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Modulation of the isoprenaline-induced membrane hyperpolarization of mouse skeletal muscle cells

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1 The hyperpolarization of the resting membrane potential, V_m, induced by isoprenaline in the lumbrical muscle fibres of the mouse, was investigated by use of intracellular microelectrodes.

2 In normal Krebs-Henseleit solution (potassium concentration: $K_o^+ = 5.7$ mM, 'control'), V_m was -74.0 ± 0.2 mV; lowering K_o^+ to 0.76 mM ('low K_o^+') resulted in either a hyperpolarization ($V_m = -95.7 \pm 2.9$ mV), or a depolarization ($V_m = -52.0 \pm 0.3$ mV).

3 Isoprenaline (≥ 200 nM) induced a hyperpolarization of V_m by $\Delta V_m = -5.6 \pm 0.4$ mV in control solution.

4 When V_m hyperpolarized after switching to low K_o^+, the addition of isoprenaline resulted in increased hyperpolarization V_m: $\Delta V_m = -16.3 \pm 3.2$ mV to a final $V_m = -110.1 \pm 3.4$ mV. Adding iso-prenaline when V_m depolarized in low K_o^+, leads to a hyperpolarization of either by -11.6 ± 0.5 mV to -63.6 ± 0.8 mV or by -51.7 ± 2.7 mV to -106.9 ± 3.9 mV.

5 Ouabain (0.1 to 1 mM) did not suppress the hyperpolarization by isoprenaline in 5.7 mM K_o^+ ($\Delta V_m = -6.7 \pm 0.4$ mV) or the hyperpolarization of the depolarized cells in low K_o^+ ($\Delta V_m = -9.7 \pm 1.5$ mV).

6 The hyperpolarization is a logarithmically decreasing function of K_o^+ in the range between 2 and 20 mM (12 mV/decade).

7 IBMX and 8Br-cyclic AMP mimicked the response to isoprenaline whereas forskolin (FSK) induced in low K_o^+ a hyperpolarization of -7.0 ± 0.7 mV that could be augmented by addition of isoprenaline ($\Delta V_m = -8.2 \pm 1.8$ mV).

8 In control and low K_o^+, Ba^{2+} (0.6 mM) inhibited the hyperpolarization induced by isoprenaline, IBMX or 8Br-cyclic AMP. Other blockers of the potassium conductance such as TEA (5 mM) and apamin (0.4 μM) had no effect.

9 We conclude that in the lumbrical muscle of the mouse the isoprenaline-induced hyperpolarization is primarily due to an increase in potassium permeability.

Keywords: Isoprenaline; propranolol; potassium; membrane potential; ouabain; barium; inward rectification; Na^+-K^+-pump; lumbrical muscle fibre

Introduction

The influence of adrenaline on skeletal and cardiac muscle has been studied extensively (see reviews by Hartzell, 1988; Williams & Barnes, 1989). Isoprenaline activates the β_2-adrenoceptor, resulting in the generation of inotropic effects in skeletal muscles (Rang & Dale, 1993; Clausen & Flatman, 1980).

The observed hyperpolarization of the membrane potential, V_m, by isoprenaline in skeletal muscle fibres, has been attributed to stimulation of the Na^+-K^+-pump by the cyclic AMP-dependent cascade (Clausen, 1986; Li & Sperelakis, 1993). From the literature on cardiac tissue however, a more complex picture emerges comprising influences on V_m by the stimulation of the Na^+-K^+-pump (Désilets & Baumgarten, 1986), K^+-conductance (Gadsby, 1983; Boyden *et al.*, 1983a), Na^+-conductance (Egan *et al.*, 1988), Ca^{2+}-conductance (Tsien *et al.*, 1986) or Cl^--conductance (Harvey & Hume, 1989; Ehara & Ishihara, 1990; Nakaya *et al.*, 1990). Also combinations of these effects have been proposed (Encina *et al.*, 1988; Harvey *et al.*, 1991).

At present some publications indicate that muscle cells may respond differently depending on the muscle type. Kuba & Nohmi (1987) presented a more complex picture for the diaphragm muscle of the rat and Pfliegler *et al.* (1983) published flux measurements from extensor digitorum longus and soleus that suggest that muscles can also differ in their responses. In this study we present evidence that in the lumbrical muscle of the mouse, the isoprenaline induced hyperpolarization, controlled by the cyclic AMP cascade, is related to an increase in potassium conductance and not primarily to an enhanced activity of the Na^+-K^+-pump.

