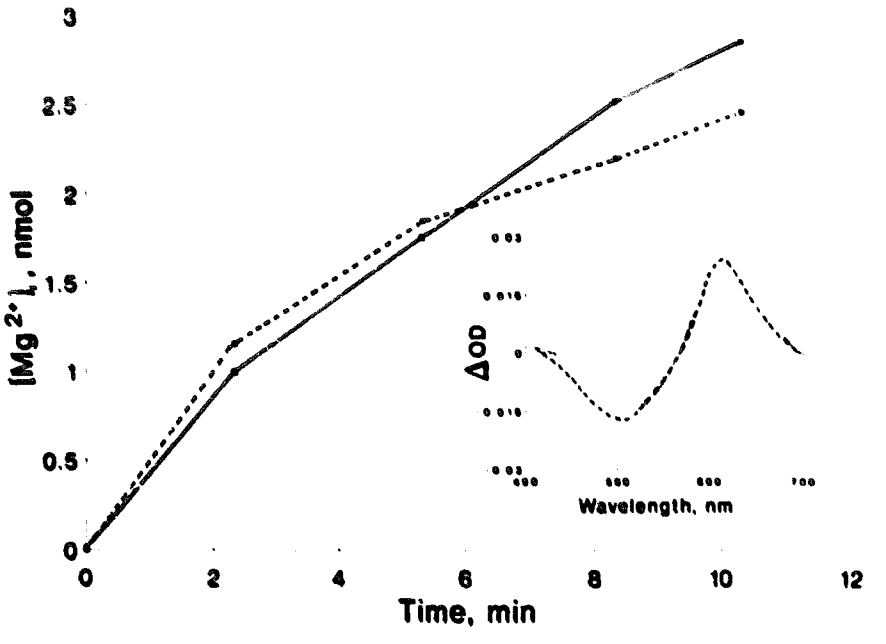


Fig. 4. $Mg^{2+}$ transport measured by the arsenazo III method. DMPC LUV containing 3.6 mM arsenazo III in 20 mM Mops (pH 7.3) and 125 mM KCl were treated in the presence of 3.5 mM external $MgCl_2$ with 30 $\mu$M of either insulin or insulin B at 37°C. The change in intravesicular $Mg^{2+}$ concentration (calculated as described in Methods) as a function of time is shown for insulin (···) and insulin B (solid line). (Inset) Difference absorbance spectra taken after 10 min with insulin (···) and insulin B (solid line).

