Potentiation of P1075-induced K$^+$ channel opening by stimulation of adenylate cyclase in rat isolated aorta

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1 The effects of analogues and stimulators of cyclic AMP on the ^{86}Rb$^+$ efflux-stimulating and binding properties of P1075, an opener of ATP-dependent potassium channels, were studied in rat aortic rings. The increase in ^{86}Rb$^+$ efflux stimulated by P1075 was taken as a qualitative measure of K$^+$ channel opening.

2 Forskolin, a direct activator of adenylate cyclase, isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, and dibutyryl-cyclic AMP (db-cyclic AMP), a membrane permeant cyclic AMP-analogue, relaxed rat aortic rings contracted by noradrenaline with EC$_{50}$ values of 0.06, 2 and 10 μM, respectively.

3 Forskolin, IBMX and db-cyclic AMP produced concentration-dependent increases of the ^{86}Rb$^+$ efflux induced by P1075 (50 nM) by up to twofold with EC$_{50}$ values of about 0.1, 1.7 and 81 μM. At these concentrations the agents had little effect on the basal rate of ^{86}Rb$^+$ efflux.

4 The ^{86}Rb$^+$ efflux produced by P1075 in the presence of the cyclic AMP stimulators was inhibited by glibenclamide, a blocker of ATP-sensitive potassium channels.

5 IBMX (100 μM) induced a leftward shift of the concentration - ^{86}Rb$^+$ efflux curve of P1075 without increasing the maximum. The enhancements of P1075-stimulated ^{86}Rb$^+$ efflux produced by combinations of forskolin and IBMX were either additive or less than additive.

6 The protein kinase A inhibitor, H-89, inhibited P1075-stimulated ^{86}Rb$^+$ efflux in the presence of IBMX significantly more than in the absence of IBMX, suggesting that the effect of increased cyclic AMP levels is mediated by protein kinase A.

7 At high concentrations, forskolin and IBMX slightly increased basal ^{86}Rb$^+$ efflux and inhibited the tracer efflux induced by P1075.

8 Binding of [^{3}H]-P1075 to rat aortic rings was either unaffected or inhibited by forskolin, IBMX and db-cyclic AMP.

9 This study shows that moderate stimulation of the cyclic AMP system potentiates the K$^+$ channel opening effect of P1075 by activation of protein kinase A. The fact that binding of [^{3}H]-P1075 remains unchanged or is diminished favours the hypothesis that the K$^+$ channel openers activate ATP-dependent K$^+$ channels by an indirect mechanism.

Keywords: Cyclic AMP; potassium channel openers; P1075; dibutyryl-cyclic AMP; isobutylmethylxanthine; forskolin; H-89; glibenclamide; ^{86}Rb$^+$ efflux; P1075 binding

Introduction

The activity of many ion channels is modulated by phosphorylation (Rosenthal & Schultz, 1987; Levitan, 1994). This holds true also for the ATP-sensitive potassium channel (K$_{ATP}$ channel), a voltage-independent K$^+$ channel which is gated by the ratio of ATP to nucleoside diphosphates like ADP and which couples the metabolic state of the cell to excitability (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993a). First discovered in the heart (Noma, 1983), K$_{ATP}$ channels form a heterogeneous group; they are found in excitable cells like pancreatic β-cells and the various muscle types (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993a) and in non-excitable cells like kidney epithelial cells (Wang et al., 1990a,b; Tsuchiya et al., 1992) and follicular cells of the Xenopus oocyte (Honoré & Lazdunski, 1991; 1993). In various tissues, particularly in smooth muscle, K$_{ATP}$ channels are opened by a heterogeneous group of organic molecules, the K$^+$ channel openers which induce smooth muscle relaxation (for reviews see Quast, 1993; Edwards & Weston, 1994a). Both the K$^+$ channel opening and the vasorelaxant effects of these compounds are inhibited by the sulphonylurea, glibenclamide, with IC$_{50}$ values ranging from 20 to 200 nM according to the concentration of the agonist used (Quast & Cook 1989; Quast, 1995).

It is generally agreed that adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent phosphorylation is necessary to keep K$_{ATP}$ channels in a functional state and to prevent 'run-down' of the channel in various tissues (for review see Ashcroft & Ashcroft, 1990; Nichols & Lederer, 1991; Terzic & Kurachi, 1995; but see de Weille (1992) for the opposite view). There is some controversy whether increased cyclic AMP levels and subsequent phosphorylation by protein kinase A are sufficient to open K$_{ATP}$ channels. In follicular cells of Xenopus oocytes where the K$_{ATP}$ channel has a pharmacological profile broadly similar to that in vascular smooth muscle cells, cyclic AMP-dependent phosphorylation is sufficient to open the channel (Honoré & Lazdunski, 1991; 1993). Similarly, in rabbit mesenteric artery cells, protein kinase A activation induced an outward K$^+$ current which was abolished by 10 μM glibenclamide, suggesting that this intervention activated K$_{ATP}$ channels (Quayle et al., 1994). On the other hand, Edwards and colleagues have shown that dephosphorylating conditions like the intracellular application of the specific protein kinase A inhibitor, PKI(6-22)amide, led to the opening of K$_{ATP}$ channels in rat isolated portal vein cells (Edwards et al., 1993) and in insulinoma cells (Edwards & Weston, 1993b).

In an earlier investigation in rat portal vein we had found that the membrane permeant cyclic AMP analogue, dibutyryl-cyclic AMP (db-cyclic AMP) enhanced the ^{86}Rb$^+$ efflux stimulating effect of the K$_{ATP}$ channel opener, cromakalim, at some agonist concentrations; this effect was considered to be

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only of minor importance (Quast, 1987). In the present study in rat isolated aorta the potent pinacidil analogue, P1075, was used as the K^+ channel opener (Steinberg et al., 1988; Quast et al., 1993) and the cyclic AMP system was stimulated by db-cyclic AMP, forskolin (a direct stimulator of adenylyl cyclase), and isobutylmethylxanthine (IBMX, a phosphodiesterase inhibitor). Opening of K_{ATP} channels was assessed by measuring the increase in the rate constant of $^{86}Rb^+$ efflux from the preparation, a method which is a reliable qualitative measure of the increase in membrane K^+ permeability produced by the openers (Quast & Baumlin, 1988; for review see Quast, 1995). A preliminary account of some of the results has been presented to the German Pharmacological Society (Linde et al., 1994).