We took advantage of our previous observations in skeletal muscle that lowering K_o^+ leads to an increased hyperpolarization of V_m by adrenaline (Siegenbeek van Heukelom, 1991). This increased hyperpolarization has also been measured in frog muscle fibres (Ling *et al.*, 1984), rat diaphragm (Kuba & Nohmi, 1987) and heart-muscle (Boyden *et al.*, 1983a; Encina *et al.*, 1988). This also applies to isoprenaline-induced hyperpolarizations.

When K_o^+ concentration is lowered, the activity of the Na^+-K^+-pump and the potassium conductances will change. It is known that the half maximal activity of the Na^+-K^+-pump is reached when K_o^+ is lowered to approximately 1-2 mM (Läuger, 1991). In normal physiological solutions the inward potassium rectifier, IKR, constitutes the major potassium conductance in the membrane. A property of the IKR, is that, due to its kinetics, it can 'switch off': voltage clamp and patch clamp measurements suggest that the major population of the channels constituting this conductance closes (Hille, 1992). Its

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conductance drops steadily with K^+_o, but on reduction of K^+_o below a critical concentration it drops to extremely low values. This concentration is reached when V_m is sufficiently less negative than E_K (the potassium equilibrium potential), and is approximately 1.4 mM in the lumbrical muscle of the mouse (Siegenbeek van Heukelom, 1994). These last properties have also been described for other mammalian (Rudy, 1988; Siegenbeek van Heukelom, 1991; 1994) and frog skeletal muscle (Standen & Stanfield, 1978b) as well as for cardiac tissue (Gadsby & Cranefield, 1977; Boyden *et al.*, 1983b; Carmeliet *et al.*, 1987). We studied the isoprenaline-induced responses above and below this critical value. The role of the potassium conductance was studied in more detail by using potassium channel blockers such as TEA, apamin and $BaCl_2$ and by measuring the hyperpolarization with varying K^+_o.

Some of these data have been presented in a preliminary form as an abstract (van Mil *et al.*, 1994).

Methods

Preparation and experimental procedures

White Swiss mice (age 8 to 18 weeks, weighing 20–40 g, either sex) were killed by cervical dislocation. The lumbrical muscles were removed from a hind foot. One bundle was cut free and cleaned of connective tissue. It had a length of 8 mm and a diameter of 250 μm and contained approximately 50 fibres. The diameter is so small that all fibres were well oxygenated (Schouten & ter Keurs, 1986). The use of a cold control solution (approximately 15°C, see specification of media) during the preparation reduced the development of ischaemia. Superficial cells were impaled with fine-tipped microelectrodes (3 M KCl: 25–80 MΩ). Impalements were considered successful and measurements were continued if V_m was more negative than −70 mV in control medium. Most data are derived from paired measurements (see statistics). When an active substance was washed out ('washout') the new steady state V_m value should differ less than 3 mV from the value before the application of the substance. Similar criteria applied when substances were used in succession. Measurements were considered successful if V_m also returned to the original value (±3 mV) after the solution was switched back to control medium. Further details were given earlier (Siegenbeek van Heukelom, 1991).

Measuring chamber, definition of V_m and chemicals

The measuring chamber, made of Sylgard 184 (Dow Corning, Mich. 48640, U.S.A.), had a volume of approximately 0.1 ml and was continuously perfused (flow velocity 3 ml min^{-1}) at 35±1°C. Solutions with K^+-concentrations differing from the control were made by equimolar replacement of NaCl by KCl or *vice versa*. The control Krebs-Henseleit solution contained (in mM): NaCl 117.5, KCl 5.7, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2 and glucose 5.6, saturated with humidified gas: 95% O_2 + 5% CO_2; pH = 7.35–7.45.

Influences of electrode junction potentials were minimized by defining V_m as the potential difference between the microelectrode in the cell and a microelectrode outside the cell, pulled in the same way. All chemicals were analytically pure; salts were bought from Janssen Chimica, ouabain was bought from Merck (g-Strophantidin krist. reinst), adrenaline (L-epinephrine, 99%) from Aldrich, methoxamine-HCl from Wellcome and all other chemicals from Sigma. Adrenaline and isoprenaline were stored in 0.1 M HCl at 0°C and added to 100 ml of salt solution to obtain the desired concentration, just before the use of the solution.

