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Ca^{2+}-dependent sensitization of adenylyl cyclase activity

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Abstract

It has been shown that intracellular Ca^{2+} concentrations have multiple modulatory influences on hormone-stimulated adenylyl cyclase activity. Here, we report that increasing intracellular Ca^{2+} concentration by treating cells with the Ca^{2+} ionophore A23187 leads to a sensitization of the β-adrenoceptor-stimulated adenylyl cyclase activity in Ltk^- cells expressing the human β_2-adrenoceptor. The ionophore treatment led to a $20 \pm 5\%$ increase of the maximal isoproterenol-stimulated cyclase activity that could be prevented by chelation of the extracellular Ca^{2+} using EGTA. A similar Ca^{2+}-mediated sensitization ($20 \pm 6\%$) was observed when the adenylyl cyclase was directly stimulated by the diterpene forskolin indicating that the catalytic activity of the enzyme itself is modulated by the change in Ca^{2+} concentration. Sensitization of both the isoproterenol- and forskolin-stimulated adenylyl cyclase activities were completely blocked by treatment with KN-62{1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine}, an inhibitor of the Ca^{2+}-calmodulin-dependent protein kinase (CamKinase). Taken together, our data reveal the existence of a CamKinase-dependent sensitization process acting at the level of the adenylyl cyclase catalytic moiety.

Keywords: β_2-Adrenoceptor; Adenylyl cyclase; Ca^{2+}; CamKinase (Ca^{2+}-calmodulin-dependent protein kinase)

1. Introduction

Cross-talk regulation between signaling systems is a well-accepted paradigm that has numerous implications on the processes of signal integration at the cellular level. Responsiveness of a given signaling pathway is under the influence of multiple regulatory systems that depend on both its own level of activity and the activation-state of other signal transduction systems (Bouvier, 1990; Weng et al., 1999). Among those, regulation of the adenylyl cyclase-coupled signaling pathways by hormones and transmitters that primarily act by regulating intracellular Ca^{2+} concentration has attracted considerable attention.

Variations in intracellular Ca^{2+} concentration have been shown to have multiple modulatory influences on hormone-stimulated adenylyl cyclase activity (for reviews; Ishikawa and Homcy, 1997; Tang and Hurley, 1998). Ca^{2+}-calmodulin can directly activate the catalytic moieties of three of the nine mammalian adenylyl cyclase isoforms (adenylyl cyclase I, III and VIII) (Cali et al., 1994; Choi et al., 1993; Tang et al., 1991). In contrast, phosphorylation of adenylyl cyclase I (Wayman et al., 1996) and III (Wayman et al., 1995; Wei et al., 1996) by the Ca^{2+}-calmodulin kinase (CamKinase) have been shown to inhibit their activity. Also, phosphorylation by another Ca^{2+}-sensitive kinase, the protein kinase C, was found to promote activation of the adenylyl cyclase type II (Jacobowitz and Iyengar, 1994) and V (Iwami et al., 1995) but to inhibit adenylyl cyclase VI (Lai et al., 1997).

Receptors represent additional potential sites of regulation. For instance, phosphorylation of the β_2-adrenoceptor by protein kinase C promotes uncoupling of the receptor from Gs and thus inhibits receptor-activated adenylyl cyclase activity (Bouvier et al., 1991). Although phosphorylation of G-protein-coupled receptors by CamKinase has not been reported, several receptors, including the β_2-adrenoceptor, harbor CamKinase consensus phosphoryla-

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tion sites (R–X–X–S; Cruzalegui and Means, 1993; Payne et al., 1983) that could potentially play regulatory roles. In addition, the G-protein-coupled receptor kinases that phosphorylate and promote the functional uncoupling and internalization of many G-protein-coupled receptors have been shown to be regulated by Ca^{2+}. For example, binding of the Ca^{2+}-sensing protein recoverin and of Ca^{2+}-calmodulin was shown to inhibit G-protein-coupled receptor kinases 1, 2, 5 and 6 (Levay et al., 1998; Pronin et al., 1997) while phosphorylation by the Ca^{2+}-dependent protein kinase C was found to inhibit G-protein receptor kinase 5 (Pronin and Benovic, 1997) and to activate G-protein-coupled receptor kinase 2 (Chuang et al., 1995).