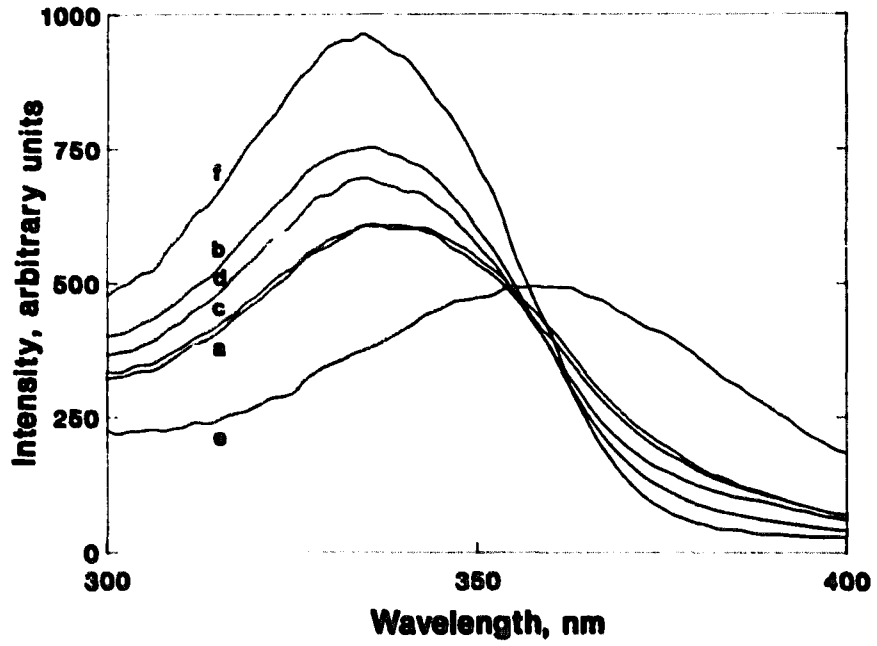


Fig. 5. Insulin-mediated $Ca^{2+}$ transport monitored by the fura-2 method using egg PC MLV at 20°C containing 100 $\mu$M fura-2 in 10 mM Hepes (pH 7.2) and 145 mM KCl. External $Ca^{2+}$: 2.0 mM; fluorescence emission wavelength: 510 nm. Excitation spectra shown correspond to: (a) 30 $\mu$M insulin, 10 min; (b) 10 nM A23187, 10 min; (c) 30 $\mu$M insulin + EGTA (final concentration: 5 mM), 10 min; (d) 10 nM A23187 + EGTA, 10 min; (e) $Ca^{2+}$-free fura-2 (used as $R_{min}$) and (f) $Ca^{2+}$-bound fura-2 (used as $R_{max}$).

spectral changes in intravesicular fura-2. It is clear that, as with the arsenazo III experiments, the hormone induces $Ca^{2+}$ influx that results in the time-dependent formation of fura-2-$Ca^{2+}$ complex inside the vesicle. The extent of dye leakage during the course of the $Ca^{2+}$ transport experiment was found to be small as shown by the data obtained in the presence of EGTA in the external buffer medium (Fig. 5). The spectra corresponding to the $Ca^{2+}$-free and $Ca^{2+}$-bound fura-2 species are also shown in Fig. 5 for comparison.

Fig. 6 shows a typical time course experiment of peptide-mediated $Ca^{2+}$ transport in the egg PC liposomal system using identical concentrations of insulin,

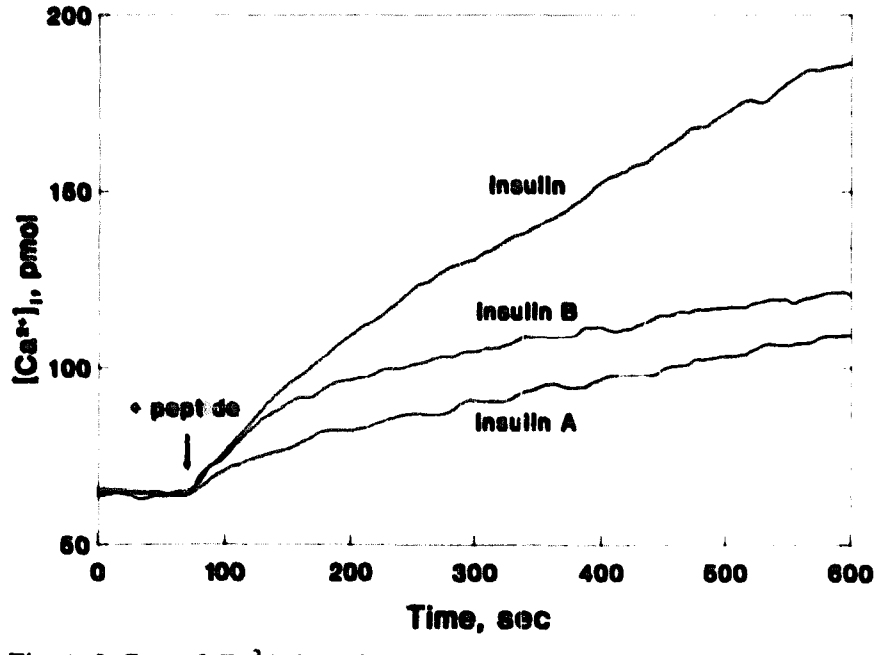


Fig. 6. Influx of $Ca^{2+}$ into fura-2-loaded MLV of egg PC induced by 30 $\mu$M insulin, insulin A, or insulin B at 20°C as a function of time. External $Ca^{2+}$ concentration was 2.0 mM. Calculation of $Ca_i^{2+}$ is described under Methods.

insulin A, and insulin B. At equivalent concentrations it is apparent that insulin A is the least effective ionophore.