Methods

Preparation of rat aortic strips

Male Sprague-Dawley rats (350–500g) were decapitated and exsanguinated. The aorta was carefully removed and washed in HEPES-buffered PSS (in mM): NaCl 139, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11 and HEPES 5, continuously gassed with 95% O_2/5% CO_2 and titrated to pH 7.4 with NaOH at 37°C; for the $^{86}Rb^+$ efflux studies the buffer was changed to (in mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11 and HEPES 20. Adherent fat and the adventitia were removed and the aorta was cut into rings.

Tension studies

The endothelium of aortic rings (4 mm in length) was removed by carefully rubbing with a rough steel pin. The rings were mounted in a thermostatic organ bath for isometric tension recordings at 37°C under continuous gassing and a resting tension of 1 g. After a 60 min equilibration period, tissues were exposed to 1 μM noradrenaline for 5 to 10 min to reach maximum tension and then allowed to relax again. After 1 h, the rings were contracted by 0.1 μM noradrenaline. A maintained contraction was allowed to develop (15 ± 0.2 mN; $n = 36$). Tissues were then exposed to substances of interest using a cumulative (IBMX, forskolin) or non-cumulative (db-cyclic AMP) protocol (cumulative application of db-cyclic AMP resulted in an unusually steep relaxation curve). Complete relaxation was induced by addition of Na-nitroprusside (10 μM). Drug-induced relaxation was corrected for spontaneous decrease in tension by comparison with time-matched solvent controls using dimethylsulphoxide or ethanol (<0.1%) as the solvent.

$^{86}Rb^+$ efflux studies

Aortic rings (15–20 mm in length) with intact endothelium were incubated for 120 min at 37°C in PSS containing $^{86}Rb^+$(5 μCi ml^{-1}). The rings were transferred to thermostatic perfusion chambers and superfused with continuously gassed PSS at a rate of 2.5 ml min^{-1} at 37°C. The perfusate was collected at 2 min intervals and counted for radioactivity as previously described in detail (Quast, 1987). The data were expressed as the efflux rate coefficient (k, in 10^{-2} min^{-1}), which represents the radioactivity released per minute expressed as a percentage of the radioactivity remaining in the tissue. Effects of the openers were assessed by the area under the curve (AUC) of the k versus time plot. AUCs were cut out and their weights compared to that of a unit square ($\Delta k = 1 \times 10^{-2}$ $min^{-1} \times$ 100 min). At concentrations $\leqslant 150$ nM, P1075 was applied for 20 min, at concentrations >150 nM for 10 min. Modulation of P1075-stimulated efflux was assessed in a double-pulse protocol by comparison of the AUCs in the presence and absence of modulator (e.g. glibenclamide or cyclic AMP-increasing substances).

Modulation of $[^3H]$-P1075 binding in rat aortic strips

$[^3H]$-P1075 binding was performed in endothelium-denuded rat aortic strips (2–4 mg of wet weight) as described (Bray & Quast, 1992). The tissues were incubated in quadruplet at 37°C for 90 min under light gassing with 95% O_2/5% CO_2 in PSS containing $[^3H]$-P1075 (0.3 nM) and the unlabelled test substance. They were then washed in ice-cold PSS for 1 min, blotted, weighed and assigned to individual vials containing 0.3 ml of Soluence-350. After 2 h at 37°C, the samples were supplemented with 50 μl 1 M HCl and 3 ml Ultima Gold and counted for ^3H. Nonspecific binding (≈ 160 d.p.m. mg^{-1} of tissue wet weight) was determined in the presence of unlabelled P1075 (1 μM) and ranged between 27 and 35% of total binding.

Drugs and solutions

$[^3H]$-P1075 (specific activity, 105 Ci $mmol^{-1}$) and $^{86}RbCl$ were obtained from Amersham (Amersham, UK). Dibutyryl-cyclic AMP (db-cyclic AMP) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (Deisenhofen, Germany) and were dissolved in dimethylsulphoxide and further diluted into ethanol. Forskolin, a generous gift of Hoechst (Frankfurt, Germany), was dissolved in ethanol; the protein kinase A inhibitor H-89 (N-[(2-(p-bromocinnamylamino)ethyl)]-5-isoquinoline sulphonamide) was from Biomol (Hamburg, Germany) and was dissolved in ethanol/dimethylsulphoxide (1 : 1). In all cases, the final concentration of solvents never exceeded 0.5% and was without any pharmacodynamic effect. P1075 was kindly donated by Leo Pharmaceuticals (Ballerup, Denmark) and was dissolved in distilled water. Soluene-350 and Ultima Gold were from Packard (Groningen, The Netherlands).

Calculations and statistics

Results are expressed as mean ± s.e.mean. Concentration-dependencies were fitted to the Hill equation according to the method of linear least squares using the programmes Fig. P (Biosoft, Cambridge, U.K) or the RS/1 (BBN Inc, Cambridge, MA, U.S.A.). Errors in the fitting parameters were estimated using the univariate approximation (Draper & Smith 1981). Differences in tissue responses were assessed using Student's two-tailed unpaired t test. In calculations involving two mean values with standard errors, propagation of errors was taken into account according to Bevington (1969).

Results

Vasorelaxant effects of forskolin, IBMX and db-cyclic AMP

In order to assess the relevant concentration range of the cyclic AMP stimulants and analogues used in this study, concentration-relaxation curves were measured in rat aortic rings precontracted with noradrenaline (0.1 μM). As shown in Figure 1, forskolin was the most potent agent ($EC_{50} \approx 0.06$ μM) followed by IBMX ($EC_{50} \approx 2$ μM) and db-cyclic AMP ($EC_{50} \approx 10$ μM). Relaxation curves were regular in shape with Hill coefficients near unity.

