Affinity isolation of imidazoline binding proteins from rat brain using 5-amino-efaroxan as a ligand

Lara K. Monks[a], Karen E. Cosgrove[c], Mark J. Dunne[c], Christopher A. Ramsden[b], Noel G. Morgan[a,*], Sue L.F. Chan[a]

[a] *Cellular Pharmacology Group, School of Life Sciences, Keele University, Staffordshire ST5 5BG, UK*
[b] *School of Chemistry and Physics, Keele University, Staffordshire ST5 5BG, UK*
[c] *Dept. of Biomedical Science and the Institute of Molecular Physiology, University of Sheffield, Sheffield S10 2TN, UK*

Received 14 January 1999; received in revised form 12 February 1999

Abstract We have employed an amino derivative of the imidazoline ligand, efaroxan, to isolate imidazoline binding proteins from solubilised extracts of rat brain, by affinity chromatography. A number of proteins were specifically retained on the affinity column and one of these was immunoreactive with an antiserum raised against the ion conducting pore component of the ATP-sensitive potassium channel. Patch clamp experiments confirmed that, like its parent compound, amino-efaroxan blocks ATP-sensitive potassium channels in human pancreatic β-cells and can stimulate the insulin secretion from these cells. The results reveal that a member of the ion conducting pore component family is strongly associated with imidazoline binding proteins in brain and in the endocrine pancreas.

© 1999 Federation of European Biochemical Societies.

Key words: I site; Imidazoline; ATP-sensitive potassium channel; Insulin secretion; Efaroxan

1. Introduction

Imidazoline binding sites are present in a range of tissues and have emerged over recent years as candidates for a new class of receptors having a unique pharmacology [1–5]. They have been subclassified according to their ligand recognition properties into two principal subtypes (I_1 and I_2 binding sites) and these are found in many regions of the central nervous system (CNS) [1–10] as well as in peripheral tissues such as the adrenal medulla, liver, kidney, platelets and placenta [11–17]. In most cases, their presence has been demonstrated by ligand binding although functional responses have also been defined in some tissues. Perhaps the best characterised of these is the stimulation of insulin secretion by imidazolines such as efaroxan and phentolamine [18–22], a response ascribed to the activation of an atypical subtype of the imidazoline binding site, recently designated I_3 [4] to differentiate it from the I_1 and I_2 subtypes expressed elsewhere.

There is evidence that the I_3-imidazoline binding site in pancreatic β-cells may be associated with ATP-sensitive potassium (K_{ATP}) channels since, when islets are treated with imidazoline insulin, secretogogues K_{ATP} channels close [19–26]. This results in membrane depolarisation, calcium influx and insulin secretion (reviewed in [4,27,28]). Thus, binding of ligands to the I_3 site appears to regulate the opening of K_{ATP} channels.

Recent work has revealed that K_{ATP} channels are multimeric proteins containing at least two different subunits, a regulatory ATP binding cassette component, designated SUR (which contains the binding site for sulphonylureas and occurs in at least three different isoforms) and a ion conducting pore component, Kir6.x (reviewed in [29–32]). Two genes have been identified which encode members of the Kir6.x family. One of these (Kir6.1) is expressed ubiquitously [33] and the second, Kir6.2 (molecular weight approximately 48 kDa), is more restricted in distribution, being localised principally in neuroendocrine cells [34]. It has not been established whether an additional subunit is required to confer sensitivity to imidazolines but there is strong evidence that the imidazoline binding site does not reside on SUR. Thus, imidazolines do not displace sulphonylurea binding in β-cells [35] and more significantly, phentolamine has been reported to induce closure of a truncated form of the pancreatic β-cell channel, Kir6.2, expressed in the absence of SUR [26]. This evidence has been interpreted to indicate that the binding site for imidazolines may lie within the ion conducting subunit of the channel, although this remains to be demonstrated directly.

