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Simulatory effect of porcine insulin on noradrenaline secretion in guinea-pig ileum myenteric nerve terminals

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1 The effect of insulin on the release of noradrenaline (NA) from nerve terminals was investigated in isolated ileal synaptosomes of guinea-pig. Release was determined as the amount of NA, quantified by h.p.l.c.-electrochemical detection, from samples incubated with insulin minus that in parallel blanks treated with some volume of vehicle.

2 Porcine insulin stimulated the secretion of NA in a concentration-dependent manner from 0.01 i.u. ml^{-1}, while the value of lactate dehydrogenase in the incubated medium was not influenced by insulin.

3 The presence of insulin receptors in this preparation was illustrated by immunoblotting with insulin receptor monoclonal antibodies.

4 The release of NA by insulin was reduced by guanethidine and bretylium and it was markedly lowered in the samples obtained from guinea-pigs that had received an intraperitoneal injection of DSP-4, the noradrenergic neurotoxin.

5 Tetrodotoxin attenuated the action of insulin at concentrations sufficient to block sodium channels. The depolarizing effect of insulin on the membrane potential was also illustrated by a concentration-dependent increase in the fluorescence of bisoxonol, a potential-sensitive dye.

6 The action of insulin was attenuated by removal of calcium chloride from the bathing medium. The induction of calcium ion influx by insulin into the synaptosomes is supported by the inhibitory effects of the calcium channel blockers ω-conotoxin GVIA (for the N-type channels) and nifedipine (for the L-type channels).

7 These findings suggest that insulin can stimulate NA release from noradrenergic terminals via activation of calcium influx.

Keywords: Insulin; noradrenaline release; bisoxonol; calcium influx; synaptosomal preparation of guinea-pig ileum

Introduction

The association between hypertension and hyperinsulinaemia/insulin resistance is well established in both human essential hypertension (Swislocki, 1990) and in spontaneously hypertensive rats (Mondon & Reaven, 1988). There is no question that insulin produces marked sympathetic activation (Anderson & Mark, 1993). Hyperinsulinaemia increases both plasma noradrenaline (NA) and muscle sympathetic nerve activity in man in the absence of hypoglycaemia (Anderson *et al*., 1991; Berne *et al*., 1992). Insulin-induced elevation of plasma NA has been observed in rats (Edwards & Tipton, 1989; Huang *et al*., 1992). Interestingly, there are marked regional differences in this sympathetic activation by insulin (Anderson & Mark, 1993). The effect of insulin on the noradrenergic neurotransmission of the gut is largely unknown. Since dysfunction of the gut is one of the common symptoms in diabetic patients, probably due to neuropathies of the autonomic nervous system (Hosking *et al*., 1978), we examined the direct effect of insulin on NA secretion in synaptosomal preparations from myenteric plexus of guinea-pig ileum.

Methods

Preparation of ileal synaptosomes

Guinea-pigs of either sex, weighing 360–430 g, were stunned by a blow to the head and bled. The segment midway between stomach and ileo-caecal junction (20–25 cm) was dissected from the mesentery and then removed from the animal. A crude synaptosomal fraction was prepared according to our previously described method (Chang & Cheng, 1993). In brief, the minced tissues of the longitudinal muscle strips, prepared as described previously (Paton & Zar, 1968), were homogenized in 0.32 M sucrose-phosphate buffer at 5 ml g^{-1} wet wt. tissue. After being centrifuged twice at 1,000 *g* for 10 min, the two supernatants were pooled and centrifuged at 17,000 *g* for 20 min to give a pellet. Synaptosomes were obtained from a discontinuous sucrose-metrizamide gradient by centrifugation at 20,000 *g* for 20 min as described previously (Briggs & Cooper, 1981). All procedures were carried out under 4°C.