Modulation of P1075-stimulated $^{86}Rb^+$ efflux by analogues and stimulators of cyclic AMP

Figure 2a shows traces where stimulation of the tissue by P1075 (50 nM, for 20 min) was followed by a recovery period and a second stimulus; in these control experiments, the second response was similar in magnitude to the first. The other traces in Figure 2 show the effect of forskolin (1 μM), IBMX (100 μM) and db-cyclic AMP (100 μM) superfused prior to the second stimulation with P1075 until to the end of the experiment. All agents increased P1075-stimulated $^{86}Rb^+$ efflux.
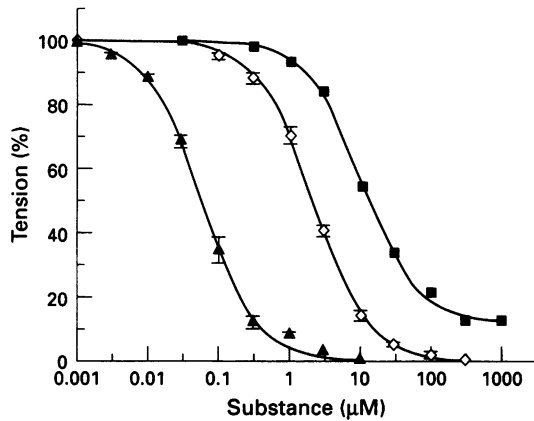
Figure 1 Relaxation of endothelium-denuded rat aortic rings precontracted by noradrenaline (100 nM) by (▲) forskolin, (◇) IBMX and (■) db-cyclic AMP. Hill analysis of the data yielded the following parameter values (forskolin/IBMX/db-cyclic AMP): Midpoints (EC$_{50}$, μM), $0.057 \pm 0.003/1.9 \pm 0.1/10 \pm 1$; maximum relaxations (% inhibition of initial tension), $97 \pm 1/98 \pm 1/88 \pm 3$; Hill coefficients, $1.11 \pm 0.06/1.13 \pm 0.07/1.09 \pm 0.09$. Data are means ± s.e.mean from 6–10 (forskolin and IBMX) and 4–5 experiments (db-cyclic AMP).
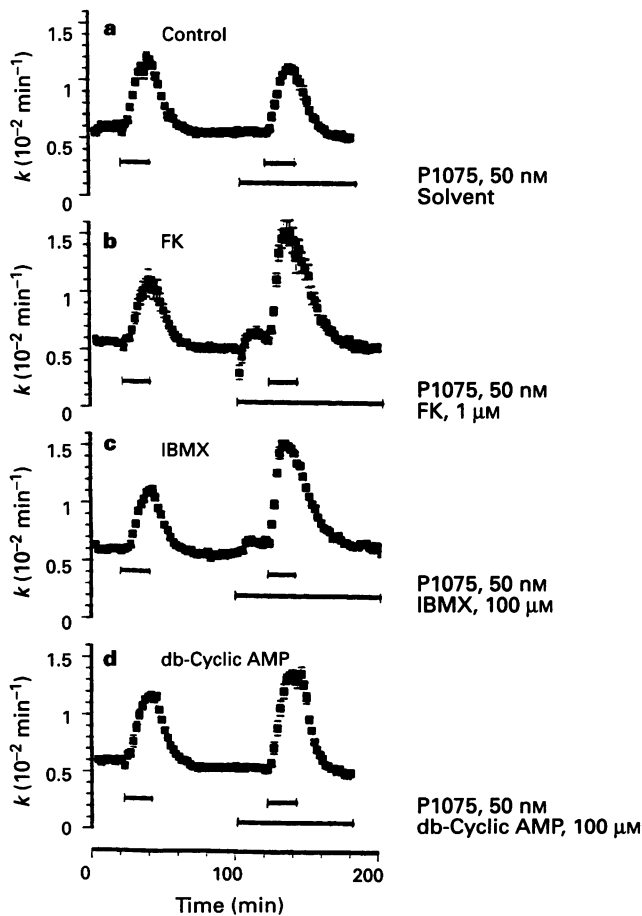

Figure 2 Mean traces showing modulation of P1075-stimulated ^{86}Rb⁺ efflux in rat aorta by analogues and stimulators of cyclic AMP. (a) Control: 50 nM P1075 was superfused for 20 min at the times indicated and gave a ratio of the areas under the curve AUC₂/AUC₁ = 1.03 ± 0.05 (9 experiments). (b-d) Effects of (b) forskolin (FK, 1 μM, 6 experiments), (c) IBMX (100 μM, 4 experiments) and (d) db-cyclic AMP (100 μM, 12 experiments) on the rate constant, k, of P1075-stimulated ^{86}Rb⁺ efflux. The agents increased the AUC ratio by $101 \pm 8\%$ (b), $96 \pm 13\%$ (c) and $56 \pm 7\%$ (d), respectively. Substances were superfused 20 min before the second application of P1075 until the end of the experiment.

Forskolin and IBMX alone induced a small increase in the rate of basal ^{86}Rb⁺ efflux which was well maintained during the application of the compound (Figure 2b and c); in the case of forskolin this was preceded by a transient inhibition. The small increase in basal ^{86}Rb⁺ efflux shown here with high concentrations of IBMX and forskolin is also produced by other interventions which massively increase cyclic AMP (Quast et al., unpublished).

The concentration-dependent modulation of P1075-stimulated tracer efflux by forskolin, IBMX and db-cyclic AMP is presented in Figure 3. The maximum effect of the three agents was an approximate doubling of the ^{86}Rb⁺ efflux produced by P1075 alone; the midpoints of the concentration-response curves were near 0.1 μM (forskolin), 1.7 μM (IBMX) and 80 μM (db-cyclic AMP). At concentrations at least 20 times higher than the respective EC$_{50}$ values, the stimulatory effects of forskolin and IBMX on P1075-induced tracer efflux were converted into inhibition; with db-cyclic AMP, such concentrations (>2 mM) could not be reached, due to limited solubility of the compound.

Figures 4 and 5 present the glibenclamide-sensitivity of the ^{86}Rb⁺ efflux produced by P1075 (50 nM) in the presence of stimulators of the cyclic AMP system. Figure 4a shows control traces of double stimulation experiments with P1075 in the presence of 100 μM db-cyclic AMP. In this case, the response to the second stimulus was slightly smaller than that to the first (AUC₂/AUC₁ = 95 ± 3%); therefore, the following results were corrected for this difference. Superfusion with glibenclamide (1 μM) 20 min prior to and during the second stimulation by P1075 completely abolished the response (Figure 4b). The concentration-dependence of this effect is shown in Figure 4, inset and yielded an IC$_{50}$ value of 79 ± 5 nM for glibenclamide. Figure 5 shows that glibenclamide (1 μM) essentially abolished the ^{86}Rb⁺ efflux response to P1075 (50 nM) also in the presence of maximally effective concentrations of forskolin (1 μM) and IBMX (100 μM). Inspection of the traces also shows that increases in basal flux induced by forskolin and IBMX were little, if at all, affected by glibenclamide.

