The role of protein kinase C in the desensitization of rat pancreatic islets to cholinergic stimulation

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Abstract

It is well known that protein kinase C (PKC) plays an important role in mediating insulin secretion in response to cholinergic stimulation. In various cells PKC also mediates a desensitization process. The role of PKC for homologous desensitization of the insulin response to repetitive stimulation with the muscarinic agonist carbachol (CCh) was investigated in perfusion experiments using isolated rat pancreatic islets. Repetitive (six times) stimulation with CCh (100 μ M) reduced insulin secretion over time (up to 50% during the second challenge). This was not a toxic effect since the desensitizing effect was mostly washed out after 45 min. When PKC was downregulated by long term preincubation (20 h) with 200 nM phorbol 12-myristate 13-acetate (TPA), the initial stimulation of insulin release by CCh was reduced by 50%, and a desensitization by further CCh stimulation was no longer obvious. In contrast, when other compounds with different mechanisms of actions for inactivating PKC were used, i.e. PKC inhibitors such as staurosporin (100 nM), Ro 31-8220 (5 μ M) or PKC peptide(19–31), the insulin secretion in response to CCh was reduced but the desensitization was not abolished. When PKC was down-

regulated or inhibited by the above methods, the PKC activator phorbol 12-myristate 13-acetate (TPA; 200 nM) was no longer able to evoke an increase in insulin secretion during static incubation, i.e. these control experiments indicate a real PKC inhibition. When heparin (50 μ g/ml), an inhibitor of G-protein coupled receptor kinase (GRK), was used, the desensitization of the cholinergic stimulation of insulin release remained unchanged. The data indicate that PKC plays a role in CCh-mediated insulin secretion and also show a desensitization of this effect after repetitive stimulation with CCh. The data further indicate that specific PKC isoenzymes that are inhibited by staurosporin or Ro 31-8220 do not take part in the desensitization process, while isoenzymes that are downregulated by TPA are involved. It may be speculated that a hitherto unknown PKC isoenzyme that is downregulated by TPA but not by the other used PKC inhibitors is involved in the desensitization process, or that a nonspecific effect of TPA is involved. Members of the GRK family are not involved in the desensitization process of CCh.

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Introduction

Protein kinase C (PKC) belongs to the serine/threonine phosphorylating kinases. It is a family of at least 12 isoenzymes that interact intracellularly by phosphorylating specific target proteins. PKC is involved in signal transduction, cellular processes such as secretion (e.g. insulin secretion), in desensitization, in gene expression and cell differentiation.

In general, desensitization can be achieved at the receptor level, by a decrease of receptor coupling factors such as G-proteins, a decrease in second messengers (e.g. downstream phospholipase C (PLC)), by the reduced efficiency of second messengers, e.g. less mobilization by inositol 1,4,5-triphosphate (IP_3) of Ca^{2+} from an intracellular pool (McArdle *et al.* 1996) or acceleration of IP_3 receptor degradation (Wojcikiewicz *et al.* 1994). PKC is

involved in the homologous desensitization of δ -opioid receptors (Ueda *et al.* 1995) but not bombesin receptors (Frankel & Viallet 1993), histamine- and ATP receptor-mediated effects (Lin & Chuang 1993, Dickenson & Hill 1993). With respect to other receptors, e.g. angiotensin II receptors, inhibition of PKC resulted in desensitization distal from the receptor itself (Tang *et al.* 1995). The atrial natriuretic peptide (ANP) receptor is desensitized in a PKC-dependent and -independent way (Potter & Garbers 1994).

In general there appears no specific rule regarding the involvement of PKC. PKC is involved in the heterologous desensitization of carbachol (CCh) binding sites mediated by cholecystokinin (Doi *et al.* 1993). The underlying mechanism of a homologous muscarinic receptor desensitization is a change in CCh receptor affinity and its regulation by G-proteins (Hishinuma *et al.* 1993). It is

already known that chronic exposure of islets to the muscarinic agonist CCh suppresses insulin release to a subsequent glucose plus CCh challenge (Zawalich & Kelley 1995). However the mechanism has not been investigated further.