The use of barium as blocker of the inward potassium rectifier

In 5.7 mM K^+_o the I_{KR} may be blocked by addition of 80 μM Ba^{2+} to the solution, which is manifested as a depolarization of V_m (Table 1) to approximately −50 mV. Standen & Stanfield (1978a) demonstrated in frog sartorius muscle that the concentration of Ba^{2+} needed to block I_{KR} is a function of the K^+_o. Using a model describing competition of Ba^{2+} and K^+_o for a site related with the I_{KR}, they explained why on raising K^+_o, the concentration of Ba^{2+}, needed for the blockade, increased quadratically. We were able to confirm their data in our preparation (see Figure 1). Blocking by Ba^{2+} was used to investigate the response of V_m to isoprenaline.

Statistics

Results are presented as mean±standard error of the mean (s.e.mean) in the tables. Changes in V_m (ΔV_m) are always expressed as the difference in the same cell between the steady state value before and after the change in solution. Groups of measurements were compared with one another either by Student's *t* test (when numbers of measurements were large and normally distributed) or Mann-Whitney (when numbers

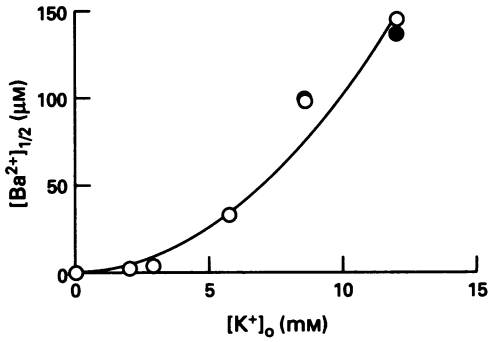

Figure 1 The block of the inward potassium rectifier by Ba^{2+}. Different concentrations of $BaCl_2$ were applied at constant K^+_o while measuring V_m in one cell (two series). The half-maximal concentrations of $BaCl_2$ thus determined for each K^+_o have been plotted. The correlation coefficient of the curve $[Ba^{2+}]_{1/2} = [K^+]^2_o$ in the figure in 0.98. The value at the origin ($[K^+]_o = 0$ and $[Ba^{2+}] = 0$) was added for the fitting of the curve.

Table 1 Effect of $BaCl_2$ on V_m

Solution K^+_o (mM)	*[$BaCl_2$] added* (μM)	*V_m before* (mV)	*V_m after* (mV)	*ΔV_m* *(mV)*	n
5.7	80	−74.0±1.6	−53.4±1.3	20.5±0.6	8
5.7	600	−75.7±0.8	−40.8±2.1	34.9±2.7	3
0.76	80	−52.7±1.9	−45.7±1.5	7.1±0.7	9
0.76	600	−52.3±1.5	−42.6±1.8	9.7±2.8	7

$BaCl_2$ (80 μM) depolarized V_m in control solutions and in low K^+_o. In a concentration of 0.6 mM (or more) it exerts the same effect and the V_m values obtained are not significantly different from those with 80 μM $BaCl_2$ ($P > 0.05$). The depolarization by $BaCl_2$ in 5.7 mM K^+_o is comparable to the depolarization due to low K^+_o. *n* = number of measurements.

were smaller than 6 or not normally distributed). When a curve was fitted to data, the quality of the fit is expressed by the correlation coefficient r.

Results

Hyperpolarization of V_m by adrenaline and isoprenaline with $K_o^+ = 5.7$ mM

In control solution saturating concentrations of adrenaline (≥ 2 μM) hyperpolarized V_m by −4.4 mV (see Table 2). Bath application of saturating (see below) concentrations of isoprenaline (≥ 200 nM) caused a −5.6 mV hyperpolarization that is comparable to observations in soleus (Clausen & Flatman, 1977) and diaphragm muscle (Kuba & Nohmi, 1987). The hyperpolarizations in response to isoprenaline and adrenaline in control solution did not differ significantly ($P > 0.05$).