Combination of forskolin and IBMX and dependence on P1075 concentration

The effects of the combined stimulation of synthesis and inhibition of degradation of cyclic AMP were studied by su-
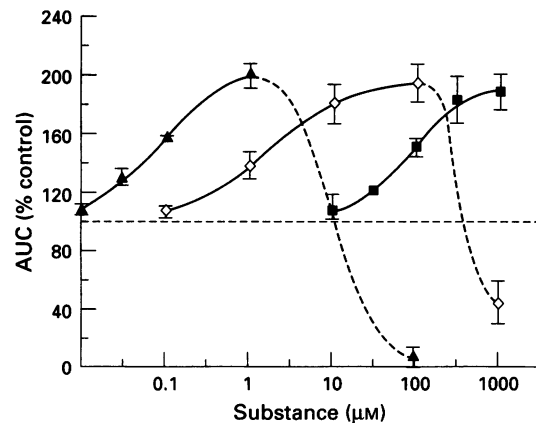

Figure 3 Concentration-dependent modulation of P1075-induced ^{86}Rb⁺ efflux by forskolin (▲), IBMX (◇) and db-cyclic AMP (■). The Hill fit of the ascending limbs of the curves yielded the following parameter values (forskolin/IBMX/db-cyclic AMP): Midpoints (EC$_{50}$, μM): $0.09 \pm 0.02/1.67 \pm 0.09/81.2 \pm 14.2$; maximum stimulation (% control): $201 \pm 9/197 \pm 13.0/191 \pm 12$; Hill coefficients: $1.10 \pm 0.08/0.88 \pm 0.03/1.21 \pm 0.19$. At higher concentrations of forskolin and IBMX, P1075-stimulated tracer efflux was inhibited. Experiments were performed as shown in Figure 2; the data are means ± s.e.mean from 4–6 (forskolin, IBMX) and 4–9 experiments (db-cyclic AMP).
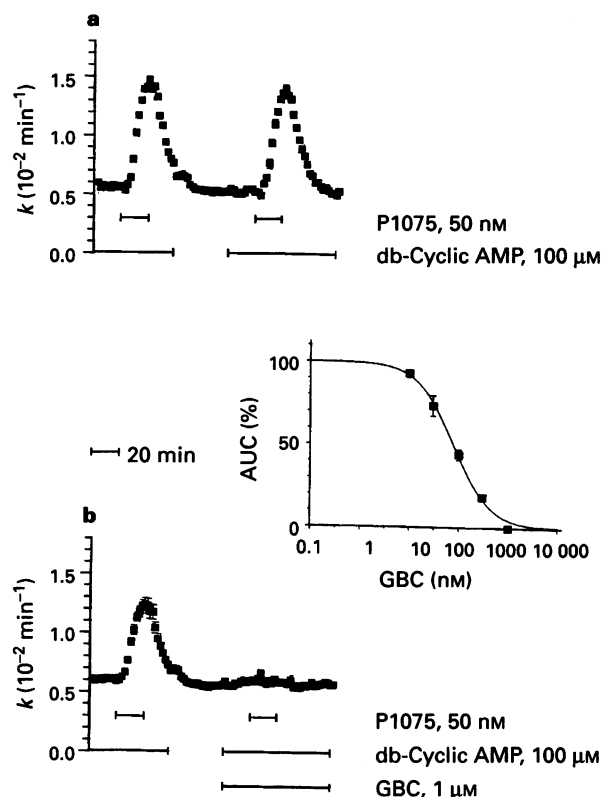
Figure 4 Inhibition by glibenclamide of P1075-stimulated $^{86}Rb^+$ efflux in the presence of 100 μM db-cyclic AMP. (a) Control traces: double stimulation of the preparation by P1075 (50 nM for 20 min) in the presence of db-cyclic AMP. db-cyclic AMP was superfused 20 min prior to, during and after the application of P1075 as indicated; the ratio of the AUCs was 95±3% (4 experiments). (b) Glibenclamide (GBC), 1 μM superfused together with the second application of db-cyclic AMP until to the end of the experiment, completely inhibited the reponse to P1075 in the presence of db-cyclic AMP (6 experiments). Inset: concentration dependence of the inhibition by glibenclamide. The fit of the data to the Hill equation gave a midpoint (IC_{50}) of 79±5 nM and a Hill coefficient of 1.18±0.08 (4 to 6 experiments per point). The data are corrected for the difference of control values ($AUC_2/AUC_1 = 0.95$).

Figure 5 Effects of glibenclamide (GBC, 1 μM) on P1075-stimulated $^{86}Rb^+$ efflux in the presence of forskolin (FK, 1 μM, a) and IBMX (100 μM, b). The inhibition amounted to 90±2 and 93±1% of the first stimulus ($n=4$). Control experiments analogous to those shown in Figure 7, upper trace, indicated that in the absence of glibenclamide, AUC_2 was less than 5% smaller than AUC_1. Note the small increase in $^{86}Rb^+$efflux due to the application of forskolin and IBMX prior to stimulation with P1075.

perfusion of forskolin (1 μM) in the presence of various concentrations of IBMX. The results, expressed as Δ % values, are listed in Table 1. At 0.1 and 1 μM IBMX, the effects of the combination with forskolin (1 μM) were additive, i.e. equal to the sum of the individual contributions. At 1 μM IBMX + 1 μM
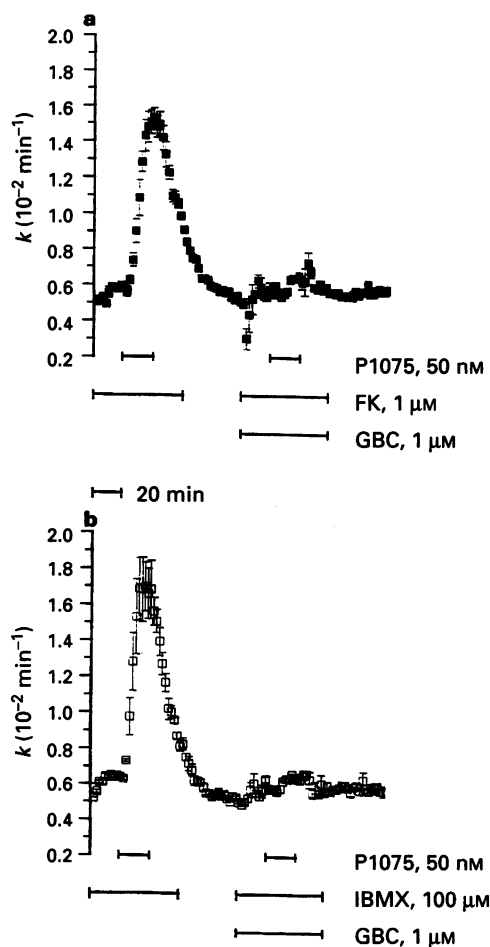
forskolin, a maximum enhancement was reached which amounted to ≈150 Δ %, i.e. AUC was 2.5 times that of control. At higher concentrations of IBMX (10 and 100 μM), the effects of the combination were smaller than the sum of the individual contributions; they were also smaller than that of