Several PKC isoenzymes are present in β -cells: α , β , δ , ϵ , ζ and ι . Involvement of PKC isoenzymes is shown in a number of ways. PKC inhibitors can be divided into three groups: inhibitors such as the indolecarbazoles (staurosporin) and bisindolylmaleinimide (Ro 31-8220) interact with the catalytic domain (Tamaoki *et al.* 1986); PKC peptide(19-31) with a pseudosubstrate sequence (same sequence as the pseudosubstrate region of PKC α) interacts with the PKC substrate binding site. In contrast to staurosporin and Ro 31-8220 which act on the C3 region of the catalytic domain of PKC close to the ATP binding site, the PKC pseudopeptide(19-31) binds to the C4 region of the catalytic domain. The phorbol ester (phorbol 12-myristate 13-acetate (TPA)) (Lee & Bell 1986) interacts with the regulatory domain of PKC. Long term activation of PKC by TPA leads to a proteolytic degradation and hence inactivation of PKC.

Different compounds have been used in our experiments since no specific PKC inhibitor is known. Using a number of approaches we investigated whether PKC is involved in homologous desensitization of rat pancreatic islets to CCh.

Materials and Methods

Animals

Adult Wistar rats of either sex weighing between 230 and 330 g were used. They were given a standard pellet diet (Altromin, Lage, Germany) and tap water and allowed to feed *ad libitum*. Animals were kept at 22 °C with a ratio of 12 h light:12 h darkness.

Chemicals

The following were purchased: soybean trypsin inhibitor (SBTI), bacitracin and Hepes (2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid) from Sigma Chemical Co. (St Louis, MO, USA); α -D-glucose was obtained from Serva Feinbiochemica (Heidelberg, Germany); bovine serum albumin (BSA) fraction V from Behringwerke AG (Marburg, Germany); and collagenase (CLS grade, 126-196 U/ml) from Worthington Biochemicals Corp. (Freehold, NJ, USA). Insulin was determined radioimmunologically using anti-insulin antibodies from Linco Research (St Louis, MO, USA), 125 I-insulin was a gift from Behringwerke AG and rat insulin (as a standard) was from Novo Research Institute (Copenhagen, Denmark). CCh, TPA, staurosporin and heparin were from Sigma (Deisenhofen, Germany); PKC peptide(19-31) was from Boehringer Mannheim (Mannheim, Germany) and Ro

31-8220 from Roche (Welwyn Garden City, Herts, UK). All other chemicals and reagents were of analytical grade supplied by E Merck (Darmstadt, Germany).

Isolation of rat pancreatic islets

Isolation of pancreatic islets was as described by Kuo *et al.* (1973) with slight modifications (Verspohl & Ammon 1980). Pancreata were isolated from the ether-anesthetized rat, minced, and washed twice with 20 ml ice-cold Krebs-Ringer buffer plus 10 mM Hepes (Krebs-Ringer-Hepes (KRH) buffer) containing 2.8 mM glucose, 1 mg/ml bacitracin, 0.2 mg/ml SBTI, and 0.02% albumin. Pancreas pieces were soaked and then shaken in a 37 °C water bath in the presence of 650 U collagenase/g tissue suspension. After 15-18 min of incubation the tissue suspension was transferred into 10 ml ice-cold Hanks' solution. Islets were separated by sedimentation and collected as described elsewhere (Kuo *et al.* 1973).

Incubation conditions and determination of insulin

To measure hormone secretion in static batch incubations, five islets were incubated for 90 min at 37 °C in 1 ml KRH buffer, pH 7.35, containing 5 mg/ml bovine albumin, 1 mg/ml bacitracin and 0.2 mg/ml SBTI and other additions as shown in the figure legends. CCh was used in the presence of 3.0 mM glucose, a substimulatory concentration, since it is an initiator of insulin release. Hormones released into the medium by islets were assayed by radioimmunoassay using rat insulin as a standard.

Perfusion studies

Fifty freshly isolated islets were used for each experiment. The perfusion medium was a KRH buffer supplemented with 0.25% BSA. The basal medium contained 8.3 mmol/l glucose and the test medium additional compounds as indicated in the legends to the figures. The medium reservoirs were maintained at 37 °C. During the first 15 min after the isolation procedure the perfusion flow rate was adjusted to 1.2 ml/min. In the next 15 min the islets were allowed to equilibrate under constant perfusion flow rate and insulin release was not determined. In the following 12 min four samples were collected to determine the basal insulin secretion. A CCh challenge was then given for 6 min by switching to the test medium. Data are shown in absolute terms (insulin concentration) and also as integrated amounts of released insulin over time (6-min area under curve (AUC)).

Electroporabilization (electroporation)

Since compounds such as PKC peptide(19-31) and heparin are not cell permeable, islets had to be permeabilized.