Hyperpolarization of V_m by adrenaline and isoprenaline with $K_o^+ = 0.76$ mM

Reduction of K_o^+ below a critical value will lead to a drastic drop in the conductance of the I_{KR} (see Introduction). A switch from control to a K_o^+ lower than this critical value will lead to a depolarization. The depolarized V_m thus obtained, does not differ significantly ($P > 0.05$) from the depolarized V_m obtained by addition of 80 μM $BaCl_2$ to the control solution, when the activity of the Na^+-K^+ pump has not been diminished by low K_o^+. Because $K_o^+ = 0.76$ mM is well below the 'switch-off'-concentration (1.4 mM for this preparation) most V_m values found were depolarized with this concentration. Saturating concentrations of adrenaline (≥ 2 μM) and of isoprenaline (≥ 200 nM) in low K_o^+ led to hyperpolarizations that were significantly higher ($P < 0.01$) than in control solutions. In these low K_o^+ conditions, adrenaline induced significantly ($P < 0.01$) larger hyperpolarizations than isoprenaline.

Three distinct types of response to isoprenaline could be observed. They were categorized as follows (for detailed information see Table 2):

Type 1 In most cases the lowering of K_o^+ to 0.76 mM led to a depolarization due to the closure of the I_{KR}. Bath-application of isoprenaline resulted in a hyperpolarization of −11.6 mV.

Type 2 When lowering K_o^+ resulted in a hyperpolarization the application of isoprenaline induced a further hyperpolarization of −16.3 mV.

Type 3 Sometimes, when reduction of K_o^+ to 0.76 mM led to a depolarization, isoprenaline induced a very large hyperpolarization (−51.7 mV). The new V_m value was comparable to the V_m obtained during type 2 hyperpolarization ($P > 0.05$).

A comparison of the results of these different types of response is given in the discussion.

In cells, depolarized to about −50 mV, calcium-dependent potassium channels might be (more) open (Cook, 1988). Therefore, we added EGTA (2–5 mM): to a low K_o^+ medium inducing an additional depolarization of 9.2±1.1 mV ($n = 13$). The responses to isoprenaline added to this medium did not differ significantly from responses without EGTA: −8.9±1.0 mV ($n = 7$; $P > 0.05$). This also excludes, in our opinion, the possibility that the 2.5 mM Ca^{2+} added routinely to the medium, without plasma proteins, influenced our observations.

Dependence of hyperpolarization on concentration of isoprenaline

Though each measurement took 45 min, we succeeded in measuring in two cells (low K_o^+) a total dose-response curve of V_m for isoprenaline (6 points), ranging from 1 nM to 1 μM. Data were fitted to an exponential curve and from this the half-maximal values 15±2 and 24±4 nM isoprenaline were found. These findings are suported by an incomplete series of data in other cells. The exact values are only indicative, as long as the events between the addition of isoprenaline and the hyperpolarization have not been fully quantified.

Time course of the response to isoprenaline

All responses to isoprenaline in low K_o^+ showed a biphasic behaviour with respect to the speed of hyperpolarization as illustrated in Figure 2. When the two phases were approximately described with two straight lines, the slopes of these lines averaged 1.98±0.14 mV min^{-1} and 0.52±0.02 mV min^{-1} ($n = 45$). Because this biphasic response was observed with low (20 nM) as well as high (1 μM) isoprenaline concentrations, it is not likely that this biphasic phenomenon is due to two independent processes with different affinities. None of the blockers or activators of the isoprenaline-response used in this study could inhibit or stimulate only one of these phases.

During the washout of isoprenaline, the depolarization of V_m was also biphasic but approximately four times slower than the induced hyperpolarization, and independent of the isoprenaline concentration used.

Table 2 The response of V_m to adrenaline and isoprenaline in control and low K_o^+ solutions

From	*to solution* K_o^+ *(mM) + addition*	V_m *before* (mV)	V_m *after* (mV)	ΔV_m (mV)	n
5.7	5.7 + adrenaline	−76.3±1.3	−80.7±1.4	−4.4±0.4	6
5.7	5.7 + isoprenaline	−72.1±0.8	−77.8±0.7	−5.6±0.4	22
5.7	0.76	−73.9±0.2	−52.0±0.3	21.9±0.3	245
5.7	0.76	−78.3±0.9	−95.7±2.9	−17.4±3.3	6
0.76	0.76 + isoprenaline (type 1)	−51.9±0.7	−63.6±0.8	−11.6±0.5	67
0.76	0.76 + isoprenaline (type 2)	−93.8±4.9	−110.1±3.4	−16.3±3.2	3
0.76	0.76 + isoprenaline (type 3)	−55.2±2.3	−106.9±3.9	−51.7±2.7	6
0.76	0.76 + adrenaline (type 1)	−51.8±1.5	−69.5±1.3	−17.7±1.4	5