Table 1 Effects of the combination of forskolin (1 μM) and isobutylmethylxanthine (IBMX) on P1075-induced $^{86}Rb^+$ efflux (AUC_2) and on specific binding (SB) of [^{3}H]-P1075[1] in rat aortic rings

Forskolin (μM)	IBMX (μM)	AUC_2 $(\Delta\%)$[2]	SB (%)	n
1	–	101±9	107±9	6
–	0.1	7±3	–	6
1	0.1	112±4	–	4
–	1	39±9	94±6	6
1	1	146±14	93±5	6
–	10	72±14	90±3	6
1	10	94±12**	98±5	6
–	100	97±13	98±3	4
1	100	51±4**	96±2	4
–	1000	−53±15	37±1	4

[1] $^{86}Rb^+$ efflux experiments were performed as shown in Figure 2. Binding assays were conducted at 0.3 nM [^{3}H]-P1075 (see Methods section); specific binding under control conditions was 1.52 fmol mg^{-1} tissue wet weight.

[2] AUC_2 values are given as Δ%, i.e. 100 × (AUC_2/AUC_1)−100, where AUC_1 and AUC_2 are the areas under the curve of the k versus time plot produced by P1075 in the absence and presence of the cyclic AMP stimulators, respectively.

**Effect of the combination is significantly smaller than the sum of the individual contributions, $P < 0.01$.

the maximally effective combination of 1 μM IBMX + 1 μM forskolin. At 1 mM IBMX alone, P1075-induced tracer efflux was strongly reduced, probably due to interference of IBMX at this concentration with the binding of P1075 to its drug receptor (Table 1).

So far, all experiments were performed at a P1075 concentration of 50 nM. Hence it was of interest to determine whether the degree of modulation of P1075-induced ^{86}Rb$^+$ efflux by cyclic AMP stimulation depended on the concentration of the opener. Figure 6 shows the concentration-dependent stimulation of ^{86}Rb$^+$ efflux by P1075 in the absence and the presence of 100 μM IBMX. IBMX induced a leftward shift of the concentration-response relationship to P1075 by a factor of two without affecting the maximum response; also the Hill slopes of the curves were not significantly different.

Effect of H-89

In order to investigate whether the effects of increased cyclic AMP levels were mediated by protein kinase A, experiments were conducted with the protein kinase A inhibitor, H-89 (Chijiwa et al., 1990). Up to 1 μM, H-89 had no effect on increased ^{86}Rb$^+$ efflux induced by P1075 in the presence of 100 μM IBMX (data not shown); at 3 and 10 μM, a substantial inhibition was seen (Figure 7). H-89 also inhibited the P1075-stimulated tracer efflux in the absence of IBMX (Figure 7) and the specific binding of [^{3}H]-P1075 to rat aortic rings (data not shown). However, the inhibition of P1075-induced ^{86}Rb$^+$ efflux by H-89 was significantly greater in the presence of IBMX than in its absence (Figure 7c).

Specific binding of [^{3}H]-P1075

Specific binding of [^{3}H]-P1075 to rat aortic rings was not affected by forskolin and IBMX at concentrations which enhanced the ^{86}Rb$^+$ efflux response to P1075 (Table 2); similarly, the combination of both compounds did not affect specific [^{3}H]-P1075 binding at concentrations which potentiated P1075-stimulated tracer efflux (Table 1). At high concentrations, however, both agents inhibited [^{3}H]-P1075 binding (Tables 1 and 2). db-cyclic AMP inhibited [^{3}H]-P1075 binding to 63% of control with a bell-shaped concentration dependence in the concentration-range where P1075-stimulated tracer efflux was potentiated (see Table 2).
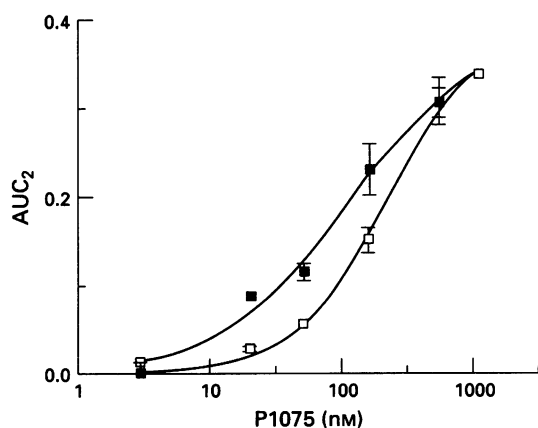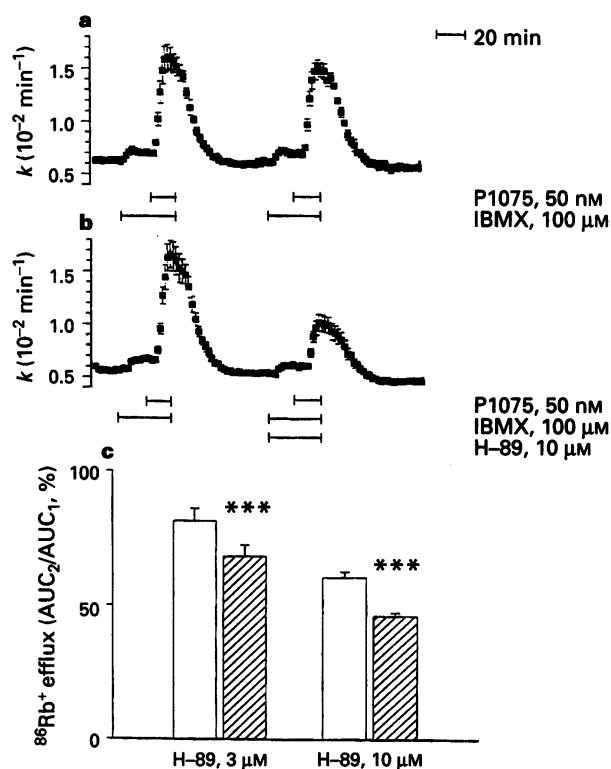

