Intracellular pH and the stimulus–secretion coupling in insulin-producing RINm5F cells

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The regulation of intracellular pH (pH_i) and its role in the insulin-secretory process were evaluated, by using the clonal insulin-secreting cell line RINm5F. Glyceraldehyde, lactate and dihydroxyacetone decreased pH_i , but only the first two released insulin. In the presence of extracellular Na^+ the cells counteracted the acidification. Blocking the Na^+/H^+ exchange in acidic cells resulted in a drastic further lowering of pH_i , an effect not obtained under basal conditions. Whereas glyceraldehyde depolarized the cells, lactate was without effect. Dihydroxyacetone hyperpolarized the cells in the presence of extracellular Na^+ , but this effect disappeared when Na^+ was excluded from the medium. Stimulation with glyceraldehyde resulted in increased free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). Dihydroxyacetone and lactate had no effect on $[Ca^{2+}]_i$ in the presence of Na^+ , but lactate induced a decrease in $[Ca^{2+}]_i$ in Na^+ -deficient medium. In RINm5F cells the activity of the Na^+/H^+ antiport could not be augmented by activation of protein kinase C (PKC). Hence, in insulin-secreting cells a PKC-insensitive Na^+/H^+ antiport is the major mechanism restoring a decrease in pH_i . Acidification itself does not affect membrane potential, but may directly interact with the mechanisms regulating exocytosis.

INTRODUCTION

In most cells intracellular pH (pH_i) is maintained in the range 7.0–7.4 [1]. This is higher than expected for passive distribution, suggesting that there are active pH_i -regulatory mechanisms operating in the cell. Known mechanisms regulating pH_i include the Na^+/H^+ and HCO_3^-/Cl^- antiporters located in the plasma membrane [1,2]. The relative importance of these mechanisms varies in different cell types [1,2].

It has been proposed that pH_i may serve as a regulator of various cellular processes such as proliferation [3,4], fertilization [2] and metabolism [2]. Also, in insulin-secreting cells changes in pH_i have been suggested to be involved in the regulation of the stimulus–secretion coupling [5]. For example, by blocking either the Na^+/H^+ or the HCO_3^-/Cl^- exchange with amiloride or 4,4'-di-isothiocyano-2,2'-stilbenedisulphonic acid ('DIDS') respectively, electrical activity and insulin release were induced at sub-threshold glucose concentrations. It was suggested that a decrease in pH_i , resulting from inhibition of the extrusion of protons generated by glucose metabolism, triggered electrical activity and insulin release [5]. However, actual changes in pH_i were never estimated under these conditions. It has been reported that an increase in the intracellular concentration of H^+ could be involved in decreasing the activity of the ATP-regulated K^+ channels [6]. Closure of these channels leads to depolarization, opening of voltage-activated Ca^{2+} channels, influx of Ca^{2+} , increase in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and eventually stimulation of insulin release [7]. Nevertheless, a direct effect of pH_i on the ATP-regulated K^+ channels is difficult to reconcile with the physiological response obtained in normal β -cells after glucose stimulation [8,9]. Under these conditions an increase in pH_i is paralleled by a decrease in channel activity [9].

The present study was performed to investigate how pH_i is regulated in insulin-secreting cells. In addition, we were interested

to clarify the extent to which changes in pH_i were directly paralleled by changes in membrane potential, $[Ca^{2+}]_i$ and thereby insulin release, with the aim of revealing a possible role of pH_i in the β -cell stimulus–secretion coupling.

MATERIALS AND METHODS

Materials

RPMI 1640 culture medium, penicillin, streptomycin and trypsin were obtained from Gibco, Paisley, Scotland, U.K. BCECF/AM and bisoxonol were bought from Molecular Probes, Junction City, OR, U.S.A. Fura-2/AM was from Calbiochem, La Jolla, CA, U.S.A. Dihydroxyacetone, glyceraldehyde, MgATP, PMA, lactate, SITS, nigericin and NH_4Cl were from Sigma, St. Louis, MO, U.S.A. Diazoxide was a gift from Schering Corp., Kenilworth, NJ, U.S.A.