Given the diversity of the regulatory processes potentially involved, it is difficult to predict the outcome that an increase in intracellular Ca^{2+} concentration will have on the adenylyl cyclase response evoked by a specific hormone. Considering the above discussion, it should depend on the type of adenylyl cyclase and regulatory proteins that are expressed in the cell type considered. The presence or absence of specific regulatory sites within the stimulated receptor could also influence the ultimate response. It follows that the effect that Ca^{2+} may have on hormone-stimulated adenylyl cyclase responsiveness should be considered independently for each receptor within a specific cellular background. The aim of the present study was to characterize the effects of an elevation of intracellular Ca^{2+} on the β_2-adrenoceptor-stimulated adenylyl cyclase in Ltk^- fibroblasts, an heterologous expression system often used to study G-protein-coupled receptors.

We found that elevation of intracellular Ca^{2+} following a treatment with the ionophore A23187 potentiates the β_2-adrenoceptor-stimulated adenylyl cyclase activity through a CamKinase-dependent sensitizing effect on the adenylyl cyclase catalytic moiety itself. The treatment also leads to a modest uncoupling of the receptor from Gs.

2. Material and methods

2.1. Materials

[^{125}I]Cyanopindolol ([^{125}I]CYP), [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from NEN Life Science Products (Boston, MA). Isoproterenol, A23187, 4-bromo A23187, isobutylmethylxanthine, ATP, GTP, cAMP, phosphoenolpyruvate, and myokinase were purchased from Sigma (St. Louis, MO) Pyruvate kinase, KN-93 (*N*-[2-[[[3-(4′-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4′-methoxy-benzenesulfonamide phosphate) and Lavendustin C 5-(*N*-2′,5′-dihydroxybenzyl)-aminosalicylic acid) were obtained from Calbiochem (La Jolla, CA), geneticin, DMEM (Dulbecco's Modified Eagle Medium), penicillin, streptomycin, amphotericin B, and trypsin were purchased from GIBCO (Gaithersburg, MD), KN-62 {1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine} was obtained from RBI (Natick, MA) and the fetal bovine serum from Immunocorp (Mount Royal, Canada).

2.2. DNA constructions, cell transfection and cell culture

Human β_2-adrenoceptor cDNA was subcloned into a pBC12BI expression vector as previously described (Suzuki et al., 1992). The resulting plasmid was co-transfected with the neomycin resistance plasmid pSV-Neo (Amersham Pharmacia Biotech, Baie-d'Urfé, Canada) by the Ca^{2+} phosphate precipitation method. Neomycin-resistant cells were selected in DMEM supplemented with 10% fetal bovine containing 450 μg/ml geneticin. Selected clones were then screened for βAR expression by binding assay using [^{125}I]CYP as the radioligand. A cell line expressing a relatively low number of βARs (193 ± 29 fmol/mg of protein) was chosen for further studies and grown as monolayer in 75-cm^2 flasks containing DMEM supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 500 U/ml penicillin, 500 U/ml streptomycin, 0.25 μg/ml amphotericin B and 450 μg/ml geneticin in 5% CO_2 at 37 °C. In some experiments, the β-adrenoceptor agonist isoproterenol, the Ca^{2+} ionophore A23187, and the Ca^{2+}-calmodulin kinase (CamKinase) II and IV inhibitor KN-62 were added to the culture 15–30 min prior to harvesting the cells.

2.3. Membrane preparations

Cells were washed three times with 10 ml of phosphate-buffered saline at 4 °C and mechanically detached in 10 ml of ice-cold buffer containing 5 mM Tris–HCl, pH 7.4, 2 mM EDTA, and protease inhibitors (5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine). Cells were then lysed with a polytron homogenizer (one burst for 7 s at maximum speed) and the lysates centrifuged at $45\,000 \times g$ for 20 min at 4 °C. The pelleted membranes were washed twice in the same buffer, resuspended in 0.5 ml of buffer containing 75 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, protease inhibitors and used immediately for adenylyl cyclase activity determination and radioligand binding assays as described below. Protein content was determined according to the method of Bradford (using the Bio-Rad system; Hercules, CA).