In order to address this issue, we have used an affinity isolation approach to identify proteins that can interact with the imidazoline efaroxan. Efaroxan binds with high affinity to I_1-imidazoline binding sites [36] but is also an effective insulin secretagogue by virtue of its activity at I_3 sites [4,28]. Thus, it represents a useful ligand for identification of imidazoline binding proteins. We have employed rat brain extracts as the source of imidazoline binding sites since I sites, Kir6.2 and SUR1 are present in this tissue.

2. Materials and methods

2.1. Affinity chromatography

An efaroxan affinity column was generated by coupling 5′-amino-efaroxan (KU08C, synthesised as described in [37]) to Aminolink (Pierce, USA), according to the manufacturer's instructions. Membranes were prepared by homogenising rat brains in 50 mM Tris buffer, pH 7.4, supplemented with protease inhibitors (final concentrations of phenylmethylsulphonyl fluoride 200 μM, aprotonin 10 μg/ml, leupeptin 1 μM, EDTA 1 mM)) (Sigma, UK). Following an initial centrifugation step at $4000 \times g$ for 10 min at 4°C, the supernatant was centrifuged at $40\,000 \times g$ for 20 min at 4°C. The membrane pellet was then solubilised in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors and a final concentration of 0.5% CHAPS (w/v) (Sigma, UK). The suspension was then centrifuged at $40\,000 \times g$ and the supernatant retained. A protein determination was performed using the BCA protein assay kit (Pierce) and bovine serum albumin as a standard.

All buffers for the affinity chromatography contained 50 mM Tris-HCl, pH 7.4, with protease inhibitors (as above) and 0.05% (w/v) CHAPS and all procedures were performed at 4°C. 50 mg of solubilised brain protein was applied to the column. The column was then

*Corresponding author. Fax: (44) (01782) 583516.
E-mail: n.g.morgan@keele.ac.uk

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)00264-1

washed to remove non-specifically bound proteins and the retained proteins were eluted sequentially with the α_2-adrenoceptor agonist UK14304 (1 mM, donated by Pfizer, UK), the mixed adrenoceptor/imidazoline ligand efaroxan (100 μM, synthesised by Dept. of Chemistry, Keele University) and finally with 1 M KCl. All three fractions were collected, dialysed, concentrated and analysed by SDS-PAGE using a 10% (v/v) polyacrylamide gel. Pre-stained molecular weight markers (10 μl Gibco BRL) were run alongside the samples. Samples were run in duplicate so that one gel could undergo silver staining for protein detection, whilst the proteins on the second gel were transferred to a PVDF membrane. The membrane was incubated for 1 h at room temperature in 2% (v/v) goat serum in Tris-buffered saline supplemented with 0.1% (v/v) Tween X-100 (TTBS) to block non-specific binding sites. It was then incubated with polyclonal anti-Kir6.2 antisera (diluted 1:1000 in 1% (v/v) goat serum in TTBS), overnight at 4°C. After several washes in TTBS, the membrane was incubated with a secondary goat anti-rabbit antisera (diluted 1:30 000 in 1% (v/v) goat serum in TTBS) for 1 h at room temperature. The membrane was washed sequentially in TTBS and then TBS and the blot was analysed by a colorimetric method (BCIP/NBT (Sigma)) according to the manufacturers protocol.

2.2. Electrophysiology

All data were obtained from primary cultured human islet cells using the inside-out recording configuration of the patch clamp technique. The patch pipette was filled with a solution consisting of (in mM) 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 $CaCl_2$, 10 *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid (HEPES), 2.5 glucose (pH 7.4 with NaOH). Efaroxan was dissolved in water and KU08C dissolved in dimethylsulphoxide (DMSO) to produce 100 mM stock solutions which were serially diluted into working solutions containing (in mM) 0.001–0.1 efaroxan or KU08C, 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 HEPES, 2.5 glucose, 1 ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) (pH 7.2 with KOH). The maximum concentration of DMSO was $< 1\%$, which has no effect on K_{ATP} channels [38]. To minimise the effects of channel run down [39], inside-out patches were continuously exposed to 0.5 mM ATP (K^+ salt, Sigma, UK) and ATP was removed only during those periods when the patch was exposed, for a minimum of 20 s, to control solutions or various concentrations of efaroxan or KU08C.