Media

The basal medium used was a Hepes buffer, pH 7.4, containing (in mM) 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 25 Hepes and 3 glucose. The bicarbonate buffer contained, in addition to 25 mM- $NaHCO_3$, the same ions, although NaCl and Hepes were decreased to 100 mM and 5 mM respectively, as the basal medium. This buffer was gassed with CO_2/O_2 (1:19) to pH 7.4. The media were supplemented with 1 mg of BSA/ml. In the measurements of membrane potential, using the fluorescent dye bisoxonol (see below), BSA was omitted. Bisoxonol is a lipophilic anion, which increases its fluorescence upon association with lipids and proteins. BSA will therefore increase the non-cell-associated fluorescence in this type of experiment. When NaCl was omitted, it was replaced equimolarly by choline chloride.

Cell culture

The studies were performed with the clonal insulin-secreting

Abbreviations used: BCECF/AM, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; bisoxonol, bis-(1,3-diethylthiobarbiturate)-trimethineoxonol; $[Ca^{2+}]_i$, free cytoplasmic Ca^{2+} concentration; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; fura-2/AM, fura-2 acetoxymethyl ester; pH_i , intracellular pH; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

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Fig. 1. Effects of glyceraldehyde and NH_4Cl on pH_i .

(a) Basal medium containing both Ca^{2+} and Na^+ ; (b) medium deficient in Na^+ ; (c) medium deficient in Ca^{2+} ; (d) medium deficient in both Ca^{2+} and Na^+ .

Fig. 2. Effects of lactate, dihydroxyacetone and NH_4Cl on pH_i in the presence (a and b) and absence (c and d) of extracellular Na^+ .

cell line RINm5F, derived from a transplantable rat islet tumour [10]. The cells were grown in RPMI 1640 culture medium supplemented with 10% (v/v) fetal-calf serum, 100 I.U. of penicillin/ml and 100 μg of streptomycin/ml in culture flasks from Nunc, at 37 °C in an incubator with 5% CO_2 in air. In the experiments the cells were either used in suspensions or attached to plastic coverslips.

Measurements of pH_i , $[\text{Ca}^{2+}]_i$ and membrane potential

pH_i was measured with the fluorescent indicator BCECF/AM. The cells were loaded with 1 μM -BCECF/AM for 30 min and washed twice with the basal medium. They were then resuspended in 1.3 ml of buffer with or without Na^+ and Ca^{2+} . Measurements of pH_i were made at 37 °C in a polystyrene cuvette, in an Aminco-Bowman or a Perkin-Elmer LS-5B spectrofluorimeter slightly modified to allow constant stirring. The excitation and emission wavelengths were set at 500 and 530 nm respectively. The experiments performed on cells in suspension were calibrated by lysing the cells with Triton X-100 and titrating pH of the

medium by addition of NaOH and HCl. From corresponding pH and fluorescence measurements a graph was constructed and used for conversion of fluorescence values into pH_i values [8,11]. When coverslips were used, pH_i was calibrated by using the K^+/H^+ ionophore nigericin, which equilibrates H^+ and K^+ gradients over the cell membrane according to the formula: $[\text{H}^+]_o \times ([\text{K}^+]_i/[\text{K}^+]_o) = [\text{H}^+]_i$ [12], where the subscript i indicates intracellular concentration and o the extracellular concentration of the ion.

$[\text{Ca}^{2+}]_i$ was analysed with the fluorescent probe fura-2/AM. Cells were loaded for 30 min with 1 μM of the indicator and washed twice with basal medium. Measurements were performed in the same way as for pH_i , but the wavelengths were set at 340 and 380 nm for excitation and 510 nm for emission. The results of the $[\text{Ca}^{2+}]_i$ measurements are presented as changes in relative fluorescence.