Experimental protocols

Aliquots of the synaptosomal preparation (about 1.8 mg protein) were washed and then suspended in 1 ml of oxygenated Tyrode solution with or without 10 μM xylamine and 0.1 mM pyrogallol. Release was initiated by incubating the preparations with insulin at the desired concentration in a continuous shaking water bath (65 strokes min^{-1}) at 37 ± 1°C for 20 min, the time required to induce maximal response, derived from preliminary experiments. The reaction was terminated by chilling the tubes in an ice bath. Following centrifugation of the tubes at 5000 *g* for 10 min, supernatants were collected for determination of NA. Release was calculated as the amount of NA from samples incubated with insulin minus the parallel blank treated with the same volume of vehicle. Treatments with blockers or other agents were started 30 min or more before incubation with insulin. Samples treated with the same volume of vehicle in parallel were taken as control. Incubation in the absence of calcium chloride was carried out in calcium-free Tyrode solution without or with 1 mM EDTA.

In order to deplete NA in the myenteric plexus, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) was employed as a noradrenergic neurotoxin (Jaim-Etcheverry &

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Zieher, 1980). NA in whole ileal strips obtained from guinea-pigs that had received an intraperitoneal injection of DSP-4 (50 mg kg^{-1}) 24 h before death was extracted with 4 volumes of 0.4 M perchloric acid (Cheng *et al*, 1990). The content of NA was quantified and compared with that from the vehicle-injected control.

Measurement of released noradrenaline (NA)

The concentration of NA in the supernatants was estimated by high performance liquid chromatography (h.p.l.c.) with an electrochemical detector (BAS 200) according to our previous study (Cheng *et al*., 1990). Samples spiked with 20 ng of dihydroxybenzylamine (DHBA), the internal standard, were adsorbed onto activated alumina by continuous shaking for 30 min. The alumina was then washed three times with 1 ml of distilled water. The catechols were eluted by 0.1 M perchloric acid by shaking for 10 min. Then, they were lyophilized and dissolved in 0.03 ml of 0.1 M perchloric acid for injection into the h.p.l.c. through an autoinjector. All values were corrected for recovery (76–81%) and expressed as pmol mg^{-1} synaptosomal protein determined as previously described (Lowry *et al*., 1951). Also, incubation of standard NA with the compounds tested in this study showed that they did not interfere with the measurement of NA.

Determination of lactate dehydrogenase (LDH) activity

Activity of lactate dehydrogenase (LDH), the enzyme located in cytosol, was determined with a commercial kit (Besteck Biotech, U.S.A.). As described previously (Mercer, 1978), the supernatant obtained (0.02 ml) was mixed with 1 ml of working solution containing 0.35 mM NADH and 0.63 mM sodium pyruvate in 0.1 M phosphate buffer (pH 7.6). After incubation at 37°C for 1 min, kinetic measurement at 340 nm for 2 min was carried out in duplicate with an u.v. spectrophotometer (Hitachi U-3210, Tokyo, Japan).

Immunoblotting of insulin receptors

The presence of insulin receptors (IR) was assessed by immunoblotting with monoclonal antibodies (White & Khan, 1986). The synaptosomal preparations obtained were lysed in buffer containing 1% Triton X-100. Discontinuous slab gels (1.0 mm thickness) containing 0.1% SDS were prepared according to Laemmli (1970) with acrylamide concentrations of 12% in the separation gel and 5% in the stacking gel. Protein samples were fractionated by gel electrophoresis run at 40 and 100 V under 4°C during the stacking and separation steps, respectively. The separated proteins were blotted onto nitrocellulose as described previously (Towbin *et al*., 1979). After treatment with anti-IR antibodies (10 μg ml^{-1}), immunostaining was performed for peroxidase activity by incubation in Tris-buffer (10 mM) by use of the enhanced chemiluminescence (ECL) development system (Amersham International plc, U.K.). This antibody, purchased from Oncogene Science, Inc. (Uniondale, N.Y., U.S.A.), is specific to the insulin receptor (Ab-3) but does not bind directly with insulin and has no reactivity with insulin-dependent protein-tyrosine kinase activity. In a preliminary study, it was found to react with insulin receptors in the liver of guinea-pigs. Identification of this response was observed at 135 Kds for the α-subunit of IR. The Western immunoblots obtained were then quantified densitometrically with a laser densitometer.