The response of V_m to adrenaline and isoprenaline in control and low K_o^+ solutions. The responses of V_m were to the addition of isoprenaline (≥ 200 nM) or adrenaline (≥ 1 μM). Column 3 gives the average of V_m in the solutions specified in column 1 and those of column 4 are related to column 2. Included are also the changes due to switching from 5.7 mM to 0.76 mM K_o^+ (row 3 and 4). The values of ΔV_m in column 5 are the averages of individual ΔV_m measurements for each transition in medium. The separation of all isoprenaline-induced hyperpolarizations in $K_o^+ = 0.76$ mM into three types is described in the text. The mean control V_m-value of the cells hyperpolarizing due to low K_o^+ is more negative than that of the depolarizing cells ($P < 0.01$) (see also Siegenbeek van Heukelom, 1991). All values of depolarized V_m with 0.76 mM K_o^+ in column 3 are comparable ($P > 0.05$). V_m with $K_o^+ = 5.7$ mM before the addition of adrenaline was more negative than the value before the switch to isoprenaline ($P < 0.05$). n = number of measurements.

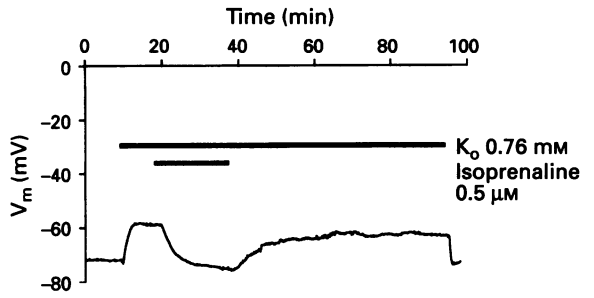

Figure 2 Typical response of V_m to the addition of 200 nM isoprenaline to the low K_o^+ superfusion medium. Two slopes can be discerned in the isoprenaline-induced hyperpolarization. The two slopes in this figure are 1.5 mV/min and 0.22 mV/min.

Effect of ouabain

To investigate the role of the Na^+-K^+-pump in the response to isoprenaline, ouabain was used to inhibit the Na^+-K^+-pump (Table 3) in control and low K_o^+ solutions. V_m depolarized 14.7 mV after the addition of ouabain (0.1–1 mM) to the control solution. When isoprenaline was added in the presence of ouabain a hyperpolarization was observed of −6.7 mV.

Also cells, depolarized in low K_o^+, depolarize further by 6.0 mV on addition of ouabain (0.1–1 mM). This indicates that the pump is still active in these low K_o^+ conditions. Addition of isoprenaline after ouabain induced a hyperpolarization of −9.8 mV ($n=12$), that is not significantly smaller than without ouabain ($P>0.05$; see Table 2). This hyperpolarization was still observable 30 min after the addition of ouabain.

Hyperpolarization is mediated via the β_2-adrenoceptor

To ascertain that the response to isoprenaline is primarily due to the stimulation of the β_2-adrenoceptor, the competitive blocker of the $\beta_{1,2}$-adrenoceptors, propranolol, in concentrations 10 times larger than the maximal isoprenaline concentration, was used to investigate the specificity at the receptor level. In this concentration range (5–10 μM) propranolol itself led to a small hyperpolarization of −1.3±0.9 mV ($n=9$) in low K_o^+. This is commented in the discussion.

Three types of experiments were performed: (1) in the presence of propranolol, isoprenaline did not induce a hyperpolarization in control or low K_o^+. (2) Addition of propranolol during the onset of the response of isoprenaline, stopped the hyperpolarization and let V_m depolarize subsequently as with the washout of isoprenaline. (3) Addition of propranolol to the solution after the hyperpolarization by isoprenaline had reached its new steady state, induced the same biphasic depolarization as observed during the washout of isoprenaline alone.

Addition of the α_1-agonist, methoxamine, did not induce a hyperpolarization, nor did it increase the hyperpolarization when added together with isoprenaline.

The stimulation of adenylylcyclase by G-proteins liberated by the binding of isoprenaline to the receptor, can to a great extent be mimicked by forskolin (FSK, Encina *et al.*, 1988). Addition of saturating concentrations of FSK (100 μM) to the cell induced a hyperpolarizing response. The response, however, was smaller than observed with isoprenaline ($P<0.01$), and isoprenaline always induced an additional hyperpolarization, when added to the FSK containing solution. Though this response was significantly smaller than without FSK (see Table 4, $P<0.05$), the combined response to FSK plus isoprenaline was not significantly different from that to isoprenaline alone ($P>0.05$). Forskolin has also been reported to block potassium channels (Hoshi *et al.*, 1988; Cook, 1988), and this might be the origin of the complex influence of FSK.

The phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX) evoked the same biphasic hyperpolarization of V_m as isoprenaline. The washout also showed the same time course. The same applies to FSK and the non-hydrolysable analogue of cyclic AMP: 8Br-cyclic AMP, that induced a ΔV_m of −8.8 mV ($n=1$). Addition of isoprenaline after the response to IBMX had reached its steady state, resulted in a small additional hyperpolarization of −1.2 mV. Similarly, IBMX, when added after the isoprenaline-response reached its steady state, induced a further hyperpolarization of −2.1 mV.

Table 3 Effect of ouabain on the response to isoprenaline

Solution-K_o^+ (mM) *+addition*	*Addition* (μM)	*V_m before* (mV)	*V_m after* (mV)	*ΔV_m* (mV)	n
5.7	Ouabain (500)	−73.8±1.0	−59.1±2.4	14.7±1.9	5
5.7+ouabain	Isoprenaline (0.5)	−58.5±2.0	−65.2±1.9	−6.7±0.4	6
0.76	Ouabain (500)	−51.6±1.9	−45.6±1.3	6.0±0.7	17
0.76+ouabain	Isoprenaline (0.5)	−45.3±1.5	−55.0±2.3	−9.8±1.4	12

Ouabain does not inhibit the isoprenaline hyperpolarization. Addition of ouabain before isoprenaline had no significant effect on the hyperpolarization by isoprenaline. Even after 30 min a hyperpolarization of –9 mV was observed. n=number of measurements.

Table 4 Effects of forskolin and IBMX, and their interaction with isoprenaline

Solution-K^+_o (mM) *+addition*	*Addition* (μM)	*V_m before* (mV)	*V_m after* (mV)	*ΔV_m* (mV)	n
0.76	FSK (10–100)	−51.9±1.8	−58.9±2.1	−7.0±0.7	10
0.76+FSK	Isoprenaline (0.2)	−56.9±2.6	−65.1±3.5	−8.2±1.8	3
0.76	IBMX (100–300)	−53.2±2.3	−69.9±2.7	−16.6±0.9	5
0.76+isoprenaline	IBMX (100)	−74.7	−77.1	−2.8	1
0.76+IBMX	Isoprenaline	−64	−65.7	−1.7	1

Effects of forskolin and IBMX, and their interaction with isoprenaline. Forskolin induced a smaller hyperpolarization than isoprenaline. When isoprenaline was added after FSK an additional hyperpolarization was found. The hyperpolarization (ΔV_m) induced by IBMX is significantly larger than the type 1 hyperpolarization by isoprenaline ($P<0.005$; see Table 2). In column 2 the values in parentheses describe the concentration-range used. n=number of measurements.

These experiments confirm that the stimulation of the β_2-adrenoceptor by isoprenaline and the inhibition of phosphodiesterase by IBMX induce hyperpolarization of V_m through a common cellular process, which is most likely the increase of the cellular concentration of cyclic AMP.

Isoprenaline-induced hyperpolarization as a function of K_o^+

Varying K_o^+ by replacement with Na^+, between 2 and 20 mM revealed an inverse semi-logarithmic dependence of the magnitude of the hyperpolarization on K_o^+ (see Figure 3). When K_o^+ was 40 mM, no depolarization was found; higher concentrations induced contractures that hampered further measurements. In four experiments we found that lowering Na_o^+, from the usual 143.7 mM to 41.2 mM (by replacing it by N-methyl glucamide), did not change the size of the hyperpolarization ($\Delta V_m = -12.5 \pm 2.9$ mV, $n=4$).

The marked dependence on K_o^+ indicates that the hyperpolarization in response to isoprenaline is related to an increased potassium conductance.

Potassium channel blockers

To investigate the properties of the K^+ conductance associated with the isoprenaline-response we used characteristic blockers. In low K_o^+, TEA (5 mM) induced a small depolarization (1.3 ± 0.6 mV, $n=4$) and isoprenaline evoked a hyperpolarization of equal size to that without TEA ($\Delta V_m = -15.1 \pm 0.4$ mV, $n=4$). Apamin (1 μM) was also ineffective.