Qualitative changes in membrane potential were measured with the fluorescent dye bisoxonol, at a final concentration of 150 nM. The excitation and emission wavelengths were set at 540

and 580 nm respectively. All traces are typical for experiments repeated at least three times.

Measurements of insulin release

The dynamics of insulin release were studied by perfusing RINm5F cells mixed with Bio-Gel P-4 polyacrylamide beads in a 0.5 ml column at 37 °C [7]. The flow rate was 0.2 ml/min, and 2 min fractions were collected and assayed for insulin radioimmunologically, with rat insulin as the standard.

RESULTS

pH_i and insulin measurements

Addition of glyceraldehyde evoked an immediate initial decrease in pH_i (Fig. 1), which was similar to that previously reported for both normal β -cells [8,13] and RINm5F cells [14]. In basal medium (a) and in Ca²⁺-deficient medium (c), the decrease

Fig. 3. Effects of glyceraldehyde (a), lactate (b) and dihydroxyacetone (c) on the dynamics of insulin release from column-perfused cell suspensions

Experiments were performed in basal medium, with additions made as indicated. The average insulin release during the first 6 min was taken as 100%, and subsequent values are given relative to this value. Results are means $\pm$ S.E.M. for three experiments.

was followed by an increase towards the basal level. In (b) Na⁺ was omitted and in (d) the medium was deficient in both Ca²⁺ and Na⁺. In neither of the latter situations was the initial decrease in pH_i followed by a slow increase. When Na⁺ is omitted from the extracellular medium, the Na⁺/H⁺ exchange is inhibited, which makes it possible to investigate the importance of this countertransport for pH_i regulation. Glyceraldehyde is often used as a glucose analogue in RINm5F cells and, in accordance with previous studies [15], it induced also insulin release under the present conditions (Fig. 3a).

In Fig. 2 the cells were stimulated with 10 mM-dihydroxyacetone or 10 mM-lactate in the presence (a and b) or absence (c and d) of extracellular Na⁺. Both substances induced a lowering in pH_i, although the effect of dihydroxyacetone was less pronounced. As in the experiments with glyceraldehyde, it was only in the Na⁺-containing media that the cells were able to extrude protons and thereby counteract the decrease in pH_i. With regard to interactions with the insulin-secretory process, the effects of lactate and dihydroxyacetone also confirmed previous work [16–19]. As evident from Fig. 3(b), lactate, like glyceraldehyde, induced insulin release, whereas dihydroxyacetone (Fig. 3c) did not stimulate release of the hormone.

In addition to inhibiting the Na⁺/H⁺ countertransport by omission of extracellular Na⁺, we used EIPA, which serves as a specific blocker of this transport [20]. Fig. 4 shows the effects of EIPA. When extracellular Na⁺ was present (Figs. 4a and 4b), blocking the Na⁺/H⁺ transporter with 50 μ M-EIPA (a) did not affect basal pH_i. However, when EIPA was added after glyceraldehyde had already induced a decrease in pH_i (b), there was a drastic further lowering in pH_i. As expected, EIPA had no effect in the absence of extracellular Na⁺. In Fig. 5, EIPA was added after dihydroxyacetone and lactate, and the same results, with a further decrease in pH_i in the Na⁺-containing medium and no effect when Na⁺ was omitted, were obtained. These experiments show that the activity of the Na⁺/H⁺ exchange is low in the clonal insulin-secreting RINm5F cells under steady-state conditions, and consequently inhibition with EIPA does not affect pH_i. Upon acidification of the cells, the Na⁺/H⁺ transport is activated, an effect then inhibited by EIPA. These effects are not unique for insulin-producing cells, but have previously been reported also in other types of cells [21–23].