The NA released in response to insulin (1 i.u. ml^{-1}) was studied in synaptosomal preparations incubated with anti-IR antibodies (10 μg ml^{-1}).

Determination of synaptosomal membrane potential variations

The lipophylic anion bisoxonol, which has been used to monitor membrane potential changes in several cell types (Lakos *et al*, 1990; Regazzi *et al*., 1990), was added at 300 nM to the quartz cuvette of the spectrofluorimeter containing 2 ml of the prewarmed medium. One min after adding bisoxonal, synaptosomal proteins (0.3–0.5 mg) were pipetted into the cuvette. Insulin at the desired concentration was then added into the cuvette during the stable state of fluorescence recorded in Hitachi F-2000 spectrophotometer; an excitation and emission wavelength of 485 and 515 nm was used, respectively (Lakos *et al*., 1990). Similar to a previous study (Taglialatela *et al*., 1990), bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values by use of the valinomycin null-point method (Rink *et al*., 1980). Data of intensity variation were expressed as the arbitrary unit of F/F_o, where F is the peak of the intensity increase in insulin and F_o is the basal fluorescence, as previously described (Rodriguez-Pascual *et al*., 1995). In preliminary experiments, insulin at the concentrations tested had no effect on the bisoxonol fluorescence in the absence of synaptosomal protein.

Drugs

Porcine insulin monocomponent for injection (Actrapid MC) was obtained from NOVO Industrias (Bagsvaerd, Denmark). Atropine sulphate, bovine serum albumin, 3,4-dihydroxybenzylamine hydrobromide, guanethidine sulphate, noradrenaline bitartrate and pyrogallol crystalline were purchased from Sigma (St. Louis, MO, U.S.A.). Bretylium tosylate, DSP-4 hydrochloride, nifedipine, ω-conotoxin GVIA, and xylamine hydrochloride were the products of Res. Biochem. Int. (RBI; Natick, MA, U.S.A.). The dye of bisoxonol was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). All chemicals used in the present study were of analytical grade. Drug solutions were prepared as stock solution and fresh dilutions were made up daily in Tyrode solution. Vehicle solutions were made in the same manner without the addition of the drug to be tested.

Statistical analysis

Values of mean $\pm$ s.e. for each group were obtained from number (N) of samples. Number (n) of experiments means the number of separate studies from different synaptosomal preparations. Statistical analysis of the differences between two mean values was assessed by Student's t test; a P value of 0.05 or less was considered significant. Where two or more of the means were compared to one control mean, analysis for significance ($P<0.05$) was carried out by use of Dunnett's multiple comparisons test (Dunnett, 1964).

Results

Effect of insulin on the release of NA from ileal synaptosomes

Incubation of ileal synaptosomes with porcine insulin induced an elevation of NA release in a time-related manner; the release of NA reached a plateau within 18 min of treatment in 8 preliminary experiments. In all subsequent experiments, insulin was incubated for 20 min. The release of NA was increased in a concentration-dependent manner by insulin (Figure 1). In order to obtain substantial NA release, at least 0.5 i.u. ml^{-1} of insulin was used in the following experiments. The effect of insulin was compared with that of velosulin U100 (Nordisk, Gentofte, Denmark), a similar product, and it showed about 10 times the activity of velosulin. In the presence of 10 μM xylamine and 0.1 mM pyrogallol, NA released by insulin (0.5 i.u. ml^{-1}) was 14.9 ± 2.8 pmol mg^{-1} synaptosomal protein ($n=8$); this was not different ($P>0.05$) from the value (14.4 ± 2.3 pmol mg^{-1} synaptosomal protein; $n=8$) obtained in the absence of xylamine, an agent that blocks the uptake of catecholamine into nerve terminals, and pyrogallol, an inhibitor of catecholamine metabolizing enzyme (COMT). Thus,

these two compounds were not added in subsequent experiments. Also, atropine at 1 μM did not modify the release of NA by insulin 0.5 i.u. ml^{-1}, release of NA was 13.6$\pm$2.6 pmol mg^{-1} synaptosomal protein (n=8) versus 14.6$\pm$3.1 pmol mg^{-1} synaptosomal protein in the vehicle-treated control (n=8).