2.4. Adenylyl cyclase assay

Adenylyl cyclase activity was measured according to the method of Salomon et al. (1974). Briefly, 5 μg of membrane proteins was added to a total volume of 50 μl of a reaction mixture containing 120 μM ATP, 0.5 μCi [α-^{32}P]-ATP, 100 μM cAMP, 53 μM GTP, 2.8 mM phosphoenolpyruvate, 0.2 U of pyruvate kinase, 1 U of

myokinase, 30 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 0.8 mM EDTA, and 0.1 mM isobutylmethylxanthine. The final free Mg^{2+} concentration in the assay was 1.1 mM as calculated by the method of Iyengar and Birnbaumer (1982). Enzyme activity was determined in duplicate in the absence (basal activity) or in the presence of activators (isoproterenol 1 nM–100 μM or forskolin 100 μM).

2.5. Radioligand binding assay

Radioligand binding assays were conducted essentially as described previously (Rousseau et al., 1997) with 5 μg of membrane proteins in a total volume of 0.5 ml. Saturation experiments were carried out using 250 pM [^{125}I]CYP, 75 mM Tris–HCl, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA in the presence or absence of 10 μM alprenolol to define nonspecific binding. For the competition experiments, triplicate assay tubes contained 50 pM [^{125}I]CYP and 0–100 μM of the agonist isoproterenol. The binding reactions were incubated at room temperature for 90 min and terminated by rapid filtration over Whatman GF/C glass fiber filters pre-incubated in a buffer containing 25 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin, and 0.3% polyethylenimine. The filtered membranes were then washed thrice with ice-cold 25 mM Tris–HCl, pH 7.4.

2.6. Identification of adenylyl cyclase subtype

Ltk$^-$ cells were washed three times with PBS and detached in Laemmli buffer containing 0.2% SDS and 10% β-mercaptoethanol. The samples were heated for 5 min at 95 °C and the proteins separated by SDS-polyacrylamide gel electrophoresis using an 8% slab gel. The proteins were then transferred electrophoretically to a nitrocellulose membranes that were blocked with 5% free-fat milk, 0.05% Tween 20 in Tris-buffer saline (TBS; pH 7.5). After three washes with TBS–Tween 20 (0.05%), membranes were incubated with anti-adenylyl cyclase ACI, ACII, ACIII, ACIV and ACV/VI (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 overnight at 4 °C, followed by four washes with TBS–Tween 20. The immunoreactivity was finally revealed using a horseradish peroxidase-conjugated anti-mouse antibody (1:2000) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) detection system.

2.7. Measurement of intracellular Ca^{2+}

Cells were loaded for 30 min at 37 °C with the fluorescent probe Fura-2 AM (2.5 μM) (Molecular probes, Eugene, OR) in DMEM without fetal bovine serum. Dye-loaded cells were then transferred onto the stage of an inverted microscope (Olympus, IMT-2, Carsen Scientific, Mississauga, Ontario) coupled to a spectrofluorometer (Deltascan 4000, Photon Technology International, Lawrenceville, NJ). The excitation wavelengths used were 350 and 380 nm, and the fluorescence emission was measured at 505 nm every 2 s. Calibration was always performed in situ at the end of an experiment by perfusion of ionomycin (10 μM) in a solution containing 4 mM EGTA (R_{min}) or 4 mM $CaCl_2$ (R_{max}). After correction for sample autofluorescence, signal ratios (F_{350}/F_{380}) were transformed into $[Ca^{2+}]_i$. The presence of nonhydrolyzed dye was periodically verified by quenching with 2 mM $MnCl_2$ and found to be negligible when compared to autofluorescence. Intracellular Ca^{2+} content was measured in cells during resting conditions or in presence of 4-bromo A23187 (10 μM).