To verify that our results did not simply reflect a lack of HCO₃⁻ in the extracellular medium, we have done control experiments in medium containing HCO₃⁻ and gassed with 5% CO₂. Even if the basal pH_i was slightly higher, glyceraldehyde induced a similar acidification to that observed when a Hepes-buffered medium was used (Fig. 6a). Also, in the HCO₃⁻-containing medium subsequent addition of EIPA induced a further lowering in pH_i (Fig. 6c). As shown in Figs. 6(b) and 6(d), glyceraldehyde and EIPA had similar effects when cells were pretreated for 1.5 h with 0.5 mM-SITS, an inhibitor of HCO₃⁻-dependent transporters [24,25].

Extracellular ATP is a purinergic-receptor agonist, which through phospholipase C stimulates the cleavage of PtdInsP₂ to InsP₃ and diacylglycerol [26–28]. Diacylglycerol activates PKC [29,30], which has been shown to enhance the activity of the Na⁺/H⁺ antiport in a variety of cells [31–33]. An activation of the Na⁺/H⁺ countertransport can also be induced by phorbol esters such as PMA [31,34–36]. In Fig. 7 the effects of ATP and PMA on pH_i in Ca²⁺-deficient (a and b) and Ca²⁺- and Na⁺-deficient (c and d) media are shown. In none of the experimental situations was there any shift in pH_i. Although the results in the present study showed that the Na⁺/H⁺ exchange activity was undetectable under basal conditions and was stimulated when pH_i was low, the effect of PMA was studied after pH_i had been decreased by addition of glyceraldehyde (Fig. 7e). PMA at a concentration

Fig. 4. Effects of glyceraldehyde and EIPA on pH_i .

(a) and (b) show experiments performed in basal medium, and (c) and (d) show experiments in medium without Na^+ .

Fig. 5. Changes in pH_i after addition of lactate or dihydroxyacetone and EIPA in Na^+ -containing medium (a and c) or in medium without Na^+ (b and d).

of 10 nM, which is known to activate PKC specifically [29], was added. As no effect could be detected, a 10-fold higher concentration was tested. However, this higher concentration did not increase the rate of alkalinization. These results therefore strongly suggest that a PKC-insensitive Na^+/H^+ antiport is the major mechanism restoring a decrease in pH_i in the insulin-secreting RINm5F cells.

Measurements of membrane potential

The effect of glyceraldehyde, dihydroxyacetone and lactate on membrane potential in a medium with (Fig. 8) or without (Fig. 9) Na^+ was studied. As shown in Fig. 8, glyceraldehyde depolarized the cells, lactate had no effect and dihydroxyacetone evoked a slight hyperpolarization. When Na^+ was omitted (Fig. 9), the effect of glyceraldehyde and lactate remained the same, but the hyperpolarization by dihydroxyacetone disappeared. These results suggest that the glyceraldehyde-induced depolarization of the RINm5F cells is not mediated by a direct effect of the acidification itself on membrane potential.

Measurements of $[Ca^{2+}]_i$

Figs. 10(a), 10(c) and 10(e) show experiments performed in basal medium. Whereas addition of glyceraldehyde increased

$[Ca^{2+}]_i$, lactate and dihydroxyacetone had no effect. After removal of extracellular Na^+ , the effect of glyceraldehyde and dihydroxyacetone remained the same (Figs 10b and 10f). However, under these conditions lactate induced a decrease in $[Ca^{2+}]_i$ (Fig. 10d). Interestingly, although both glyceraldehyde and lactate induced acidification and insulin release, the latter compound caused neither depolarization nor an increase in $[Ca^{2+}]_i$. Hence, a lowering in pH_i does not automatically interfere with the ionic events involved in the stimulus–secretion coupling of insulin-secreting cells.

DISCUSSION

Metabolism of glucose leads to increased levels of cellular ATP and thereby closure of the specific ATP-regulated K^+ -channels [37–39]. Interestingly, the ATP-regulated K^+ channels are also the site of action of hypoglycaemic sulphonylureas [37]. Hence a decreased activity of the ATP-regulated K^+ channels can be viewed as a key event triggering the insulin-secreting process. It is noteworthy that the activity of the ATP-regulated K^+ channels is influenced not only by ATP but in a much more complex manner by a variety of other factors [37,40–42]. Of particular interest for the present study are previous observations

Fig. 6. Effects of glyceraldehyde and EIPA on pH_i in medium containing HCO_3^-

In (b) and (d) the cells were pretreated for 1.5 h with SITS.