Lactate dehydrogenase (LDH) activity was not influenced in samples incubated with insulin. The activity in samples incubated with 3 i.u. ml^{-1} insulin, the maximal concentration tested, was 0.15$\pm$0.09 u ml^{-1} (n=8), which was not different from the control (0.16$\pm$0.03 u ml^{-1}; n=8).

Presence of insulin receptors in synaptosomal preparations

The presence of insulin receptors (IR) in ileal synaptosomes was illustrated by immunoblotting with monoclonal antibodies. Figure 2 shows that the increase in IR-immunoprecipitant was parallel with the increase in incubated synaptosomal protein (from 25 mg to 75 mg) yielding a positive correlation (r=0.934) between the elevation of density and the increase of synaptosomal protein. Moreover, in the presence of IR antibodies (10 μg ml^{-1}), the release of NA by insulin (1 i.u. ml^{-1}) was 7.2$\pm$1.7 pmol mg^{-1} synaptosomal protein (n=6), which was significantly lower (P<0.01) than in the plasma-treated control (22.4 $\pm$3.2 pmol mg^{-1} synaptosomal protein, n=6). However, spontaneous secretion of NA was not modified by this

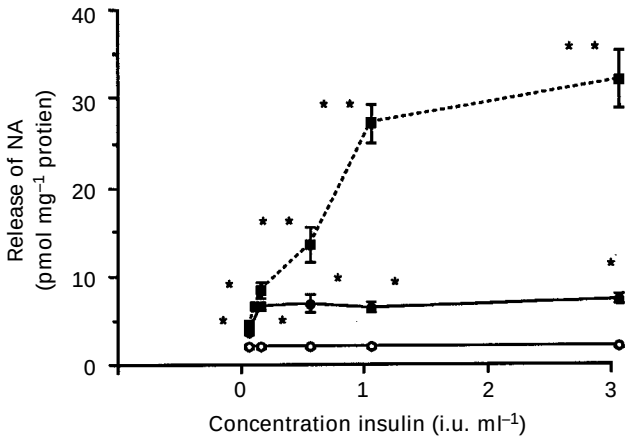

Figure 1 Concentration-response curves for the effect of insulin on noradrenaline (NA) release from isolated myenteric synaptosomes of guinea-pig. The NA release was measured in samples, incubated for 20 min with insulin at the indicated concentration; 0 indicates the spontaneous release of NA. Each point on the broken line (■) represents the mean of 8 separate experiments in normal medium; vertical lines show s.e. Samples treated in calcium chloride-free bathing medium with (○) or without (●) the addition of 1 mM EDTA are indicated by the solid lines and each point represents the mean of 6 experiments with vertical lines showing s.e. *P<0.05 and **P<0.01 versus corresponding value obtained in EDTA-containing calcium-free medium (○), by unpaired Student's t test.

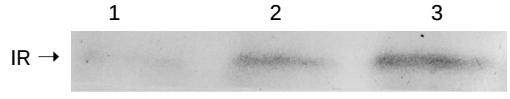

Figure 2 Identification of insulin receptors (IR) on isolated ileal synaptosomes of guinea-pig. The synaptosomal proteins at concentrations of 25 mg (lane 1), 50 mg (lane 2), and 75 mg (lane 3) were incubated with 10 μg ml^{-1} anti-IR monoclonal antibodies. The density from the Western blotting increased in parallel (r=0.93) with the amount of synaptosomal proteins (y=82.67+0.82x).