Data were recorded at 0 mV voltage clamp using a List L/M EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). All experiments were carried out at room temperature (19–25°C). Single channel currents were filtered at 70 Hz low pass and changes in K_{ATP} channel open state probability were estimated 'off-line' by analysis of 25–30 s stretches of continuous data using a CED 1401 interface and designated software (Cambridge Electronic Design, Cambridge, UK) as described previously [38].

Average concentration/response data are expressed as a percentage of the initial control value and plotted using Excel (Microsoft Corporation, USA) as mean values ± S.E.M. Unpaired Student's *t*-tests were carried out to determine the significance of differences in the K_{ATP} channel activity at equivalent concentrations of efaroxan and KU08C. All current traces are displayed as upward deflections, which represent outward current events.

2.3. Insulin secretion studies

Isolated human pancreatic islets were provided by Drs. R.F.L. James, S. Swift and H. Clayton (Dept. of Surgery, University of Leicester) and were incubated in groups of 3 in 0.5 ml of incubation medium as described previously [40]. Following incubation at 37°C with appropriate test reagents, samples of the medium were removed and assayed for insulin by a radioimmunoassay. Crystalline biosynthetic human insulin was used as a standard.

3. Results and discussion

3.1. The Effect of KU08C on insulin secretion

The imidazoline derivative efaroxan is a potent antagonist at imidazoline I_1 sites [36] whereas it shows a significant agonist activity at the putative imidazoline I_3 sites expressed in pancreatic β-cells [4,19,20,22]. Thus it seems likely that derivatives of efaroxan will be useful reagents for affinity purification of these, still elusive, imidazoline receptor classes. In the present work, we have coupled efaroxan to an affinity resin via an amino group substituted at position five of the parent molecule to generate the derivative KU08C [37]. KU08C was previously shown to retain the ability of efaroxan to stimulate insulin secretion from isolated rat islets, albeit with a slightly reduced potency [37]. We now demonstrate that KU08C is also agonistic in human islets (Fig. 1) since it was able to dose-dependently reverse the inhibition of glucose-induced insulin secretion mediated by the K_{ATP} channel agonist diazoxide. This effect is characteristic of imidazoline insulin secretagogues and the results are consistent with the possibility that KU08C is able to regulate the opening of K_{ATP} channels in human pancreatic β-cells.

3.2. Patch clamp analysis of the effects of KU08C on human pancreatic β-cell K_{ATP} channels

In order to confirm that KU08C is able to inhibit K_{ATP} channels in human pancreatic β-cells, electrophysiological recordings were performed on isolated, cultured human β-cells. These were derived from the same preparations of islets used in the insulin secretion studies described above, to allow direct comparison of the data. A total of 22 recordings were made using cell-free inside-out membrane patches. Under the experimental conditions described, K_{ATP} channels were spontaneously active and could be reversibly inhibited by ATP (0.5 mM) applied to the inside of the patch of the membrane. Direct application of either efaroxan (0.1 mM) or KU08C (0.1 mM) produced a reversible inhibition of channel activity in $n = 8/8$ and $n = 5/5$ patches, respectively. To minimise run down of K_{ATP} channels, inside-out patches were exposed to 0.5 mM ATP unless the actions of efaroxan or KU08C were