Experiments describing this procedure have been recently published (Verspohl *et al.* 1997). For some experiments islets were electrically permeabilized in a permeabilization buffer containing 70 mM 2-hydroxyethanesulfonic acid, 70 mM potassium chloride, 10 mM Hepes, 1 mM MgCl_2 , 1 mM ATP, 3 mM glucose, 1 mM EGTA and 0.5 mg/ml BSA. The Ca^{2+} concentration (5×10^{-8} M) was calculated according to the method described by Fabiato & Fabiato (1979). Islets were permeabilized by five exposures (15-s intervals, τ 200 μs) to an electric field of 1.36 kV/0.4 cm. As shown recently using the same electroporation protocol, all of the cells within the islet appear to be permeabilized to a similar extent (Jones *et al.* 1992). We obtained similar results when the fluorescence of cells was visualized both spectrophotometrically and microscopically for uptake of cell membrane impermeable propidium iodide, fluorescein isothiocyanate (FITC)-labeled antibodies and FITC-labeled dextran (Verspohl *et al.* 1997). Using propidium iodide as a marker of cell viability and effectiveness of electroporation, an effectiveness of electroporation of 81.5% was found, and a cell viability of 64% of all cells either electroporated or not was shown. This confirms the data of Yaseen *et al.* (1982). The cells appear to reseal since control experiments indicated a rapid outflow of lactate dehydrogenase for the first 15–20 min which levels off thereafter and is no longer observed after 45 min (Verspohl *et al.* 1997). Since the molecular weight of lactate dehydrogenase (140 000) is in the range of that of immunoglobulins it is possible that immunoglobulins significantly cross the membrane due to the permeabilization procedure (see Verspohl *et al.* (1997)).

In order to block the effects of PKC by intracellularly presenting PKC peptide(19–31), 50 islets were incubated in 400 μl permeabilization buffer containing the compound. After electroporation islets were kept untreated at 22 °C for 60 min to seal the pores. Then the 50 islets were either perfused for different periods or five islets were batch incubated each in the presence of either stimulator for 90 min at 37 °C as mentioned above using KRH buffer, pH 7.35, containing 5 mg/ml bovine albumin, 1 mg/ml bacitracin, and 0.1 mg/ml SBTI. The incubation conditions and medium composition were the same whether the islet had been electroporated before or not.

Insulin assay

Insulin concentrations in the effluent and the reservoir, or as released during static incubation, were measured in duplicate by a double-antibody method using specific antibodies and rat insulin as a standard.

Compounds used and their incubation conditions (a critical reflection)

To address the problem of desensitization we used PKC inhibitors which differed in their mechanism of action on

the enzyme: TPA on a long run inactivates the enzyme by proteolysis; staurosporin and Ro 31–8220 interact with the catalytic domain of PKC, and PKC peptide(19–31) has a pseudosubstrate sequence which interacts with the substrate binding site of PKC. The outcome depends on the concentration of inhibitor used. Reports differ with respect to the concentration and/or time used of the various PKC inhibitors, e.g. the IC_{50} for staurosporin with respect to the isoform PKC δ is 100-fold different depending on the substrate used (McGlynn *et al.* 1992, Mizuno *et al.* 1993, Birchall *et al.* 1994).

The optimum time and concentration were checked for rat pancreatic islets by static incubation to be sure that under the conditions used in our study, PKC was inhibited (Fig. 1), i.e. there was no acute TPA stimulatory effect on insulin release. A total of 200 nM TPA acutely increased insulin release. A 20-h preincubation with 200 nM TPA as well as 15-min preincubation with either 100 nM staurosporin, 5 or 10 μM but not 2 μM Ro 31–8220, or addition of approximately 10 μM PKC peptide(19–31) into electroporated islets, led to an inhibition of the effect of TPA on insulin release (Fig. 1) indicating the effectiveness of treating the cells with either compound leading to inactivation of PKC. Other concentrations were also tried (data not shown).

The optimum concentration of the compounds was: 200 nM for TPA, 100 nM for staurosporin and 5–10 μM for Ro 31–8220. The exact intracellular concentration of the PKC peptide(19–31) after electroporation could not be determined; the extracellular concentration during the electroporation process was 10 μM .

Statistics

For statistical evaluation multiple comparisons of means were carried out by one-way analysis of variance followed by a post-hoc test (Newmans–Keul test).

Results

In Fig. 2 (upper panel) a homologous desensitization of the CCh effect is shown for perfused rat pancreatic islets. When the islets were challenged six times there was a decrease in the CCh effect on insulin release over time. When challenges were interrupted for 45 min, there was a recovery of the stimulatory effects of CCh in the seventh challenge.