30 min pretreatment with IR antibodies (10 μg ml^{-1}) (3.9$\pm$1.3 pmol mg^{-1} synaptosomal protein, n=6, in antibody-treated synaptosomes versus 3.4$\pm$1.1 pmol mg^{-1} synaptosomal protein in the plasma-treated samples, n=6).

Effect of noradrenergic blockers on the action of insulin

Guanethidine produced a concentration-dependent inhibition of insulin-induced NA release (Table 1), as did bretylium (Table 1). Also, bretylium and guanethidine reduced the spontaneous release of NA (Table 1). Release of NA (pmol mg^{-1} synaptosomal protein) by insulin (1 i.u. ml^{-1}) was 3.7$\pm$0.5 (n=8) in samples incubated with 20 μM guanethidine and was 3.8$\pm$0.7 (n=8) in bretylium (0.1 mM)-treated group, which was not statistically different from the spontaneous release of NA (Table 1). Spontaneous release of NA (pmol mg^{-1} synaptosomal protein) was lowered by guanethidine (20 μM) to 1.8$\pm$0.2 (n=8) and to 1.9$\pm$0.3 (n=6) by bretylium (0.1 mM). The content of NA in whole ileal strips obtained from guinea-pigs that had received an intraperitoneal injection of DSP-4 (50 mg kg^{-1} of body weight) 24 h before the experiments was 22.1$\pm$2.4 pmol mg^{-1} protein of ileum (n=8) which was significantly (P<0.05) lower than that of vehicle-treated control (30.2$\pm$4.8 pmol mg^{-1} protein ileum; n=8). The release of NA by insulin from synaptosomal preparations in DSP-4 (50 mg kg^{-1} body weight) treated guinea-pigs was significantly decreased (Figure 3), while the vehicle of DSP-4 did not influence insulin-induced NA release. The amount of NA released by insulin (3 i.u. ml^{-1}) was about 61% of the endogenous NA contained in synaptosomes, prepared from vehicle-treated animals (50$\pm$7.3 pmol of NA mg^{-1} synaptosomal protein; n=8).

Effect of insulin on the membrane potential

The NA release by insulin was markedly reduced in samples pretreated (30 min) with tetrodotoxin (TTX); NA release (pmol mg^{-1} synaptosomal protein) by 0.5 i.u. ml^{-1} insulin was 8.2$\pm$2.3 (n=6) in samples treated with 1 μM TTX and 6.9$\pm$2.1 (n=6) in 2 μM TTX-treated synaptosomes, which was significantly (P<0.05) different from the release in vehicle-treated samples (14.6$\pm$3.2 pmol mg^{-1} synaptosomal protein, n=6). However, the NA release by insulin was only reduced but not totally abolished by TTX. Even at 3 μM, TTX did not produce a more marked inhibition; NA release (pmol mg^{-1} synaptosomal protein) by 0.5 i.u. ml^{-1} insulin was 7.2$\pm$1.5 (n=6), which was not statistically different from the value obtaining in 2 μM TTX-treated synaptosomes. The spontaneous release of NA was not influenced by TTX; it was 3.2$\pm$0.3 pmol mg^{-1} of synaptosomal protein (n=6) in 3 μM

Table 1 Effects of bretylium and guanethidine on insulin (1 i.u. ml^{-1})-induced release of noradrenaline (NA) from isolated ileal synaptosomes of guinea-pigs

Groups	*Spontaneous*	*Insulin*
Control	3.3$\pm$0.2 (n=8)	21.3$\pm$1.1 (n=8)
Bretylium		
5 μM	3.1$\pm$0.6 (n=6)	18.4$\pm$3.2 (n=8)
10 μM	2.9$\pm$0.4 (n=6)	14.7$\pm$2.8** (n=8)
50 μM	2.2$\pm$0.5* (n=6)	12.3$\pm$2.3*** (n=8)
Guanethidine		
1 μM	3.2$\pm$0.7 (n=6)	16.3$\pm$2.4* (n=8)
5 μM	2.3$\pm$0.5* (n=6)	13.8$\pm$2.1** (n=8)
10 μM	1.9$\pm$0.3** (n=6)	10.9$\pm$2.7*** (n=8)

All values shown are means$\pm$s.e. of NA secretion (pmol mg^{-1} protein) from n separate experiments. Insulin (1 i.u. ml^{-1}) was incubated for 20 min while bretylium or guanethidine were added 30 min before insulin. *P<0.05, **P<0.01 and ***P<0.001 versus corresponding control, respectively, via unpaired comparison by Dunnett's multiple comparisons test.