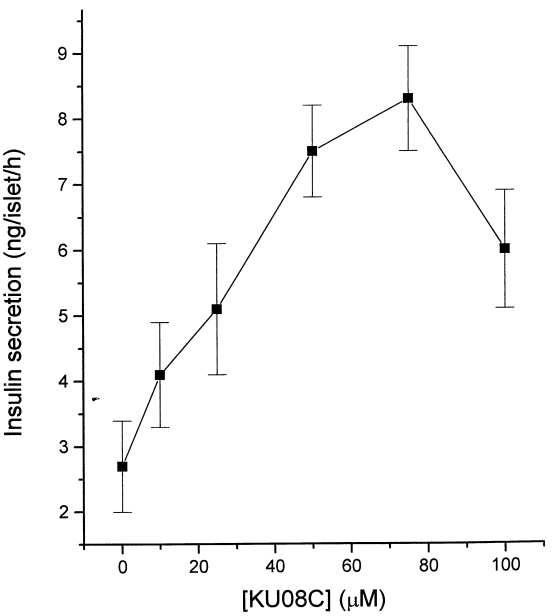

Fig. 1. Stimulation of insulin secretion from isolated human islets by KU08C. Groups of three isolated human pancreatic islets were incubated with 20 mM glucose and 200 μM diazoxide and then increasing concentrations of KU08C, as shown. Following incubation for 1 h at 37°C, samples of the medium were removed and assayed for insulin content by a radioimmunoassay. Each point represents the mean rate of insulin secretion ± S.E.M. for six observations. Similar results were obtained with two separate batches of islets.

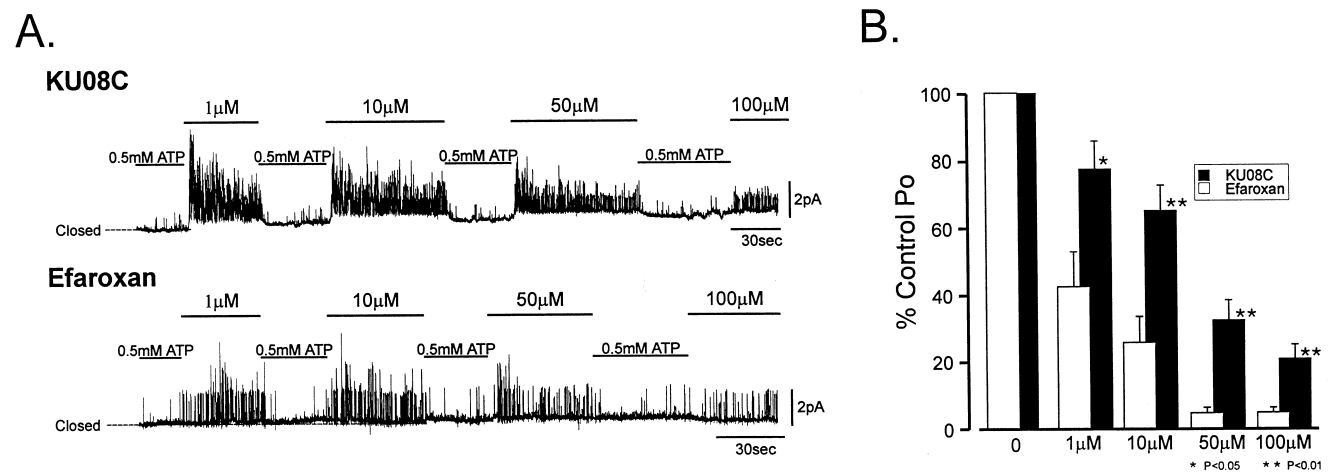

Fig. 2. Effects of KU08C and efaroxan on ATP-sensitive potassium channel activity in isolated human pancreatic β-cells. The opening of human β-cell K_{ATP} channels was monitored by patch clamp recording in the presence and absence of 0.5 mM ATP and increasing concentrations of either KU08C (A, upper trace) or efaroxan (A, lower trace). The summated results from a number of experiments is shown in B which allows a comparison of the dose-response relationship for channel closure by each drug. The level of channel opening in the absence of drug (Po) is given as 100%. $*P < 0.05$; $**P < 0.01$ versus absence of KU08C.

studied (Fig. 2A). Under these conditions, concentration/response (0.001–0.1 mM) profiles were generated for both efaroxan ($n = 9$) and KU08C ($n = 7$). Analysis of these data ($n = 7$, Fig. 2B) indicated that in human islet cells, the approximate half maximal blocking concentrations were 1 μM for efaroxan and 25 μM for KU08C. Thus, these electrophysiological studies confirm that the mode of action of KU08C involves inhibition of K_{ATP} channels in β-cells. Overall, KU08C is somewhat less potent than equivalent concentrations of efaroxan, but nevertheless the compound is a significant modulator of β-cell K_{ATP} channels.