Fig. 7. Effects of ATP and the phorbol ester PMA on pH_i in a Ca^{2+} -deficient medium (a and b) and in both Ca^{2+} - and Na^{+} -deficient medium (c and d)

In (e) two concentrations of PMA were tested on cells at low pH_i in basal medium.

Fig. 8. Effects of glyceraldehyde, lactate and dihydroxyacetone on membrane potential in basal medium as measured with bisoxonol

Fig. 10. Effects of glyceraldehyde, dihydroxyacetone and lactate on $[Ca^{2+}]_i$

In (a), (c) and (e) basal medium was used, and in (b), (d) and (f) Na^+ was omitted from the medium.

Fig. 9. Effects of glyceraldehyde, lactate and dihydroxyacetone on membrane potential in medium where Na^+ was omitted

demonstrating that a lowering of pH_i decreases and a rise increases the activity of these channels [6].

Most cells appear to have more than one system regulating pH_i , and the interaction between the different systems is complex.

According to Madhus [1], there is a general agreement that all cells possess a Na^+/H^+ antiport. In addition, most cells seem to have HCO_3^-/Cl^- exchangers, these anion exchangers being dependent on or independent of Na^+ [25,43]. The relative importance of different systems in regulating pH_i probably varies between different cell types [2,7,44]. In order to have access to a large number of homogeneous cells, we took the advantage of using the clonal insulin-producing cell line RINm5F. This cell line is often used as a model when investigating the mechanisms regulating the insulin-secretory process. RINm5F cells differ from normal β -cells in not responding to glucose [15], and therefore glyceraldehyde was used as an insulin secretagogue. In these cells stimulation with glyceraldehyde leads to acidification [14]. Whereas such an effect of glyceraldehyde is evoked also in normal β -cells [13], glucose in this case induces an alkalization [8,9]. Insulin secretion from glyceraldehyde-stimulated RINm5F cells involves basal mechanisms similar to those of glucose-induced insulin secretion in normal β -cells, and the clonal cells contain both ATP-regulated K^+ channels and voltage-dependent Ca^{2+} channels [37,46,47].

The alkalization induced by glucose stimulation in normal pancreatic β -cells mainly reflects activation of a Na^+/H^+ antiport that is not sensitive to PKC [9] and not a HCO_3^-/Cl^- exchanger [8,48]. To investigate further the role of the Na^+/H^+ transporter in insulin-secreting cells, Na^+ was omitted from the extracellular medium, resulting in blockage of the Na^+/H^+ exchange. This exchange can also be blocked by the amiloride analogue EIPA [20]. Whereas omission of Na^+ only promoted a slightly lower pH_i , this parameter was not affected to any major extent in the presence of EIPA. These results suggest that the activity of the Na^+/H^+ antiport is low under basal conditions. In other cells, such as normal pancreatic β -cells [9], lymphocytes [49], human fibroblasts [22] and rabbit gastric-gland cells [50], a varying degree of acidification was obtained under similar conditions. When Na^+/H^+ exchange was blocked by omission of extracellular Na^+ , the RINm5F cells were not able to counteract the intracellular acidification induced by glyceraldehyde, dihydroxy-

acetone or lactate. Moreover, blockage of Na^+/H^+ antiport with EIPA in acidified cells in the presence of extracellular Na^+ led to a further decrease in pH_i . These findings are compatible with the notion that the Na^+/H^+ antiport is activated to restore pH_i in RINm5F cells upon acidification. Similar results have been obtained in human fibroblasts [21,22] and rat thymic lymphocytes [23]. Hence, although the $\text{HCO}_3^-/\text{Cl}^-$ exchange is present in the insulin-secreting RINm5F cells, the Na^+/H^+ exchanger, as in the normal pancreatic β -cells [8,9], is the main mechanism restoring changes in pH_i .