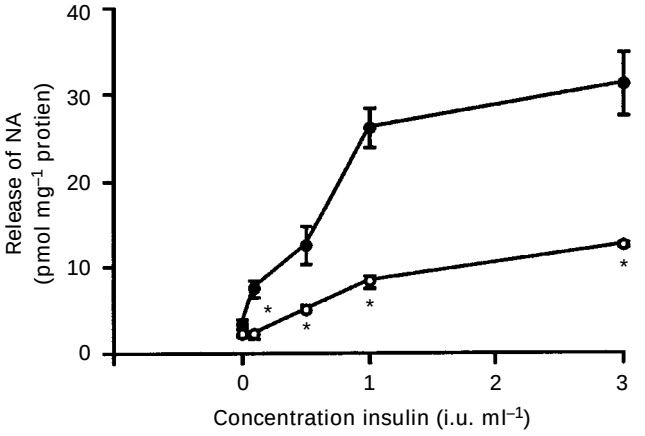

Figure 3 Concentration-response curve for insulin stimulation of noradrenaline (NA) release from isolated myenteric synaptosomes of guinea-pigs that had received a DSP-4 injection (○) or the same volume of vehicle (●). Each point represents the mean of 8 animals with vertical lines indicating s.e. Values obtained from animals that received an intraperitoneal injection of DSP-4 (50 mg kg^{-1}) 24 h before the experiments (○) are statistically significant from the values of vehicle-treated control (●) (*$P<0.05$; unpaired Student's t test).

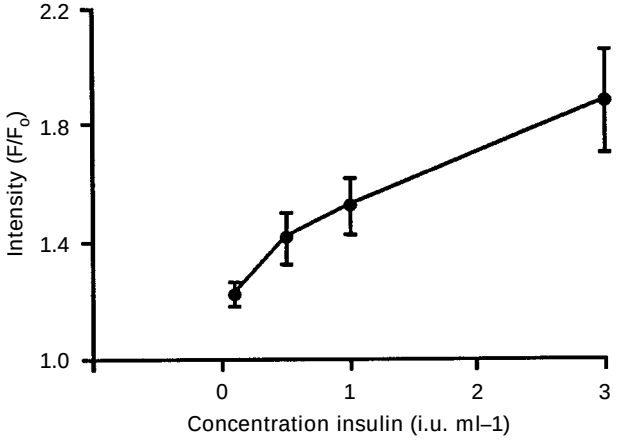

Figure 4 Effect of insulin on membrane potential in ileal synaptosomes of guinea-pig monitored by bisoxonol dye. Values are the arbitrary unit of F/F_o where F represents the peak of intensity induced by insulin and F_o the basal fluorescence. Each point represents the mean of 6 separate experiments with vertical lines indicating s.e.

TTX-treated synaptosomes versus 3.7 ± 0.4 pmol mg^{-1} synaptosomal protein ($n=6$) in the vehicle-treated samples. When synaptosomal preparations were exposed to insulin, a concentration-dependent depolarization, monitored as an increase in bisoxonol fluorescence, was obtained (Figure 4). Similar incubation with distilled water at the same volume did not depolarize the synaptosomes and no marked changes of basal fluorescence were observed.