3.3. *Affinity chromatography with KU08C*

Pancreatic islets are available in only relatively small quantities and are unlikely to serve as a useful source of protein for affinity purification purposes unless the target is expressed at very high levels. By contrast, rat brain is an abundant source of tissue and it expresses both I_1- and I_2-imidazoline binding sites, as well as K_{ATP} channels. Thus, it represents an appropriate source of starting material for affinity purification of imidazoline binding proteins.

Previous work has shown that KU08C possesses a negligible affinity for I_2 sites and is only weakly potent as an α_2-adrenoceptor ligand [41]. Thus, affinity resins based on KU08C are unlikely to specifically retain either of these types of receptor. However, it is possible that both I_1- and I_3-imidazoline binding sites could be retained. When solubilised extracts of rat brain were applied to an affinity column derived from KU08C, a number of proteins were retained which could then be eluted on subsequent application of imidazoline reagents or high salt concentrations (Fig. 3A). A major species with an approximate molecular weight of 60 kDa was detected in all eluates and silver staining of SDS-PAGE gels revealed a range of additional proteins (30 kDa–70 kDa). None of these proteins was immunoreactive with an anti-I_2-imidazoline receptor serum (kindly provided by Dr D. Reis, Cornell University Medical School) nor with an anti-α_2-adrenoceptor antibody (from Santa-Cruz) consistent with the expectation that such molecules would not be readily retained on the resin. However, Western blotting of eluted proteins with an antiserum raised against the pore forming subunit of β-cell K_{ATP} channels, Kir6.2 (kindly provided by Prof. S. Seino, Chiba University, Japan) revealed a single immunoreactive species (molecular weight ~50 kDa) which could be eluted from the column by efaroxan (Fig. 3B).

These results suggest that efaroxan can interact with Kir6.2 or with a closely related member of the Kir6.x family in rat brain extracts. We suggest that this is likely to be Kir6.2 itself since the antibody used to detect the protein was raised against the C-terminal sequence of Kir6.2 (S. Seino, personal communication) and this region represents that over which Kir6.2 and Kir6.1 are most divergent. Moreover, the finding

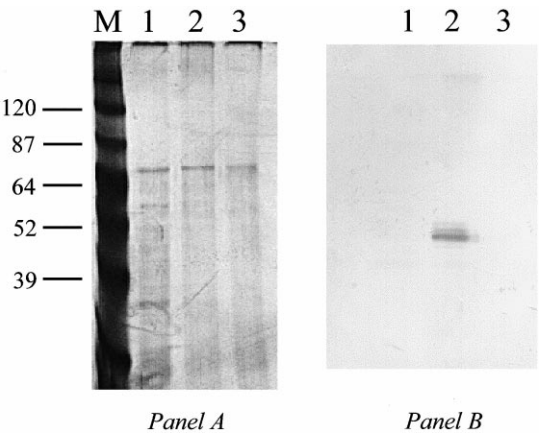

Fig. 3. Retention of proteins present in extracts of rat brain on a KU08C affinity column. Solubilised extracts of rat brain were applied to an affinity matrix containing KU08C and, after extensive washing, retained proteins were eluted with UK14304 (1 mM, lane 1) efaroxan (100 μM, lane 2) or KCl (1 M, lane 3). Eluted proteins were dialysed and concentrated prior to electrophoresis on SDS gels. A shows the protein profile of each eluate after silver staining of the gel. B shows a Western blot of each eluate probed with a polyclonal antiserum raised against Kir6.2 (diluted 1:2000) after transfer to a PVDF membrane. Immunoreactive bands were detected colorimetrically using a secondary antibody coupled to alkaline phosphatase. Similar results were obtained with three different brain preparations.

that KU08C exhibits a significant functional activity in pancreatic β-cells supports the involvement of Kir6.2 as a site of action, since Kir6.2 forms the ion conducting pore component of β-cell K_{ATP} channels.