2.8. Statistical analysis

All results are expressed as mean ± standard error of the mean (S.E.M.). Student's paired *t*-tests were used to assess the statistical significance of the differences between control and treatment groups. Data from competition and saturation experiments were analyzed by non-linear least squares regression using the computer program SCATFIT (De Léan et al., 1982). For comparisons, we used an analysis of variance followed by a Dunnett test. Data from adenylyl cyclase activity were analyzed with the use of a non-linear least-squares regression with a slope factor fixed at 1. Basal and maximal stimulation values presented in tables were estimated with regression analysis. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Expression of the β_2-adrenoceptor in Ltk$^-$ cells

Ltk$^-$ cells with no detectable β-adrenoceptor-stimulated adenylyl cyclase activity were transfected with cDNAs encoding human β_2-adrenoceptor. Cellular clones were selected by virtue of co-transfection with G418 resistance marker (450 μg/ml) and β-adrenoceptor expression was assessed in the resistant clones with the use of [^{125}I]CYP binding assays. A stable cell line expressing 193 ± 29 fmol/mg of membrane protein was chosen for further study.

3.2. Effect of A23187 treatment on β_2-adrenoceptor-stimulated adenylyl cyclase activity

Fig. 1A illustrates the classical dose-dependent increase in adenylyl cyclase activity measured in membrane preparation derived from Ltk$^-$ cells expressing the β_2-adrenoceptor upon stimulation with the β-adrenoceptor agonist, isoproterenol. In an effort to assess the influence of an increased intracellular Ca^{2+} concentration on β-adrenoceptor responsiveness, cells were treated with the Ca^{2+}

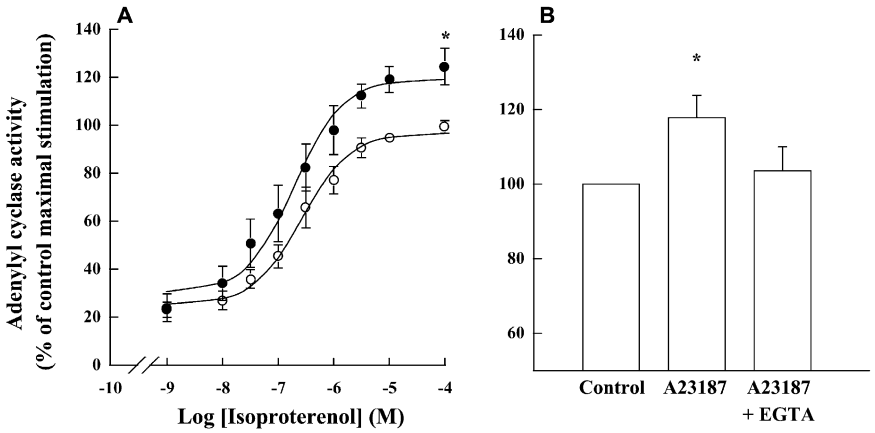

Fig. 1. A23187 effects on isoproterenol-stimulated adenylyl cyclase activity. (A) Ltk$^-$ cells expressing human β_2-adrenoceptor were incubated in the absence (○) or the presence of 10 μM A23187 (●) for 15 min at 37 °C. Adenylyl cyclase activity was measured in membrane preparations derived from these cells and expressed as the percentage of maximal stimulation observed in membranes derived from control cells. Data represent the mean ± S.E.M. of eight independent experiments. (B) Maximal isoproterenol-stimulated activity expressed as the percentage of the response observed in control membranes. In the presence of EGTA (1 mM, 60 min), positive effect of Ca^{2+} ionophore on isoproterenol-stimulated adenylyl cyclase was abolished. The data represent the mean ± S.E.M. of 7–8 distinct experiments. $^*P < 0.05$.

ionophore A23187. Pretreatment of the cells with A23187 for 15 min prior to membrane preparation led to a significant ($P < 0.05$) 20 ± 5% increase in the maximal isoproterenol-stimulated adenylyl cyclase activity. This effect truly resulted from an elevation in intracellular Ca^{2+} as it could be blocked by the addition of the cationic chelator EGTA (Fig. 1B). The magnitude of the Ca^{2+} elevation promoted by the A23187 treatment was documented by measuring the intracellular Ca^{2+} content using Fura-2 AM as a Ca^{2+} indicator. In resting conditions, the intracellular Ca^{2+} concentration was 77 ± 31 nM. The treatment with A23187 (10 μM) promotes a global 44% elevation of the intracellular Ca^{2+} content; an increase that is well within the range of Ca^{2+} changes observed in physiological conditions.