Role of calcium ions in the action of insulin

In the absence of calcium ions (calcium chloride-free medium), both the spontaneous NA release and the action of insulin were lowered markedly as compared with that in the presence of calcium ions (Figure 1). When 1 mM EDTA was further added into the bathing medium, as shown in Figure 1, the action of insulin disappeared completely. Also nifedipine and ω-conotoxin GVIA lowered the insulin-induced release of NA in a concentration-dependent manner (Table 2). In samples treated with a combination of nifedipine (100 nM) and ω-conotoxin GVIA (80 mM), the NA release (pmol mg^{-1} synaptosomal protein) by 1 i.u. ml^{-1} insulin was 6.9 ± 1.1 ($n=8$) versus 22.2 ± 2.9 ($n=8$) in the vehicle-treated control. The value in the presence of nifedipine plus ω-conotoxin GVIA was significantly ($P<0.05$) different from the values shown in Table 2, after treatment with nifedipine (100 nM) or ω-conotoxin GVIA (80 nM) only. However, the spontaneous release of NA (3.4 ± 0.8 pmol mg^{-1} protein, $n=6$) was not affected by nifedipine or ω-conotoxin GVIA at the highest concentration used (0.1 μM); 3.1 ± 0.6 pmol mg^{-1} protein in nifedpine-treated samples ($n=6$) and 2.9 ± 0.7 pmol mg^{-1} protein in ω-conotoxin GVIA-incubated synaptosomes ($n=6$).

Table 2 Effects of calcium channel blockers on insulin-induced release of noradrenaline (NA) from isolated ileal synaptosomes of guinea-pigs

Group	*Insulin (0.5 i.u.)*	*Insulin (1 i.u.)*
Control	12.6 ± 2.2 ($n=6$)	22.6 ± 1.7 ($n=8$)
ω-Conotoxin GVIA		
20 nM	9.2 ± 1.6* ($n=6$)	18.3 ± 1.2* ($n=8$)
40 nM	7.6 ± 1.2** ($n=6$)	15.8 ± 0.9** ($n=8$)
80 nM	5.4 ± 0.8*** ($n=6$)	14.1 ± 1.4*** ($n=8$)
Nifedipine		
50 nM	11.8 ± 1.9 ($n=6$)	18.8 ± 1.7 ($n=8$)
100 nM	8.1 ± 1.3* ($n=6$)	15.7 ± 1.2* ($n=8$)
500 nM	6.2 ± 0.7** ($n=6$)	13.7 ± 0.5** ($n=8$)

All values shown are mean$\pm$s.e. of NA release (pmol mg^{-1} protein) from n separate experiments in different synaptosomal preparations incubated with insulin for 20 min. Each of the calcium channel blockers was incubated at the indicated concentration for 30 min before the addition of insulin. *$P<0.05$ and **$P<0.01$ versus corresponding control, respectively, via unpaired comparison by Dunnett's multiple comparisons test.

Discussion

In the present study, we found that porcine insulin stimulates the secretion of NA from noradrenergic terminals of guinea-pig ileum. Insulin stimulated NA release in a concentration-dependent manner from 0.01 i.u. ml^{-1}. At these concentrations, insulin did not affect the value of LDH, one of the cytosolic enzymes, suggesting a specific action rather than damage of the cell membrane. Hence, the involvement of insulin receptors (IR) seems likely. Indeed, immunoblotting with IR monoclonal antibodies illustrated the presence of IR in the synaptosomal preparations and the NA release induced by insulin was reduced by these antibodies.

Guanethidine attenuated the NA releasing action of insulin in a concentration-dependent manner and abolished it at a concentration sufficient to block noradrenergic nerve terminals (Starke, 1972). Similar results were obtained in samples treated with bretylium, another adrenergic neurone blocking agent (Hertting *et al.*, 1962). Another piece of evidence that insulin stimulates NA release from noradrenergic terminals is the observation that its effect was markedly reduced in synaptosomes prepared from guinea-pigs that received an injection of DSP-4, a useful tool for denervation of noradrenergic neurones (Jonsson *et al.*, 1981), with a significant lowering of endogenous NA in the ileum. Also, as atropine at a concentration sufficient to block cholinoceptors failed to modify the NA releasing action of insulin, the participation of acetylcholine seems unlikely. We did try to study the role of neuropeptide Y, a co-transmitter in noradrenergic nerve terminals,

but failed to identify it with a radioimmunoassay kit (data not shown). Specific release of NA by insulin can thus be considered to occur.