Ba^{2+} (80 μM) induced a depolarization of V_m in the control solution (see Table 1) to -52.8 mV. A significantly greater hyperpolarization was induced by isoprenaline than was observed without Ba^{2+} (see Table 5; $P<0.05$). When V_m was depolarized in low K_o^+, Ba^{2+} induced a further depolarization, and the hyperpolarization by isoprenaline was smaller than that observed without Ba^{2+} ($P<0.05$; see Table 2, type 1).

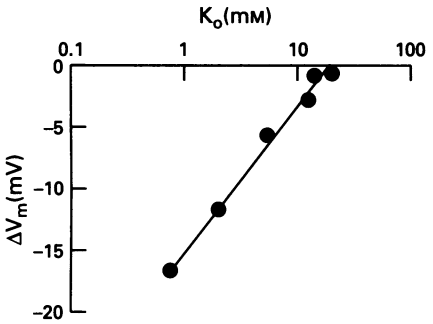

Figure 3 Type 2 hyperpolarizations induced by isoprenaline as a function of K_o^+. The data were fitted in a semi-logarithmic plot with the straight line: $\Delta V_m = -15 + 11.6 \log[K^+]_o$ mV ($r=0.99$). All the measurements were performed at saturating concentrations (>200 nM) of isoprenaline.

When $[Ba^{2+}]$ was increased to 0.6 mM or higher, the response to isoprenaline was fully blocked. At this concentration, Ba^{2+} also blocked the response to IBMX (see Table 5) or 8Br-cyclic AMP (not shown). When the hyperpolarization was completely blocked in low K_o^+ by 0.6 mM Ba^{2+}, the simultaneous washout of both isoprenaline and Ba^{2+} resulted in a significantly ($P<0.05$, paired data, $n=6$) larger hyperpolarization (-13.6 ± 2.2 mV) than could be accounted for by the depolarization by Ba^{2+} alone (7.8 ± 2.5 mV) (see Figure 4). The additional transient hyperpolarization of -5.8 ± 1.1 mV is attributed to an effect evoked by isoprenaline in the cell, that manifests itself after the simultaneous washout. This hyperpolarization behaved like a normally evoked hyperpolarization in low K_o^+ (compare Figure 2). Figure 4 also demonstrates that re-admission of Ba^{2+} (0.6 mM) depolarized the cell again after this hyperpolarization had reached its steady state. This is in line with the observations that Ba^{2+} (0.6 mM) also suppressed the isoprenaline response in low and normal K_o^+ (see Table 5). Apparently, the block by Ba^{2+} of the membrane conductance responsible for the observed hyperpolarization was direct and reversible (see Figure 4) and much faster than the isoprenaline washout.

To exclude the possibility, that during the washout period small amounts of isoprenaline, left behind somewhere in the perfusion system, could reach the cell and stimulate it, propranolol (10 μM) was also added to the washout medium in some experiments. This produced no different results.

Discussion

Measurements in low potassium solutions

Most of the experiments in this study were performed with a low concentration of extracellular potassium (0.76 mM). Un-

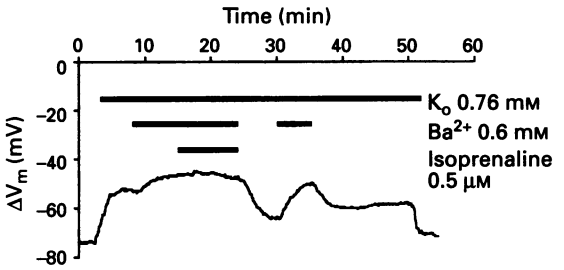

Figure 4 Inhibition of isoprenaline effect by high Ba^{2+}. In a cell, depolarized in low K_o^+, application of 0.6 mM Ba^{2+} resulted in a further depolarization. In the new steady state 0.5 μM isoprenaline could not elicit a response. The simultaneous washout of both chemicals was followed by a hyperpolarization that was greater than the depolarization due to Ba^{2+}. Normally the disappearance of this hyperpolarization was comparable to the normal washout of isoprenaline. This figure also shows that application of 0.6 mM Ba^{2+} during this washout period resulted in a reversible depolarization to a level that is comparable to V_m in low K_o^+ plus 0.6 mM Ba^{2+}.