In addition to a 'true' desensitization (alteration in stimulus-secretion coupling) there is a depletion of intracellular insulin stores ('exhaustion') typical for a perfusion system as a consequence of chronic stimulation. Due to this 'run down' of the system data are also expressed as % AUC (integrated amounts of insulin secretion over time) to compensate for this effect (Fig. 2, middle panel). As a control, the % increase in AUC is also shown for

stimulation by 8.3 mM glucose (Fig. 2, lower panel); in contrast to CCh, glucose does not show a desensitizing effect.

The effect of 20-h TPA pretreatment was also investigated. Figure 3 shows a significant decrease in the stimulatory effect of CCh (first challenge). A further decrease of insulin release (desensitization) was not observed during another five CCh challenges.

When, however, PKC was inhibited by other methods, i.e. using 100 nM staurosporin, 5 μ M Ro 31-8220 and 10 μ M PKC peptide(19-31), there was an inhibition of insulin release at the first CCh challenge (Fig. 4). The homologous desensitization to the CCh effect was not interrupted by either compound.

The effect of 50 μ g/ml heparin on the CCh-mediated desensitization was also investigated; heparin is known to inhibit G-protein coupled receptor kinase (GRK) (Benovic & Gomez 1993). The CCh-induced desensitization action of the system was not affected by the addition of 50 μ g/ml heparin in the extracellular medium during electroporation of the islets (when the first challenge was normalized to 100% because of the electroporation procedure, the second challenge tended to be lower: 43 ± 30 – 19 ± 11 ; the third challenge 46 ± 15 – 21 ± 7 ; fourth challenge 18 ± 9 – 21 ± 8 ; all data were not significantly different from their individual control, i.e. no desensitization was obvious).

Discussion

PKC involvement in CCh-induced insulin release

CCh is known to potentiate the glucose (8.3 mM)-mediated insulin release and specific M_3 receptors have been demonstrated in rat pancreatic islets (Verspohl *et al.* 1990). PKC is involved in the stimulatory effect of CCh (Persaud *et al.* 1989, Wollheim & Regazzi 1990) which is confirmed by our data using TPA. This inhibitory effect was weaker with respect to PKC peptide(19-31) and staurosporin; since staurosporin is known to inhibit tyrosine kinases as well (Guma *et al.* 1992) this non-selectivity may result in a counter regulatory increase as it was recently shown for tyrosine kinase inhibitors (Verspohl *et al.* 1995). In islets acetylcholine-induced IP_3 levels are also influenced by TPA pretreatment (Gao *et al.* 1994) and an acute addition of TPA decreases CCh-induced increases in Ca^{2+} concentration and production of IP_3 (Arkhammar *et al.* 1994).

Figure 1 Effect of a long term (20 h) TPA (200 nM) preincubation and preincubation (15 min) with various concentrations of staurosporin, Ro 31-8220 and PKC peptide(19-31) on the insulinotropic effect of 200 nM TPA (static experiments). Five rat pancreatic islets were incubated with 8.3 mM glucose plus 200 nM TPA acutely for 90 min after pretreatment with the indicated compounds. Data are expressed as means $\pm$ S.E.M. of 3–12 experiments; * $P < 0.05$ vs absence of TPA (acutely).

Figure 2 Desensitization of CCh-induced insulin release after repetitive CCh addition (perfusion experiment). Fifty rat pancreatic islets were perfused at a glucose concentration of 8.3 mM. They were challenged seven times as indicated. Basal secretion is calculated from the last 3 min prior to the next CCh challenge. The top panel shows the absolute released amounts of insulin for every 3 min (6 min=challenge time indicated by the hatched area). Since the insulin baseline shows a run down (upper panel), the data are recalculated: the stimulatory effect is also shown as a percentage increase over basal at the individual challenge time point, AUC (integrated area under the curve) (middle panel). In control experiments islets were challenged seven times by switching from 3.0 to 8.3 mM glucose (lower panel). Data are expressed as means $\pm$ S.E.M. of 3–7 experiments; One-way ANOVA was performed: $P < 0.01$; * $P < 0.05$ vs first stimulation; ** $P < 0.05$ vs sixth stimulation.