Despite the considerations above, it should be emphasised that the data do not formally exclude the possibility that the imidazoline binding site controlling K_{ATP} channels may reside on a separate protein which is tightly associated with Kir6.2 in the brain, rather than with Kir6.2 itself. In either case, the present data offer the first direct biochemical evidence that components of the K_{ATP} channel can represent important targets for imidazoline molecules.

Acknowledgements: Financial support from the Wellcome Trust (LKM, NGM, SLFC) and British Diabetic Association (NGM, CAR, MJD) is gratefully acknowledged. KE Cosgrove was supported by an MRC research studentship. We also thank Drs Roger James, Sue Swift and Heather Clayton for the provision of human islets and Prof. S. Seino for a gift of the anti-Kir6.2 serum.

References

[1] Bousquet, P. (1995) J. Cardiovasc. Pharmacol. 26, S1–S6.
[2] Molderings, G.J. (1997) Drugs Future 22, 757–772.
[3] Parini, A., Moudanos, C.G., Pizzinat, N. and Lanier, S.M. (1996) Trends Pharmacol. Sci. 17, 13–16.
[4] Eglen, R.M., Hudson, A.L., Kendall, D.A., Nutt, D.J., Morgan, N.G., Wilson, V.G. and Dillon, M.P. (1998) Trends Pharmacol. Sci. 19, 381–390.
[5] French, N. (1995) Pharmacol. Ther. 68, 175–208.
[6] Escriba, P.V., Ozaita, A., Miralles, A., Reis, D.J. and Garcia-Sevilla, J.A. (1995) Mol. Brain Res. 32, 187–196.
[7] Greney, H., Bennai, F., Molines, A., Belcourt, A., Dontenwill, M. and Bousquet, P. (1994) Eur. J. Pharmacol. 265, R1–R2.
[8] Lione, L.A., Nutt, D.J. and Hudson, A.L. (1998) Eur. J. Pharmacol. 353, 123–135.
[9] Ernsberger, P.R., Graves, M.E., Graff, L.M., Zakieh, N., Nguyen, P., Collins, L.A., Westbrooks, K.L. and Johnson, G.G. (1995) Ann. New York Acad. Sci. 763, 22–42.
[10] Ugedo, L., Pineda, J., Ruiz-Ortega, J.A. and Martin-Ruiz, R. (1998) Br. J. Pharmacol. 125, 1685–1694.
[11] Gothert, M. and Molderings, G.J. (1991) Naunyn-Schmiedeberg's Arch. Pharmacol. 343, 271–282.
[12] Coupry, I., Podevin, R.A., Dausse, J.P. and Parini, A. (1987) Biochem. Biophys. Res. Commun. 147, 1055–1060.
[13] Evans, R.G. and Haynes, J.M. (1994) Clin. Exp. Pharmacol. Physiol. 21, 649–658.
[14] Allan, D.R., Penner, S.B. and Smyth, D.D. (1993) Br. J. Pharmacol. 108, 875.
[15] Head, G.A. (1995) Ann. New York Acad. Sci. 763, 531–540.
[16] Piletz, J.E., Andorn, A.C., Unnerstall, J.R. and Halaris, A. (1991) Biochem. Pharmacol. 42, 569–584.
[17] Diamant, S., Eldar-Geva, T. and Atlas, D. (1992) Br. J. Pharmacol. 106, 101–108.
[18] Schulz, A. and Hasselblatt, A. (1988) Naunyn-Schmiedeberg's Arch. Pharmacol. 337, 637–643.