Moreover, TTX markedly decreased the insulin-stimulated NA release TTX is well-known as a specific blocker for sodium channels (Narahashi, 1974) and the TTX-sensitive release of neurotransmitter is usually due to the excitation of nervous cells (Ritchie & Rogart, 1977). Depolarization of the membrane potential by insulin was also observed in synaptosomes, as monitored with bisoxonol, a fluorescence dye (Figure 4). This dye is a lipophilic anion which freely permeates the cell, its distribution across the cell membrane being dependent upon membrane potential (Lakos *et al*., 1990). When the membrane potential is depolarized, it allows more of this negatively charged dye to enter the cells leading to an increase of fluorescence. Similar experiments have been performed in cerebral synaptosomes of the rat (Taglialatela *et al*., 1990). Thus, the concentration-dependent increase of bisoxonol fluorescence in ileal synaptosomes incubated with insulin indicates an induction of membrane depolarization. This finding is consistent with the action of TTX. However, the action of insulin was only partially reduced by TTX even at 3 μM. The involvement of a TTX-insensitive mechanism in this effect of insulin can thus not be ruled out. This might be related to the presence of TTX-resistant sodium channels (Rogart *et al*., 1989; Backx *et al*., 1992), although TTX-resistance does not necessarily imply TTX-insensitive sodium channels.

The increase of NA secretion by insulin was attenuated by the removal of calcium chloride from the bathing medium indicating its dependence on calcium ions. Moreover, ω-conotoxin GVIA suppressed this NA releasing action of insulin in a concentration-dependent manner within the range effective at blocking N-type calcium channels (Dooley *et al*., 1987). Also, nifedipine at concentrations sufficient to block L-type calcium channels (Nachshen & Blaustein, 1979) reduced this action of insulin (Table 2). Thus, one can assume that insulin causes influx of calcium ions through N-type and L-type calcium channels, as further illustrated by the additive effect of nifedipine and ω-conotoxin GIVA. Calcium influx via N-type and L-type calcium channels has been shown to be associated with neurotransmitter release (Miller, 1987). The action of insulin was attenuated by the addition of EDTA in the calcium chloride-free bathing medium (Figure 1). Nifedipine and ω-conotoxin GVIA, specific for voltage-dependent calcium channels (Dooley *et al*., 1987), did not modify the spontaneous release of NA and did not completely suppress the stimulating effect of insulin on NA release as was the case in the calcium-free solution with EDTA. This might suggest the involvement of passive influx of calcium ions through a voltage-independent mechanism. However, it is not easy to rule out a nonspecific action of EDTA because this chelator may bind metals at random.

The effective concentrations of insulin obtained in the present study are higher than those found in plasma (20–30 μu ml^{-1}) by Suzuki *et al*. (1976), although concentrations up to 4 i.u. ml^{-1} insulin have been used to stimulate catecholamine release in hypothalamic slices (Sauter *et al*., 1983). One possible explanation is that porcine insulin was employed to stimulate the receptors in guinea-pig ileum synaptosomes. The concentration of insulin required in the present study is higher than that for a physiological action and the effect thus reflects a pharmacological action, although a marked regional difference in sympathetic activation by insulin has been found previously (Anderson & Mark, 1993). An investigation into the subcellular signalling (Hunter, 1987) in insulin stimulated synaptosomes may be helpful in understanding the reason why a high concentration is essential for this action of insulin. Nevertheless, there is no doubt that insulin can be used to stimulate the release of NA from noradrenergic nerve terminals of guinea-pig ileum in *in vitro* studies.

In conclusion, our results suggest that high concentrations of insulin can stimulate NA secretion from noradrenergic nerve terminals in the guinea-pig ileum in a calcium-dependent manner.

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