Homologous desensitization of the CCh-modulated insulin release

Desensitization includes the decrease in the biologic response to a specific agent and the restoration of the effect after regenerating the system. The CCh effect desensitizes very quickly, already after the first challenge. The CCh effect was restored indicating that no simple toxic effect mimicked desensitization. This was carefully controlled by using 8.3 mM glucose since a run down typical for a perfusion system might have obscured the interpretation of our data.

PKC involvement in desensitization

Some data hint to the possibility that the activation of PKC by CCh leads to a desensitization: 20-h TPA pretreatment which degrades PKC leads to an inhibition of the desensitization process in islets. This has already been shown for M_3 receptors in other cell systems (Yamatani *et al.* 1988, Maruyama 1989, Tan & Marty 1991, reviewed by Berrie & Elliott 1994). However, the observation that desensitization of PLC-coupled muscarinic receptors is mediated by PKC (Haga *et al.* 1990) is not supported by all studies as described by Benya *et al.* (1995).

In contrast to TPA, all other compounds used to inhibit PKC such as staurosporin, Ro 31-8220 and PKC peptide (19–31) were not able to abolish inhibition of CCh-induced desensitization. With respect to staurosporin this is surprising since this compound is able to do so with other PLC-coupled receptor systems such as cholecystikinin (Willems *et al.* 1993), or with muscarinic receptors in other systems such as the lacrimal gland (Tan & Marty 1991). However, staurosporin did not desensitize those receptors which are not linked to PLC, e.g. histamine-1 receptors (Zamani *et al.* 1995). Concerning Ro 31-8220 our data are in line with those for M_3 receptors in other cell lines (Tobin *et al.* 1993) and reflect what is known for other receptors coupled to PLC (Barr & Watson 1994, Lattion *et al.* 1994). With respect to other receptors other groups also showed a difference when either TPA or staurosporin for PKC downregulation were used in rat pancreatic islets, i.e. the cholecystikinin-induced insulin release is inhibited by long term pretreatment with TPA but not with staurosporin (Karlsson & Åhrén 1991).

Selectivity of PKC inhibitors (a critical reflection)

Maximum selectivity of some compounds used was not achieved, e.g. staurosporin inhibits other protein kinases. The lack of selectivity is due to the huge homologies in the catalytic domain of many kinases (Bruns *et al.* 1991). Staurosporin is the most potent inhibitor ($K_i \sim 2$ nM) (Kase *et al.* 1987); however using a 10-fold higher concentration leads to inhibition of PKA, of CaMKII (calcium-calmodulin dependent kinase II) and of phosphorylase

Figure 3 Effect of long term TPA preincubation on CCh effects on insulin release, especially with respect to desensitization. After 20-h preincubation with or without 200 nM TPA, 50 rat pancreatic islets were perfused at a glucose concentration of 8.3 mM. The percentage increase of insulin secretion over basal (last 3 min prior to challenge) is shown for six CCh challenges. Data are expressed as means $\pm$ S.E.M. of 5–8 experiments; one-way ANOVA $P < 0.001$ for desensitization in the absence of TPA-preincubation; $P = 0.9$ (not significant (NS)) for desensitization after 20-h TPA pretreatment; * $P < 0.05$ insulin release with or without TPA pretreatment.

kinase (Nixon *et al.* 1992). A total of 1000 nM staurosporin leads to 90% inhibition of insulin receptor tyrosine kinase (Guma *et al.* 1992) which is involved in the feedback of insulin release on its release (Verspohl *et al.* 1995). The bisindolylmaleinimide-derivative Ro 31-8220 inhibits PKC α , β , ϵ and ζ (Wilkinson *et al.* 1993, Persaud & Jones 1994) and ι (Persaud & Jones 1994) (only those isoenzymes are mentioned which are present in islets). Nonspecific effects of TPA cannot be excluded since no reliable control compound exists. The inactive phorbol esters do not possess all the nonspecific effects of the active phorbol esters (Kachintorn *et al.* 1992). The PKC isoenzyme selectivity of the different PKC inhibitors is important when regarding interpretation of the data. Isoenzymes are not thought to have redundant but specific effects. One way to interpret the data is to introduce specific antibodies to either isoenzyme into islets by the electroporation method (Verspohl & Herrmann 1996, Verspohl *et al.* 1997) to knock out their effects; unfortunately, we did not get reproducible results by this method.

From our results it appears that the desensitization is affected by a specific isoenzyme which is present in rat pancreatic islets and is inhibited by TPA but not by staurosporin, Ro 31-8220 and PKC peptide(19–31). However, none of the enzymes found in islets (PKC α , PKC β , PKC δ , PKC ϵ , PKC ζ and PKC ι) are inhibited by TPA, and none are simultaneously inhibited by any of the other inhibitors we have used. Thus a hitherto unknown PKC isoenzyme is involved in the desensitization process.