[19] Chan, S.L.F. and Morgan, N.G. (1990) Eur. J. Pharmacol. 176, 97–101.
[20] Berdeu, D., Gross, R., Ribes, G., Loubatieres-Mariani, M.-M. and Bertrand, G. (1994) Eur. J. Pharmacol. 254, 119–125.
[21] Rustenbeck, I., Kopp, M., Polzin, C. and Hasselblatt, A. (1998) Naunyn-Schmiedeberg's Arch. Pharmacol. 358, 390–393.
[22] Mourtada, M., Smith, S.A. and Morgan, N.G. (1998) Eur. J. Pharmacol. 350, 251–258.
[23] Dunne, M.J. (1991) Br. J. Pharmacol. 103, 1847–1850.
[24] Plant, T.D., Jonas, J.-C. and Henquin, J.-C. (1991) Br. J. Pharmacol. 104, 385–390.
[25] Jonas, J.-C., Plant, T.D. and Henquin, J.-C. (1992) Br. J. Pharmacol. 107, 8–14.
[26] Proks, P. and Ashcroft, F.M. (1997) Proc. Natl. Acad. Sci. USA 94, 11716–11720.
[27] Dunne, M.J., Harding, E., Jagger, J., Squires, P., Liang, R., Kane, C., London, N.J.M. and James, R.F.L. (1995) Ann. New York Acad. Sci. 763, 243–262.
[28] Morgan, N.G., Chan, S.L.F., Mourtada, M., Monks, L.M. and Ramsden, C.A. (1999) Ann. N.Y. Acad. Sci. (in press).
[29] Babenko, A.P., Aguilar-Bryan, L. and Bryan, J. (1998) Ann. Rev. Physiol. 60, 667–687.
[30] Clement, J.P., Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguilar-Bryan, L. and Bryan, J. (1997) Neuron 18, 827–838.
[31] Aguilar-Bryan, L., Clement, J.P., Gonzalez, G., Kunjilwar, K., Babenko, A. and Bryan, J. (1998) Physiol. Rev. 78, 227–245.
[32] Ashcroft, F.M. and Gribble, F.M. (1998) Trends Neurosci. 21, 288–294.
[33] Inagaki, N., Tsuura, Y., Namba, N., Masuda, K., Gonoi, T., Horie, M., Seino, Y., Mizuta, M. and Seino, S. (1995) J. Biol. Chem. 270, 5691–5694.
[34] Inagaki, N., Gonoi, T., Clement, J.P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S. and Bryan, J. (1995) Science 270, 1166–1170.
[35] Brown, C.A., Chan, S.L.F., Stillings, M.R., Smith, S.A. and Morgan, N.G. (1993) Br. J. Pharmacol. 110, 1017–1022.
[36] Ernsberger, P., Graves, M.E., Graff, L.M., Zakieh, N., Nguyen, P., Collins, L.A., Westbrooks, K.L. and Johnson, G.D. (1995) Ann. New York Acad. Sci. 763, 22–42.
[37] Chan, S.L.F., Pallet, A.L., Clews, J., Ramsden, C.A., Chapman, J.C., Kane, C., Dunne, M.J. and Morgan, N.G. (1998) Eur. J. Pharmacol. 355, 67–76.
[38] Dunne, M.J., Illot, M.C. and Peterson, O.H. (1987) J. Membr. Biol. 99, 215–224.
[39] Findlay, I. and Dunne, M.J. (1986) Pflug. Arch. 407, 238–240.
[40] Lacey, R.J., Cable, H.C., James, R.F., London, N.J.M., Scarpello, J.H.B. and Morgan, N.G. (1993) J. Endocrinol. 138, 555–563.
[41] Tyacke, R.J., Nutt, D.J., Chan, S.L.F., Ramsden, C.A., Morgan, N.G. and Hudson, A.L. (1997) Br. J. Pharmacol. 122, SS66.