Given that Ca^{2+}-dependent inhibition of G-protein receptor kinases has been documented, one could propose that the increased β_2-adrenoceptor-stimulated adenylyl cyclase activity promoted by the increased intracellular Ca^{2+} concentration results from a blunted homologous desensitization. To test this hypothesis, the effect of the Ca^{2+} ionophore treatment was directly assessed on agonist-promoted desensitization. Pretreatment of cells with 1 μM isoproterenol for 15 min led to a marked desensitization of

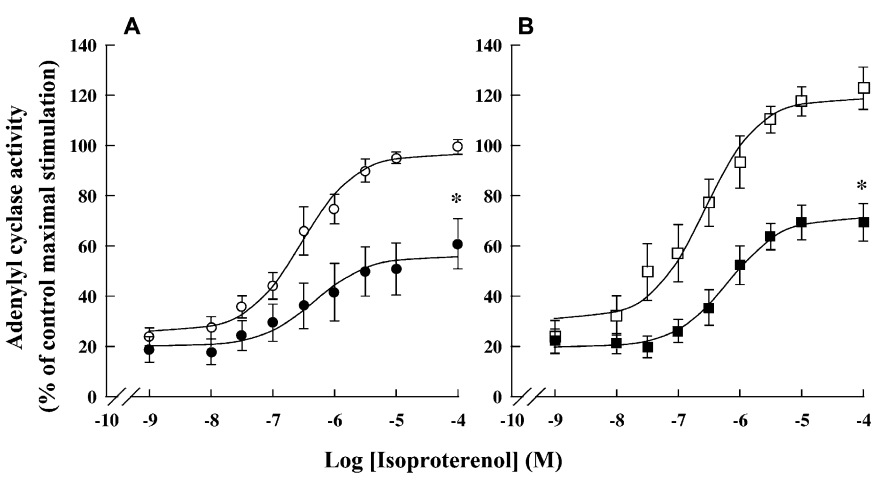

Fig. 2. Isoproterenol-induced desensitization of β_2-adrenoceptor expressing cells. (A) Ltk$^-$ cells were incubated in the absence (○) or the presence of 1 μM isoproterenol (●) for 15 min at 37 °C. Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. The data are expressed as the percentage of the response observed in control membranes ($n = 7$). (B) Isoproterenol-induced desensitization of β_2-adrenoceptor expressing cells incubated with A23187 for 15 min at 37 °C in absence (□) or the presence of 1 μM isoproterenol (■). Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. The data are expressed as the percentage of the response observed in control membranes ($n = 7$). $^*P < 0.05$.

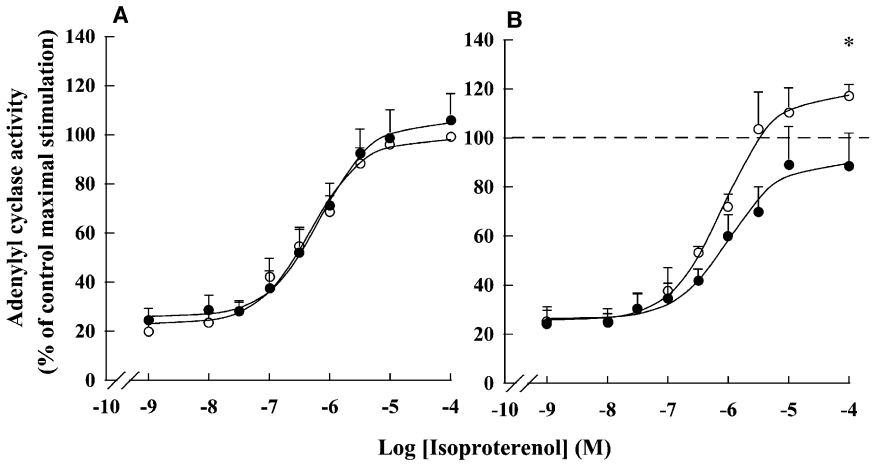

Fig. 3. KN-62 effects on the β_2-adrenoceptor agonist-induced adenylyl cyclase activity. (A)Ltk$^-$ cells expressing β_2AR were incubated in the absence (○) or the presence of 1 μM KN-62 (●) for 30 min at 37 °C. Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. The data are expressed as the percentage of the maximal isoproterenol-stimulated adenylyl cyclase response observed in control membranes ($n = 7$). (B) Ltk$^-$ cells expressing β_2-adrenoceptor were incubated with A23187 for 15 min at 37 °C in the absence (○) or the presence of 1 μM KN-62(●) for 30 min at 37 °C. Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. The data are expressed as the percentage of the maximal isoproterenol-stimulated adenylyl cyclase response observed in control membranes. To facilitate comparison, we indicated the 100% with a dashed line ($^* P < 0.05$; $n = 7$).