Figure 7 Inhibition of P1075-stimulated ^{86}Rb$^+$ efflux by H-89 in the presence of IBMX. (a) Double stimulation protocol with P1075 in the presence of IBMX; the ratio AUC$_2$/AUC$_1$ was 0.97 ± 0.05 (n = 4). (b) Application of H-89 (10 μM) before the second stimulus reduced this ratio to 0.46 ± 0.01 (n = 5). Note that the small effect of IBMX on the basal rate of ^{86}Rb$^+$ efflux persisted in the presence of H-89. (c) Comparison of the effects of H-89 on P1075-induced ^{86}Rb$^+$ efflux in the absence (open columns) and presence (hatched columns) of IBMX (100 μM); the difference is significant (P < 0.005; n = 5–6).

Table 2 Specific binding (% of control) of [^{3}H]-P1075 in presence of analogues and stimulators of cyclic AMP

[μM]	Forskolin	IBMX	db-cyclic AMP
0.1	93 ± 12	–	–
0.3	89 ± 1	–	–
1.0	107 ± 9	94 ± 6	94 ± 6
3.0	97 ± 4	–	79 ± 3
10.0	100 ± 8	90 ± 3	63 ± 3
30.0	81 ± 7	–	88 ± 5
100.0	53 ± 3	98 ± 3	84 ± 4
300.0	–	–	101 ± 6
1000.0	–	37 ± 1	85 ± 6

Values are means ± s.e.mean of 4 to 21 experiments. Binding assays were conducted at 0.3 nM [^{3}H]-P1075 as described in the Methods section; specific binding under control conditions was 1.52 fmol mg^{-1} tissue wet weight.

Figure 6 Effect of IBMX (100 μM) on the concentration-dependent ^{86}Rb$^+$ efflux stimulated by P1075. The efflux response is represented by the AUC values normalized to 10 min application of P1075 (see Methods); values are means ± s.e.mean from 4 to 9 experiments. Hill analysis of the concentration-response curves in the absence (□) and presence (■) of IBMX, respectively, yielded EC$_{50}$ values of 200 ± 40/99 ± 62 nM, maximum values of 0.39 ± 0.03/0.38 ± 0.08 and Hill slopes of 1.26 ± 0.18/0.92 ± 0.29. The EC$_{50}$ values of the two curves are significantly different.

Discussion

This study shows that moderate stimulation of the cyclic AMP signalling chain potentiates the ^{86}Rb$^+$ efflux generating effect of the K$_{ATP}$ channel opener, P1075, whereas excessive stimulation leads to an inhibition. Under conditions which cause potentiation of the P1075-induced tracer efflux, specific binding of [^{3}H]-P1075 to its drug receptor is either not affected or reduced.

Facilitation of P1075-induced K⁺ channel opening

The potentiation of P1075-induced tracer efflux by cyclic AMP stimulants was observed at concentrations which are similar to (or at most 10 times higher than) those which induce vaso-relaxation. This finding may be of pharmacotherapeutical interest if combinations of cyclic AMP increasing drugs with K⁺ channel openers are to be used. The ^{86}Rb⁺ efflux stimulated by P1075 in the presence of increased intracellular levels of cyclic AMP and db-cyclic AMP has two remarkable properties. First, it is inhibited by glibenclamide at relatively low concentrations similar to those which inhibit P1075-induced ^{86}Rb⁺ efflux in the absence of cyclic AMP stimulation (Quast & Cook, 1989; Quast, 1995). This suggests that the P1075-induced ^{86}Rb⁺ efflux also in the presence of cyclic AMP stimulation is passing entirely through K$_{ATP}$ channels. Second, the facilitatory effect of increased cyclic AMP levels on P1075-induced ^{86}Rb⁺ efflux is more pronounced at lower concentrations of the opener, the effect disappears at saturating levels of the opener such that the maximum P1075-induced ^{86}Rb⁺ efflux is not increased (Figure 6). Hence, in rat aorta, increased cyclic AMP levels seemingly sensitize the system for the K$_{ATP}$ channel opener. These results also suggest that this sensitization does not involve an increase in the number of channels that can be activated by P1075 at maximally effective concentrations nor does it involve modifications of the channel that lead to the permeation of more Rb⁺ ions per channel per unit time. Since we have observed that low concentrations of forskolin also augment the ^{86}Rb⁺ efflux induced by the K$_{ATP}$ channel opener, cromakalim (Quast et al., unpublished), it appears that increased cyclic AMP levels sensitize the pathway by which the chemically different classes of openers activate the K$_{ATP}$ channel in rat aorta.

Reversal of stimulation by high concentrations of forskolin and IBMX

The enhancement of P1075-induced ^{86}Rb⁺ efflux by the combination of forskolin and IBMX was additive at low concentrations; at higher concentrations it was less than additive and lower than the maximum effect, thus showing a bell-shaped concentration-dependence. This result requires further investigation.

At very high concentrations, forskolin and IBMX inhibited P1075-stimulated ^{86}Rb⁺ efflux. In the case of IBMX, this inhibition (to ≈50% of control at 1000 μM) is quantitatively explained by the inhibition of binding of P1075 to its drug receptor. The almost complete inhibition of P1075-induced tracer efflux observed at 100 μM forskolin is in agreement with earlier observations where cromakalim was used as the agonist (Quast, 1988; Quast et al., 1994). The binding data show that this inhibition cannot be attributed solely to interference with P1075 binding; in addition one has to take into consideration that forskolin inhibits several ion channels, including voltage-dependent K⁺ channels, by an action unrelated to stimulation of adenylyl cyclase (Laurenza et al., 1989).