## Discussion

There is much evidence in the literature for the involvement of $Ca^{2+}$ in hormone-mediated signal transduction [24]. In the specific case of insulin, several studies point to the importance of extracellular $Ca^{2+}$ in insulin-elicited cellular responses [1–6], although the nature and role of any *intra*cellular levels messenger(s) for insulin-mediated events are not yet clear [25,26]. A role for extracellular $Ca^{2+}$ at the level of the interaction of insulin with its plasma membrane-bound receptor is a possibility that had been suggested quite a while ago [27,28]. This was pursued by Williams and co-workers [7] who, by means of $Tb^{3+}$ displacement experiments, showed that the insulin receptor has a $Ca^{2+}$-binding domain which may be involved in the binding of the hormone. They also found that $Mg^{2+}$ can compete for the $Tb^{3+}$ bound to the receptor though less efficiently than $Ca^{2+}$. Interestingly, along side these observations on the receptor, spectroscopic [29], including X-ray diffraction [6], studies from the laboratory of Dunn and his collegues have demonstrated that insulin also contains a $Ca^{2+}$-binding site aside from the well-known $Zn^{2+}$-binding site.

In this study, we have examined the interaction of insulin and its constituent polypeptide chains, A and B, with $Ca^{2+}$ (as well as $Mg^{2+}$) at the water/lipid interface of the lipid bilayer using model liposomal systems. We have assessed the ability of these peptides to translocate the cations from the exterior aqueous phase across the nonpolar lipid bilayer, where these ions are normally insoluble, into the interior aqueous phase of the lipid vesicles. Such a translocation implies that the test peptide binds the cation of interest in the aqueous phase as well as in the lipid phase, the binding in the latter solvent being stronger than the former so as to effect cation transport along a concentration gradient. The data presented in this study demonstrate that insulin and its B-chain exhibit such binding characteristics with respect to $Ca^{2+}$ and $Mg^{2+}$. While a large number of cation transport studies have been carried out with many cyclic peptides and non-peptidic organic compounds, very few studies have examined the ionophoretic properties of peptide hormones [30,31].

Since it is possible that $Ca^{2+}$ influx may be caused by peptide hormones by mechanisms other than ionophore-mediated transport, we have, in this study, carried out auxilliary experiments to test this possibility. First, we have studied the ion transport in two types of lipid systems, one containing a pure lipid (DMPC) and another (egg PC) containing a mixture of lipids, each involving a different monitoring method for cation flux. Both the arsenazo III and fura-2-based methods have been well-documented [10,12,16,17] and were tested for their effectiveness in our hands by carrying out detailed experiments on the well-known ionophores A23187 and ionomycin. The values obtained from these experiments for the stoichiometries of the ionophore-$Ca^{2+}$ complexes were in close agreement with those reported in the literature. (We have earlier carried out qualitative experiments on insulin using $Pr^{3+}$ as a $Ca^{2+}$ mimic and following the coalescence of the proton NMR resonances of the lipid head group as an index of cation transport in a manner reported for iononophoretic synthetic peptides [32]. These results (Taylor, L. and Ananthanarayanan, V.S., unpublished data) also showed significant cation transport by insulin and its B but not A chain). Next, we have assessed the peptide-induced leak of the cations using three different methods as described in the previous sections and found this to be far less in magnitude compared to the peptide-mediated ion transport. Our results show that insulin and the B chain of insulin possess significant $Ca^{2+}$ and $Mg^{2+}$ ionophoretic character. This suggests that these peptides bind to the cations stronger in a lipid environment than in water. The A chain of the hormone shows a rather weak ion-transport ability in our liposomal systems. We find that at higher concentrations ($> 30\ \mu M$), the B chain is a more effective transporter of $Ca^{2+}$ than the intact hormone. This is analogous to the observation made by Grasso et al. on human folicle-stimulating hormone (hFSH) where the fragments 1–15 and 51–65 of the $\beta$-subunit of the hormone were 7-times more potent than the full hormone in causing $Ca^{2+}$ flux in model liposomes made of a mixture of pure lipids and cholesterol [31]. Putative $Ca^{2+}$-binding motifs have been identified in the above hFSH fragments [31]. Interestingly, the $Ca^{2+}$ binding domain in insulin has also been found to reside in the B chain [6]. An estimate of the stoichiometry of the $Ca^{2+}$ complex of the B chain from the transport data suggests an 1 : 1 complex formation (Fig. 3). It would thus appear that the ion translocating ability of insulin is derived from that of its B chain. Studies by others have indicated that the B chain, particularly the residues at its carboxyl end, may be involved in interaction with insulin receptor [33,34]. The fact that $Ca^{2+}$ transport by insulin and the B chain was negligible below the liquid-crystalline-to-gel phase transition temperature of DMPC ($\approx 21°C$) suggests that these peptides act by a carrier-mediated mechanism rather than by forming ion channels as reported for the hFSH peptides [31].