When discussing possible mechanisms regulating pH_i , it is noteworthy that the Ca^{2+} concentration in the extracellular medium influenced neither cytoplasmic H^+ concentration nor Na^+/H^+ exchange, suggesting that transport of Ca^{2+} across the plasma membrane does not interfere with the regulation of pH_i . This is in accordance with previous investigations showing that the effect of glyceraldehyde on pH_i persisted in the presence of D-600, a blocker of the voltage-activated Ca^{2+} channels [8]. On the other hand, a decrease in pH_i may displace intracellularly bound Ca^{2+} and thus be responsible for the increase in $[\text{Ca}^{2+}]_i$ observed in RINm5F cells stimulated with glyceraldehyde in the presence of verapamil [46]. This fits well with the data from Prentki *et al.* [51], demonstrating that a decrease in pH_i promoted mobilization of Ca^{2+} from rat insulinoma mitochondria.

PKC has been shown to increase the activity of the Na^+/H^+ antiport in various cells, and the resulting increase in pH_i has been proposed to be the signal for mitogenesis [3,4,33]. Indeed, stimulation of the Na^+/H^+ exchange is a consistent and virtually universal finding early in the mitogenic process (for review see [52]). On the contrary, it has also been suggested that pH_i is too fundamental a parameter to act as an intracellular messenger [44]. Moreover, the significance of the stimulated Na^+/H^+ exchange is unclear under physiological conditions, and it has even been demonstrated that this stimulatory effect is not essential for the development of the growth response in mammalian cells in physiological media [52]. Nevertheless, as pointed out by Grinstein *et al.* [52], it is highly unlikely that this ubiquitous stimulation of the Na^+/H^+ exchange is functionally meaningless. More likely, the increase in antiport activity facilitates some of the reactions in the proliferative cascade. To test if the PKC system activates Na^+/H^+ exchange in RINm5F cells, this system was activated by stimulation with either extracellular ATP or the phorbol ester PMA. It should be noted that previous studies have demonstrated that PMA indeed activates PKC in these cells [53,54]. Since we were not able to detect an increased activity of the Na^+/H^+ antiport, after stimulation of the enzyme with either of these agonists, PKC-induced activation of the Na^+/H^+ exchange process is probably of minor importance in regulating pH_i in the clonal insulin-secreting cells. Moreover, we have recently obtained similar results in normal pancreatic β -cells [9].

In the present study, glyceraldehyde, lactate and dihydroxyacetone all decreased pH_i , but only the first two induced insulin secretion. Glyceraldehyde increased $[\text{Ca}^{2+}]_i$ and depolarized the cell, whereas lactate did not affect any of these parameters. It is noteworthy that diverging results can be found in the literature. Wollheim *et al.* [14] reported that lactate neither depolarized their RINm5F cells nor stimulated insulin release. On the contrary, in HIT cells lactate induced depolarization, increased $[\text{Ca}^{2+}]_i$ and stimulated insulin release [17]. These effects persisted when the Ca^{2+} - and ATP-regulated K^+ channels were blocked with tetraethylammonium and tolbutamide respectively. It was suggested that an electrogenic flux of lactate across the plasma membrane was the mediator of these effects [17,18]. Obviously we cannot fully confirm these results. This may indicate not only that HIT and RINm5F cells are different from each other and also from normal β -cells [9], with regard to changes in pH_i , but

also that various clones of RINm5F cells have a different response pattern. Of course this is a complicating factor, indeed emphasizing the difficulties arising when working with tumour cell lines instead of normal β -cells. Nevertheless, our results suggest that an acidification itself does not directly interfere with the regulation of membrane potential in insulin-secreting cells. However, our data do not exclude the possibility that changes in pH_i may still modulate the activity of the ATP-regulated K^+ channels and thereby influence the stimulus-secretion coupling [6]. Moreover, drastic changes in pH_i may interact with the molecular mechanisms directly regulating exocytosis. The latter hypothesis is supported by the fact that lactate induced insulin secretion without concomitant changes in membrane potential and $[\text{Ca}^{2+}]_i$. Interestingly, previous studies in both normal β -cells and macrophages have demonstrated that pH_i and intralysosomal pH respectively directly affect the secretory process [9,55].