Involvement of protein kinase A

There are several ion channels which are activated directly by cyclic nucleotides (Latorre et al., 1991; Matthews, 1991); in most cases, however, the effects of cyclic nucleotides on ion channels are mediated by protein phosphorylation (see Introduction). In order to distinguish between these possibilities we have used the potent ($K_i = 48$ nM), relatively selective and membrane permeant, protein kinase A inhibitor H-89 (Chijiwa et al., 1990). This inhibitor is competitive with ATP (Chijiwa et al., 1990); thus, for inhibition of protein kinase A to occur, the ratio of inhibitor concentration over K_i must exceed the ratio of ATP concentration over K_m(ATP), the Michaelis constant of the kinase for ATP. Since the ATP concentration in vascular smooth muscle is approximately 2 mM (Butler & Davies, 1980) and K_m (ATP) = 8 μM (Whitehouse et al., 1983), the con-

centration of H-89 in the cell should exceed the μM range in order to obtain inhibition of protein kinase A. Indeed, we found that H-89 was inactive up to 1 μM and only at 3 and 10 μM, was substantial inhibition of P1075-stimulated ^{86}Rb⁺ efflux in presence of 100 μM IBMX observed. At these concentrations, however, H-89 loses its selectivity for protein kinase A (Chijiwa et al., 1990); in agreement with this point we observed that H-89 also inhibited binding of [^{3}H]-P1075 and, consequently, P1075-induced efflux in the absence of IBMX. However, these effects of H-89 were significantly smaller than the reduction of P1075-induced efflux in the presence of IBMX. This strongly suggests that the potentiating effect of increased cyclic AMP levels on P1075-induced ^{86}Rb⁺ efflux is mediated by protein kinase A.

Obviously, this study does not give any indication of the target which is phosphorylated. At present it is not known whether the K$_{ATP}$ channel openers activate the channel by directly binding to it or indirectly by initiating a complex chain of events (see below); hence the results presented here should be interpreted with caution. Indirect mechanisms like a phosphorylation-induced decrease in intracellular Ca^{2+} concentration which is known to favour K$_{ATP}$ channel opener-induced ^{86}Rb⁺ efflux (Coldwell & Howlett, 1988) might also be envisaged. However, the finding that moderate stimulation of cyclic AMP in rat aorta sensitizes the preparation for the K$_{ATP}$ channel opening action of P1075 is in agreement with electrophysiological studies in rabbit mesenteric artery (Quayle et al., 1994) and in follicle-enclosed Xenopus oocytes (Honoré & Lazdunski, 1991; 1993); these studies also show that activation of protein kinase A by increased cyclic AMP facilitates K$_{ATP}$ channel opening. However, in rat portal vein it has been found that dephosphorylating conditions open the K$_{ATP}$ channel and that the openers may activate the channel activation by inducing dephosphorylating conditions (Edwards et al., 1993, Edwards & Weston, 1994b). At present there is no convincing explanation for this apparent discrepancy.

Modulation of [³H]-P1075 binding

A surprising finding of this study was the observation that moderate stimulation of cyclic AMP levels, which enhanced P1075-induced K$_{ATP}$ channel opening, left specific [^{3}H]-P1075 binding unchanged (forskolin, IBMX) or reduced binding with a biphasic concentration dependence (db-cyclic AMP). This was quite unexpected since previous experiments had shown an excellent correlation between the potencies of major K$_{ATP}$ channel openers in binding and ^{86}Rb⁺ efflux assays (Bray & Quast, 1992; Quast et al., 1993).

In the case of voltage-dependent Na⁺ and dihydropyridine-sensitive Ca^{2+} channels, ligands which induce a certain state of the channel (e.g. open or desensitized) have the highest affinity for the channel in this particular state (Catterall, 1984; Glossmann et al., 1984). Obviously, this is not the case here, where stimulation of cyclic AMP promotes K$_{ATP}$ channel opening but not the binding of the opener. We feel that this tends to support an indirect mechanism of action of the K$_{ATP}$ channel openers where channel opening is some steps further downstream from binding, resembling the hypothesis of Edwards & Weston (1993b) in this aspect. Such a mechanism could also explain apparent discrepancies between the K⁺ channel opening and the vasorelaxant properties of these compounds (Quast, 1993). On the other hand, it has recently been shown that the rat cardiac K$_{ATP}$ channel expressed in a kidney epithelial cell line, can be activated by the opener, pinacidil, in isolated inside-out patches (Ashford et al., 1994). This may be considered evidence for a direct mode of action of this compound although it cannot be ruled out that the observed sensitivity to pinacidil may have been conferred to the cloned channel by another protein present in the patch. Undoubtedly, the ongoing effort in channel cloning and expression in combination with the use of electrophysiological and biochemical techniques will soon elucidate the mechanism of action of the K$_{ATP}$ channel openers.

References

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell. Signal.*, **2**, 197–214.

ASHFORD, M.L.J., BOND, C.T., BLAIR, T.A. & ADELMAN, J.P. (1994). Cloning and functional expression of a rat heart K_{ATP} channel. *Nature*, **370**, 456–459.

BEVINGTON, P.R. (1969). *Data Reduction and Error Analysis for the Physical Sciences*. pp. 55–65 and 92–118. New York: McGraw-Hill.

BRAY, K.M. & QUAST, U. (1992). A specific binding site for K^+ channel openers in rat aorta. *J. Biol. Chem.*, **267**, 11689–11692.

BUTLER, T.M. & DAVIES, R.W. (1980). High-energy phosphates in smooth muscle. In *Handbook of Physiology, Section 2: Cardiovascular System, Vascular Smooth Muscle*. ed. Bohr, D.F., Somlyo, A.P. & Sparks, H.V. Vol. 2, pp. 237–252. Bethesda, MD: Am. Physiol. Soc.

CATTERALL, W.A. (1984). The molecular basis of neuronal excitability. *Science*, **223**, 653–661.

CHIJIWA, T., MISHIMA, A., HAGIWARA, M., SANO, M., HAYASHI, K., INOUE, T., NAITO, K., TOSHIOKA, T. & HIDAKA, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.*, **265**, 5267–5272.

COLDWELL, M.C. & HOWLETT, D.R. (1988). Potassium efflux enhancement by cromakalim (BRL 34915) in rabbit mesenteric artery: an indirect effect independent of calcium? *Biochem. Pharmacol.*, **37**, 4105–4110.

DE WEILLE, J.R. (1992). Modulation of ATP sensitive potassium channels. *Cardiovasc. Res.*, **26**, 1017–1020.

DRAPER, N.B. & SMITH, H. (1981). *Applied Regression Analysis*. pp. 85–96, 458–517. New York: Wiley.

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K^+-current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.*, **110**, 1037–1048.