the β_2-adrenoceptor-stimulated adenylyl cyclase activity that is reflected by a reduction of $43 \pm 8\%$ of the maximal response (Fig. 2A). The extent of desensitization promoted by the agonist was not affected by the ionophore treatment as indicated by a similar reduction in maximal β-adrenoceptor stimulated adenylyl cyclase activity ($42 \pm 5\%$) observed in cells concomitantly treated with A23187 (Fig. 2B). This indicates that elevated Ca^{2+} concentration did not inhibit agonist-promoted desensitization and thus, the sensitizing effect of Ca^{2+} cannot be attributed to an inhibition of G-protein receptor kinase activity.

To determine if Ca^{2+}-calmodulin kinase could be involved in the sensitizing effect promoted by the ionophore treatment, the effect of the KN-62, an inhibitor of CamKinase II and IV was investigated. As shown in Fig. 3A, treatment of cells with this inhibitor alone was without effect on the isoproterenol-stimulated adenylyl cyclase activity. However, when co-administered with A23187, it completely blocked the sensitizing effect of the ionophore (Fig. 3B) thus suggesting the involvement of CamKinase-dependent phosphorylation events. Identical results were obtained using two additional CamKinase inhibitors KN-93 (1 μM) and lavendustin-C (1 μM) (data not shown). Interestingly, the three inhibitors have clearly distinct chemical structures making it unlikely that the observed inhibitory effects could reflect a non-specific action of the pharmacological agents.

3.3. Effect of A23187 treatment on forskolin-stimulated adenylyl cyclase activity

To identify the site of action of the CamKinase, we assessed the effect of the A23187 treatment on the activity of the adenylyl cyclase catalytic moiety directly stimulated by the diterpene forskolin. As illustrated in Fig. 4, the ionophore treatment potentiated the forskolin-stimulated adenylyl cyclase activity by $20 \pm 6\%$. Similarly to what was observed for the β_2-adrenoceptor-stimulated activity, this sensitizing effect of A23187 was completely blocked by KN-62 while this inhibitor was without effect on the forskolin-stimulated adenylyl cyclase activity when administered alone. The sensitizing effect was also blocked by the two other CamKinase inhibitors KN-93 and lavendustin C (data not shown). These data indicate that the

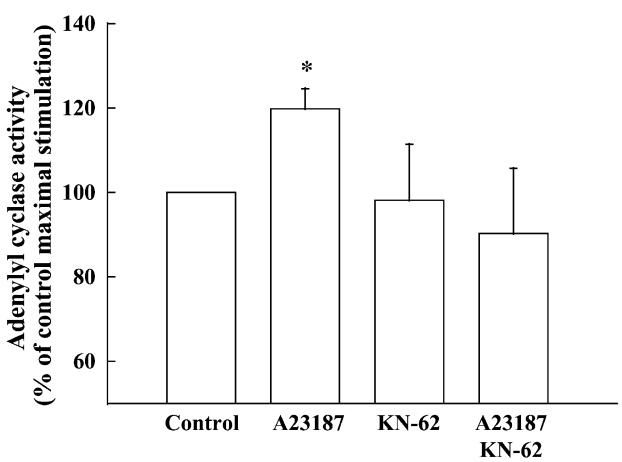

Fig. 4. A23187 and KN-62 effects on forskolin-induced adenylyl cyclase activity. Forskolin-induced stimulation of adenylyl cyclase in membrane preparation derived from Ltk$^-$ cells expressing β_2-adrenoceptor. Cells were pretreated with either A23187 (10 μM, 15 min at 37 °C), KN-62 (10 μM, 30 min at 37 °C) or both (KN-62, A23187). The data are expressed as the percentage of the response observed in control membranes ($n = 7$–8).