Another possible explanation for the different effects between TPA pretreatment and the different PKC inhibitors could be that protein kinase M is formed by the proteolytic degradation of PKC due to phorbol esters. In contrast the competitive inhibitors staurosporin, Ro 31-8220 and PKC peptide(19–31) do not produce this kinase. It might be speculated that this kinase is involved in the desensitization mediated by TPA.

A third explanation for the divergent effect of TPA could be that TPA exhibits additional nonspecific effects since TPA is known to induce a depolarization of plasma membranes (Yada *et al.* 1989), stimulation of phospholipase D (PLD) (Guillemain & Rossignol 1994) and PLA₂ (Conklin *et al.* 1988) and an inhibition of epidermal growth factor (EGF) receptor tyrosine kinase (Lin *et al.* 1986). It is well known in the latter case that tyrosine kinase inhibition increases insulin release (Jonas *et al.* 1995, Verspohl *et al.* 1995).

In conclusion, the data indicates the involvement of PKC, although a hitherto unidentified specific isoenzyme of PKC may be involved in the homologous desensitization of the CCh-induced insulin release in pancreatic islets.

β ARK (β -adrenergic receptor kinase)

Members of the GRK family are receptor-specific kinases. They phosphorylate the receptor in an agonist-dependent way and are involved in the homologous desensitization of

Figure 4 Effects of staurosporin, Ro 31-8220 and PKC peptide(19-31) on CCh effects on insulin release, with respect to desensitization. In the absence or presence of different PKC inhibitors (staurosporin upper panel; Ro 31-8220 middle panel; PKC peptide(19-31) lower panel), 50 rat pancreatic islets were perfused at a glucose concentration of 8.3 mM. The percentage increase of insulin secretion over basal (last 3 min prior to challenge) is shown for four CCh challenges. Data are expressed as means $\pm$ S.E.M. of four experiments; one-way ANOVA $P < 0.002$ for desensitization in the absence of staurosporin; multi-way ANOVA $P < 0.05$ for desensitization in the absence of Ro 31-8220; one-way ANOVA $P < 0.05$ for desensitization in the absence of PKC peptide(19-31); * $P < 0.05$ denotes the difference in the presence and absence of either staurosporin or Ro 31-8220 during the first challenge. Note that the data of the first CCh challenge with respect to PKC peptide(19-31) are normalized to 100% since the electroporation procedure resulted in day-to-day slightly variable basic secretion.

some G-protein coupled receptors. Recent studies show that the M_3 receptor is a substrate of GRK2 (Debburman *et al.* 1995), and GRK2 was shown to be present in the pancreas (Benovic & Gomez 1993). It is known that the β ARK inhibitor heparin prevents the augmenting effect of TPA on β ARK desensitization and that β ARK activity can be increased through phosphorylation by PKC (Chuang *et al.* 1995).

An involvement of GRK, however, does not seem to play a role with respect to M_3 receptor desensitization in islets since the GRK inhibitor heparin was not effective in our system, even though a high extracellular concentration (50 μ g/ml) during electroporation was used. Heparin is also known to inhibit the IP_3 receptor which is important for insulin release. The IP_3 receptor being a calcium channel is involved in the desensitization in the way that an activation of PLC by CCh and the resulting increase in IP_3 results in a proteolysis of the receptor (Simpson *et al.* 1994, Wojcikiewicz *et al.* 1993, 1994). However, since the heparin concentration used is able to inhibit IP_3 receptors and since there was no effect of heparin pretreatment, this mechanism is unlikely to be involved in the CCh desensitization observed.

In conclusion there is clear evidence that in rat pancreatic islets CCh effects on insulin release desensitize quickly. Whereas PKC isoenzyme downregulation or inhibition is involved in diminished acute insulin response to CCh, it is questionable which PKC isoenzyme is involved in the homologous CCh desensitization; three out of four different PKC inhibitors are unable to inhibit desensitization. This may be due to an unknown isoenzyme downregulated by TPA and not the other compounds used or by a nonspecific effect of TPA. The data are in contrast to a PKC involvement in homologous M_3 -subtype receptor desensitization, e.g. in pancreatic acini and lacrimal gland cells. Other possible mechanisms of desensitization have to be investigated, e.g. at the level of G proteins.

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