EDWARDS, G. & WESTON, A.H. (1993a). The pharmacology of ATP-sensitive K^+ channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

EDWARDS, G. & WESTON, A.H. (1993b). Induction of a glibenclamide-sensitive K-current by modification of a delayed rectifier channel in rat portal vein. *Br. J. Pharmacol.*, **110**, 1280–1281.

EDWARDS, G. & WESTON, A.H. (1994a). Effect of potassium channel modulating drugs on isolated smooth muscle. In *Handbook of Experimental Pharmacology Volume 111*. ed. Szerkeres, L. & Gy Papp, J. pp. 469–531. Heidelberg: Springer.

EDWARDS, G. & WESTON, A.H. (1994b). K_{ATP} - fact or artefact? New thoughts on the mode of action of the potassium channel openers. *Cardiovasc. Res.*, **28**, 735–737.

GLOSSMANN, H., FERRY, D.R., GOLL, A. & ROMBUSCH, M. (1984). Molecular pharmacology of the calcium channel: Evidence for subtypes, multiple drug-receptor sites, channel subunits, and the development of a radioiodinated, 1,4-dihydropyridine calcium channel label, [125I]iodipine. *J. Cardiovasc. Pharmacol.*, **6**, Suppl. 6, S608–S621.

HONORÉ, E. & LAZDUNSKI, M. (1991). Hormone-regulated K^+ channels in follicle enclosed oocytes are activated by vasorelaxing K^+ channel openers and blocked by antidiabetic sulfonylureas. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 5438–5442.

HONORÉ, E. & LAZDUNSKI, M. (1993). Single-channel properties and regulation of pinacidil/glibenclamide-sensitive K^+ channels in follicular cells from Xenopus oocytes. *Pflügers Arch.*, **424**, 113–121.

LATORRE, R., BACIGALUPO, J., DELGADO, R. & LABARCA, P. (1991). Four cases of direct ion channel gating by cyclic nucleotides. *J. Bioenerg. Biomembr.*, **23**, 577–597.

LAURENZA, A., MCHUGH SUTKOWSKI, E. & SEAMON, K.B. (1989). Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol. Sci.*, **10**, 442–447.

LEVITAN, I.B. (1994). Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.*, **56**, 193–212.

LINDE, C., LÖFFLER, C. & QUAST, U. (1994). Phosphorylation: Effects on K^+ channel opening and K^+ channel openers in rat isolated aorta. *Naunyn Schmied. Arch. Pharmacol.*, **349**, R47.

MATTHEWS, G. (1991). Ion channels that are directly activated by cyclic nucleotides. *Trends Pharmacol. Sci.*, **12**, 245–247.

NICHOLS, C.G. & LEDERER, W.K. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.*, **261**, H1675–H1686.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature*, **305**, 147–148.

QUAST, U. (1987). Effect of the K^+ efflux stimulating vasodilator BRL34915 on $^{86}Rb^+$ efflux and spontaneous activity in guinea-pig portal vein. *Br. J. Pharmacol.*, **91**, 569–578.

QUAST, U. (1988). Inhibition of the effects of the K^+ channel stimulator cromakalim (BRL 34915) in vascular smooth muscle by glibenclaminde and forskolin. *Naunyn-Schmied. Arch. Pharmacol.*, **337**, R72.

QUAST, U. (1993). Do the K^+ channel openers relax smooth muscle by opening K^+ channels? *Trends Pharmacol. Sci.*, **14**, 332–336.

QUAST, U. (1995). Effects of potassium channel activators in isolated blood vessels. In *Potassium Channels and their Modulators*. ed. Evans, J.M., Hamilton, T.C., Longman, S.D. & Stamp, G. Prentice Hall: Ellis Horwood, (in press).

QUAST, U. & BAUMLIN, Y. (1988). Comparison of the effluxes of $^{42}K^+$ and $^{86}Rb^+$ elicited by cromakalim (BRL34915) in tonic and phasic vascular tissue. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 319–326.

QUAST, U., BAUMLIN, Y. & DOSOGNE, J. (1994). Forskolin blocks and opens K^+ channels in rat isolated aorta. *Can. J. Physiol. Pharmacol.*, **72**, Suppl. 1, 518.

QUAST, U., BRAY, K.M., ANDRES, H., MANLEY, P.W., BAUMLIN, Y. & DOSOGNE, J. (1993). Binding of the K^+ channel opener [3H]-P1075 in rat isolated aorta: relationship to functional effects of openers and blockers. *Mol. Pharmacol.*, **43**, 474–481.

QUAST, U. & COOK, N.S. (1989). In vitro and in vivo comparison of two K^+ channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. *J. Pharmacol. Exp. Ther.*, **250**, 261–270.

QUAYLE, J.M., BONEV, A.D., BRAYDEN, J.E. & NELSON, M.T. (1994). Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase. *Am. J. Physiol.*, **475**, 9–13.

ROSENTHAL, W. & SCHULTZ, G. (1987). Modulations of voltage-dependent ion channels by extracellular signals. *Trends Pharmacol. Sci.*, **8**, 351–354.

STEINBERG, M.I., ERTEL, P., SMALLWOOD, J.K., WYSS, V. & ZIMMERMAN, K. (1988). The relation between vascular relaxant and cardiac electrophysiological effects of pinacidil. *J. Cardiovasc. Pharmacol.*, **12**, Suppl. 2, S30–S40.

TERZIC, A. & KURACHI, Y. (1995). Potassium channel opening drugs activate cardiac ATP-sensitive K^+ channels through distinct molecular mechanisms: A novel basis for a functional classification. *Trends Pharmacol. Sci.*, (in press).

TSUCHIYA, K., WANG, W., GIEBISCH, G. & WELLING, P.A. (1992). ATP is a coupling modulator of parallel Na, K-ATPase-K-channel activity in the renal proximal tubule. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6418–6422.

WANG, W., SCHWAB, A. & GIEBISCH, G. (1990a). Regulation of small-conductance K^+ channel in apical membrane of rat cortical collecting tubule. *Am. J. Physiol.*, **259**, F494–F502.

WANG, W., WHITE, S., GEIBEL, J. & GIEBISCH, G. (1990b). A potassium channel in the apical membrane of rabbit thick ascending limb of Henle's loop. *Am. J. Physiol.*, **258**, F244–F253.

WHITEHOUSE, S., FERAMISCO, J.R., CASNELLIE, J.E., KREBS, E.G. & WALSH, D.A. (1983). Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase. *J. Biol. Chem.*, **258**, 3693–3701.