CamKinase-dependent sensitizing effect resulted at least in part from an increased responsiveness of the adenylyl cyclase itself.

3.4. Identification of the adenylyl cyclase subtypes in Ltk⁻ cells

In order to further document the identity of the adenylyl cyclase isoform(s) that could be involved in the sensitization process, antibodies raised against the adenylyl cyclase I to VI were used. Western blot analysis performed on total extracts from Ltk⁻ cells revealed the presence of adenylyl cyclase I and III but not II, IV, V or VI (Fig. 5). The efficacies of the antibodies to recognize the various isoforms were verified in control tissues (data not shown).

3.5. Effect of A23187 treatment on receptor coupling

To determine if alterations in receptor coupling to Gs could also contribute to the sensitization, the agonist high-affinity binding state was used as an index of receptor–Gs interaction (De Léan et al., 1980). Competition of [^{125}I]CYP binding by isoproterenol carried out in membrane derived from control cells resulted in characteristic biphasic curves that can be best resolved by a two-affinity state model. Analysis using the program SCATFIT (De Léan et al., 1982) revealed that 70 ± 4% of the receptor population are in a high affinity state for agonists (Table 1). Treatment of cells with A23187 reduced this proportion to 55 ± 5% without significantly changing the affinity of the receptors for isoproterenol, indicating that the ionophore treatment promoted a modest uncoupling of the receptor from Gs. The uncoupling induced by A23187 was not blocked by pretreatment with KN-62 suggesting that this effect is not CamKinase-dependent.

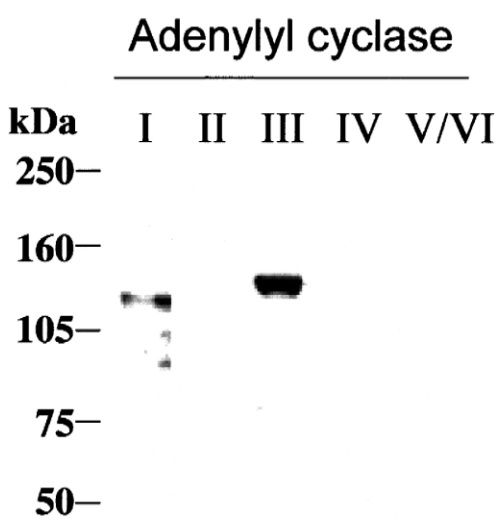

Fig. 5. Immunodetection of adenylyl cyclase isoforms expressed in Ltk⁻ cells. Total cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and the adenylyl cyclase immunoreactivity assessed with the specific anti-adenylyl cylase I, II, III, IV and V/VI antibodies. Protein one hundred micro (μ)g of proteins was loaded in each well. The results shown are representative of two independent experiments.

Table 1
Isoproterenol binding parameters for β_2AR
Isoproterenol binding parameters were determined by competition of [^{125}I]CYP binding using increasing concentrations of isoproterenol (0–100 μM). The estimates were obtained from analysis of 3–5 competition experiments using the program Ligand ((De Léan et al., 1982)).

	Control	A23187 (10 μM)	KN-62 + A23187 (1 μM + 10 μM)
$K_{i(H)}$ (nM)[a,b]	0.3 ± 0.1	0.5 ± 0.2	2.6 ± 1.4
$K_{i(L)}$ (μM)[a,b]	0.2 ± 0.2	0.3 ± 0.1	2.3 ± 2.1
$\%R_H^{a,c}$	70 ± 4	55 ± 5[d]	43 ± 7[d]

[a] H and L refer to high- and low-affinity binding parameters of isoproterenol, respectively.
[b] Equilibrium inhibition constant for isoproterenol.
[c] Percentage of site in the high-affinity state for isoproterenol.
[d] Significantly different ($P < 0.05$) vs. control condition.