It may be noted that the minimum concentration needed to discern the ionophoretic ability of insulin and its B chain in our systems is about 1 $\mu$M. While this value is dictated by the sensitivity of the detecting system employed, it is lower than that used to demon-

strate the ionophoretic nature of the nonpeptide ionophore chlortetracycline [35] but is close to that used in studies on $Ca^{2+}$ transport by cyclic octapeptides [36] and hFSH [31] and $Mn^{2+}$ transport by angiotensin II [30]. While these comparisons serve to establish insulin and its B chain among moderately potent $Ca^{2+}$ and $Mg^{2+}$ ionophores, the concentration range of the hormone used in our study with model liposomes is still orders of magnitude higher than the physiological concentration of the hormone. This may preclude any direct extrapolation of our data to $Ca^{2+}$ flux, if any, mediated by insulin in vivo. However, it is possible that the ability of the hormone to interact at the lipid/water interface with bound $Ca^{2+}$ which is manifested in its ionophoretic character and demonstrated in this study may have biological significance at the level of the interaction of insulin with its receptor. For example, the $Ca^{2+}$-bound hormone rather than the free hormone may be the species that actually interacts with the receptor with the cation serving the role of a cofactor that links the $Ca^{2+}$ binding sites in the hormone and the receptor [37]. Whether this interaction would happen in or near the membrane is, however, not clear at present since insulin binding is presumed to occur in the extracellular $\alpha$-subunit of the receptor [38]. An interesting analogy is seen in the interaction of the RGD ligands with the integrin receptors. The $Ca^{2+}$ or $Mg^{2+}$ ion required for this interaction has been suggested to link the RGD motifs of the ligand with the two large extracellular parts of the receptor [39]. Interestingly, we have found several RGD peptides to be capable of translocating the above cations in our DMPC liposome system (Ananthanarayanan, V.S. and Pathinathan, B., to be published). It may be noted here that Williams et al. [7] have suggested a hydrophobic binding pocket for $Ca^{2+}$ in the insulin receptor and Yip et al. [38] have observed a binding site for insulin in the receptor that is normally inaccessible to an antibody raised against the peptide segment in this site. As $Ca^{2+}$ binding would enhance the amphiphilicity of the insulin molecule as in other ionophores and proteins [37,40], we might, in this context, conceive a necessity for the penetration of the $Ca^{2+}$-bound part of the insulin molecule (as happens when the hormone translocates $Ca^{2+}$ in the absence of the receptor in our model system) so as to interact at the receptor's hydrophobic hormone binding site. It is also plausible that the $Ca^{2+}$-binding site of the insulin receptor is the site of interaction of the $Ca^{2+}$-bound part of insulin and this is allosterically linked to the binding of the rest of the insulin molecule to the receptor. This would be in line with the suggestions made by Williams et al. [7]. Studies using liposomes containing reconstituted insulin receptor may prove useful for a further analysis of these possibilities.

The involvement of $Ca^{2+}$ in insulin–receptor interaction as envisaged above would provide a rationale for the observed requirement for extracellular $Ca^{2+}$ for insulin action. We are currently studying the specific details of the insulin–receptor interaction using human insulin receptor expressed in the frog oocyte in vivo system and examining the effects of insulin and specific insulin B fragments in the absence and presence of extracellular $Ca^{2+}$ and $Mg^{2+}$ (Vassilakos, A., Andrews, D. and Ananthanarayanan, V.S., to be published). It is of interest to note the need for extracellular $Ca^{2+}$ in the action of other polypeptide hormones such as glucagon [41,42] and cholecystokinin [43] as well as insulin-like growth factor [44] which shares structural and functional characteristics with insulin [45].

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