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REFERENCES

- Madhus, I. H. (1988) *Biochem. J.* **250**, 1-8
- Roos, A. & Boron, W. (1981) *Physiol. Rev.* **61**, 296-434
- Pouyssegur, J., Chambard, J. C., Franchi, A., Paris, S. & van Obberghen-Schilling, E. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3935-3939
- Moolenaar, W. H., Tsien, R. Y., van der Saag, P. T. & de Laat, S. W. (1983) *Nature (London)* **304**, 645-648
- Pace, C. S. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 2379-2384
- Misler, S., Gilles, K. & Tabcharani, J. (1989) *J. Membr. Biol.* **109**, 135-143
- Arkhammar, P., Nilsson, T., Rorsman, P. & Berggren, P.-O. (1987) *J. Biol. Chem.* **262**, 5448-5454
- Arkhammar, P., Berggren, P.-O. & Rorsman, P. (1986) *Biosci. Rep.* **6**, 355-361
- Juntti-Berggren, L., Arkhammar, P., Nilsson, T., Rorsman, P. & Berggren, P.-O. (1991) *J. Biol. Chem.* **266**, 23537-23541
- Oie, H. K., Gazdar, A. F., Minna, J. D., Weir, G. C. & Baylin, S. B. (1983) *Endocrinology (Baltimore)* **112**, 1070-1075
- Rogers, J., Hesketh, T. R., Smith, G. A. & Metcalfe, J. C. (1983) *J. Biol. Chem.* **258**, 5994-5997
- Thomas, J. A., Buchsbaum, R. N., Zimniak, A. & Racker, E. (1979) *Biochemistry* **18**, 2210-2218
- Best, L., Bone, E. A., Meats, J. E. & Tomkinson, S. (1988) *J. Mol. Endocrinol.* **1**, 33-38
- Wollheim, C. B., Ullrich, S. J., Salomon, D. C., Halban, P. A., Tsien, R. Y. & Pozzan, T. (1984) *Diabetologia* **27**, 347A
- Praz, G. A., Halban, P. A., Wollheim, C. B., Blondel, B., Strauss, A. J. & Renold, A. E. (1983) *Biochem. J.* **210**, 345-352
- Malaisse, W. J., Kawazu, S., Herchuelz, A., Hutton, J. C., Somers, G., Devis, G. & Sener, A. (1979) *Arch. Biochem. Biophys.* **194**, 49-62
- Meats, J. E., Tuersley, M. D., Best, L., Lynch, A. M. & Tomlinson, S. (1989) *J. Mol. Endocrinol.* **3**, 121-128
- Best, L., Yates, A. P., Meats, J. E. & Tomlinson, S. (1989) *Biochem. J.* **259**, 507-511
- Malaisse, W. J., Herchuelz, A., Levy, J., Sener, A., Pipeleers, D., Devis, G., Somers, G. & van Obberghen, E. (1976) *Mol. Cell. Endocrinol.* **4**, 1-12
- Vigne, P., Frelin, C., Cragoe, E. J., Jr. & Lazdunski, B. (1983) *Biochem. Biophys. Res. Commun.* **116**, 86-90
- Moolenaar, W. H. (1986) *Trends Biochem. Sci.* **11**, 141-143

22. Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *J. Biol. Chem.* **259**, 7563–7569
23. Grinstein, S., Cohen, S. & Rothstein, A. (1984) *J. Gen. Physiol.* **83**, 341–369
24. Boyarsky, G., Ganz, M. B., Sterzel, R. B. & Boron, W. F. (1988) *Am. J. Physiol.* **255**, C844–C856
25. Boyarsky, G., Ganz, M. B., Sterzel, R. B. & Boron, W. F. (1988) *Am. J. Physiol.* **255**, C857–C869
26. Dubyak, G. R. & De Young, M. B. (1985) *J. Biol. Chem.* **260**, 10653–10661
27. Dubyak, G. R. (1987) *Arch. Biochem. Biophys.* **245**, 84–95
28. Arkhammar, P., Hallberg, A., Kindmark, H., Nilsson, T., Rorsman, P. & Berggren, P.-O. (1990) *Biochem. J.* **265**, 203–211
29. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
30. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
31. Grinstein, S., Cohen, S., Goetz, J. D., Rothstein, A. & Gelfand, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1429–1430
32. Zavoico, G. B., Cragoe, E. J., Jr. & Feinstein, M. B. (1986) *J. Biol. Chem.* **261**, 13160–13167
33. Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *Nature (London)* **312**, 371–374
34. Vara, F., Schneider, J. A. & Rozengurt, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2384–2388
35. Siffert, W., Siffert, G. & Scheid, P. (1987) *Biochem. J.* **241**, 301–303
36. Rosoff, P. M., Stein, L. F. & Cantley, L. C. (1984) *J. Biol. Chem.* **259**, 7056–7060
37. Rorsman, P. & Trube, G. (1989) in *Potassium Channels: Structure, Classification, Function and Therapeutic Potential* (Cook, N. S., ed.), pp. 96–116, Ellis Horwood, Chichester
38. Ashcroft, F. M., Harrison, D. E. & Ashcroft, S. J. H. (1984) *Nature (London)* **312**, 446–448
39. Cook, D. L. & Hales, C. N. (1984) *Nature (London)* **311**, 271–273
40. Bokvist, K., Ashcroft, F. M., Berggren, P.-O. & Rorsman, P. (1990) *Diabetologia* **33**, A78
41. Wollheim, C. B., Dunne, M. J., Peter-Riesch, B., Bruzzone, R., Pozzan, T. & Petersen, O. H. (1988) *EMBO J.* **7**, 2443–2449
42. Ribalet, B., Eddlestone, G. T. & Ciani, S. (1988) *J. Gen. Physiol.* **92**, 219–237
43. Ganz, M. B., Boyarsky, G., Sterzel, R. B. & Boron, W. F. (1989) *Nature (London)* **337**, 648–651
44. Thomas, R. C. (1989) *Nature (London)* **337**, 601
45. Reference deleted
46. Wollheim, C. B. & Pozzan, T. (1984) *J. Biol. Chem.* **259**, 2262–2267
47. Prentki, M. & Wollheim, C. B. (1983) *Experientia* **40**, 1052–1069
48. Lindström, P. & Sehlin, J. (1984) *Biochem. J.* **218**, 887–892
49. Grinstein, S. & Dixon, J. (1989) *Physiol. Rev.* **69**, 417–481
50. Paradiso, A. M., Tsien, R. Y. & Machen, T. E. (1987) *Nature (London)* **325**, 447–450
51. Prentki, M., Janjic, D. & Wollheim, C. B. (1983) *J. Biol. Chem.* **258**, 7597–7602
52. Grinstein, S., Rotin, D. & Mason, M. J. (1989) *Biochim. Biophys. Acta* **988**, 73–97
53. Regazzi, R., Li, G., Ullrich, S., Jaggi, C. & Wollheim, C. B. (1989) *J. Biol. Chem.* **264**, 9939–9944
54. Li, G., Regazzi, R., Ullrich, S., Pralong, W. F. & Wollheim, C. B. (1990) *Biochem. J.* **272**, 637–645
55. Tapper, H. & Sandler, R. (1990) *Biochem. J.* **272**, 407–414

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