4. Discussion

The results presented in this study indicate that increased intracellular Ca^{2+} concentrations sensitize both β_2-adrenoceptor- and forskolin-stimulated adenylyl cyclase activation in Ltk⁻ mouse fibroblasts. This effect most likely results from an action on the enzyme itself since the Ca^{2+}-promoted increases were virtually identical whether adenylyl cyclase was stimulated through the activation of the β-adrenoceptor (using isoproterenol) or directly by the diterpene forskolin. In fact, any effect on receptor responsiveness appears to be in the opposite direction since the treatment with the ionophore promoted a modest uncoupling of the receptor from Gs as revealed by the 15% reduction of sites in the high-affinity state for agonist.

Sensitizing effect of Ca^{2+} on adenylyl cyclase reactivity has been observed in several studies (Cali et al., 1996; Choi et al., 1992; Villacres et al., 1995). In most cases, however, it was attributed to a direct action of Ca^{2+}-calmodulin binding on the cyclase (Cali et al., 1994; Choi et al., 1993; Tang et al., 1991) and not to the action of the CamKinase. This contrasts with our observation that selective inhibition of the CamKinase by three distinct inhibitors entirely blocked the sensitizing effect of the Ca^{2+} ionophore. Although CamKinase was previously shown to modulate adenylyl cyclase activity in some cells, it was always reported as being inhibitory rather than stimulatory. In fact, for the adenylyl cyclase type I, the inhibitory action was attributed to the direct phosphorylation of Ser^{545} and Ser^{552} within the C1b domain of the adenylyl cyclase by CamKinase IV (Wayman et al., 1996). An

inhibitory effect of CamKinase I on the activity of adenylyl cyclase type III was also reported (Wei et al., 1996). Whether other isoforms of the enzyme can also be negatively regulated by CamKinase remains to be investigated. Interestingly, although several consensus phosphorylation sites for CamKinase exist in all nine mammalian adenylyl cyclases, the inhibitory site proposed for adenylyl cyclase type I (Ser^{545}, Ser^{552}) is absent in all other types and only a few sites are conserved in identical positions in all isoforms. This raises the possibility of differential regulation of the distinct isoforms by the kinase. Whether the CamKinase-dependent sensitization that we observed in the present study could result from the phosphorylation of one or more consensus sites present in a specific adenylyl cyclase isoform(s) remains to be investigated. Western blot analysis revealed the expression of adenylyl cyclase I and III in the Ltk^- cell used in the present study. Given the inhibitory effect that has been previously reported, it is highly unlikely that CamKinase-dependent sensitization observed could involve the phosphorylation of these isoforms. Since adenylyl cyclase II, IV, V and VI were not detected, this leaves us with adenylyl cyclase VII, VIII or IX as potential candidates. Unfortunately, the unavailability of good antibodies against these isoforms prevents us to determine if they are present in Ltk^- cells.

Opposite effects of a kinase on distinct adenylyl cyclase isoforms would not be unique. Indeed, phosphorylation by yet another Ca^{2+}-sensitive kinase, protein kinase C, was shown to promote activation of the adenylyl cyclase type II (Jacobowitz and Iyengar, 1994), V (Iwami et al., 1995) and VII (Chern, 2000; Hellevuo et al., 1995) but to inhibit adenylyl cyclase type VI (Lai et al., 1997).

The modest uncoupling of β_2-adrenoceptor observed in the competition studies, which was found not to be blocked by KN-62, indicates that it did not result from CamKinase activation. In fact, it did not seem to have a major impact on the adenylyl cyclase activation since the effects of the Ca^{2+} ionophore and KN-62 on receptor-stimulated adenylyl cyclase were found to be very similar to those observed for the forskolin-stimulated activity.

The Ca^{2+}-binding proteins recoverin and calmodulin were previously reported to modulate the activity of the G-protein-coupled receptor kinases (Levay et al., 1998; Pronin et al., 1997). However, we found no evidence for a regulatory action of Ca^{2+} on the agonist-promoted desensitization of the β_2-adrenoceptor in Ltk^- cells, suggesting that the activity of the G-protein-coupled receptor kinases involved in this process is not significantly affected by the increase in intracellular Ca^{2+}.

In summary, we revealed the existence of a novel adenylyl cyclase sensitizing process mediated by CamKinase activation in Ltk^- cells. The identity of the adenylyl cyclase and CamKinase isoforms involved in this process still remain to be determined. Whether this regulatory mechanism is restricted to Ltk^- cells or is a more general phenomenon also remains an open question.

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