Table 5 Effects of $BaCl_2$ on the response to isoprenaline

Solution-K^+_o (mM) + *$BaCl_2$* (µM)	*Addition* (µM)	*V_m before* (mV)	*V_m after* (mV)	*ΔV_m* (mV)	n
5.7 + $BaCl_2$ (80)	Isoprenaline (0.2)	−52.8 ± 1.4	−61.6 ± 2.1	−8.8 ± 1.2	7
5.7 + $BaCl_2$ (600)	Isoprenaline (0.2)	−40.8 ± 2.7	−40 ± 2.1	0.8 ± 0.5	3
0.76 + $BaCl_2$ (80)	Isoprenaline (0.2)	−44.3 ± 2.2	−52.7 ± 2.5	−8.4 ± 1.4	6
0.76 + $BaCl_2$ (600)	Isoprenaline (0.2)	−44.1 ± 1.6	−44.1 ± 1.0	0 ± 0	5
0.76 + $BaCl_2$ (600)	IBMX (100)	−43.2 ± 0.9	−42.3 ± 0.5	0.9 ± 0.5	3
0.76 + isoprenaline	$BaCl_2$ (600)	−60.2 ± 2.1	−47.9 ± 2.1	14.7 ± 0.5	6

Effects of $BaCl_2$ on the response to isoprenaline. All initial mean V_m-values of cells depolarized by $BaCl_2$ in low K_o^+ are comparable to the mean value presented in Table 1 (3rd row in column 4). When 0.6 mM $BaCl_2$ (or more) was present in low K_o^+ isoprenaline did not induce changes in V_m ($P<0.05$). n = number of measurements.

der these conditions the activity of the pump and the potassium conductivity of the membrane differ from their control values in 5.7 mM K_o^+.

Different isoforms of the Na^+-K^+-pump with different affinities for the substrates ATP, Na^+ and K^+, and different sensitivities to endogenous regulators have been reported. Clearly the concentrations of K_o^+, in the low K_o^+ experiments, are below the generally accepted affinity for K_o^+ (1–2 mM, Läuger, 1991). In addition, the kinetics of the Na^+-K^+-pump are voltage-dependent and increase when V_m becomes less negative (Apell, 1989). Experimentally we found an instantaneous depolarization of V_m in low K_o^+ as well as in control solution, on addition of ouabain to the perfusion buffer. We conclude that this inhibition indicated that the Na^+-K^+-pump was still operative and actively transporting in low K_o^+. The close qualitative resemblance of the responses to isoprenaline in 5.7 mM and 0.76 mM K_o^+ also supports the conclusion that we measured essentially the same phenomena in low K_o^+ as in control solutions, but with larger responses.

Depolarization associated with reduction of K_o^+ to 0.76 mM is associated with the decrease of the I_{KR} conductance and as a consequence with the increase in the permeability ratio P_{Na}/P_K (Siegenbeek van Heukelom, 1991, and literature cited therein). The enlargement of the hyperpolarizations of V_m, induced by isoprenaline or adrenaline, is corroborated by the observations of Boyden *et al.* (1983a), and Encina *et al.* (1988) in heart tissues and of Ling *et al.* (1984) in frog muscle. Measurements in low K_o^+ also unveiled a distinctly biphasic speed of hyperpolarization (Figure 2), and subsequent repolarization during washout similar to observations by Boyden *et al.* (1983a), though with a different time scale. The observed small hyperpolarizations induced by propranolol might be related to secondary effects due to the high concentrations (5–10 μM). No interaction with 5-hydroxytryptamine (5-HT) (Rang & Dale, 1993) was found. A less specific effect, such as membrane perturbations, like mild local anaesthetics, cannot be excluded. It is not likely that the membrane depolarization as such is the cause of the increased response to isoprenaline in low K_o^+, because the type 1 and type 2 responses are comparable in size (−11.6 vs. −16.3 mV, see Table 1) and the response disappeared in 20 mM K_o^+, when V_m was comparable to V_m in low K_o^+, type 1.

Isoprenaline-induced hyperpolarization is not due to Na^+-K^+-pump activation

Though the Na^+-K^+-pump generates the gradients for Na^+ and K^+ across the cell membrane, arresting it with ouabain does not dissipate these gradients immediately (Mullins & Noda, 1963; Thomas, 1972; Läuger, 1991). In this study, on the mouse lumbrical muscle, we showed that inhibition of the Na^+-K^+ pump with ouabain did not suppress the hyperpolarization induced by adrenaline or isoprenaline, as was observed by Clausen & Flatman (1977) in rat soleus and by Kuba & Nohmi (1987) in rat diaphragm. Even after a long exposure to ouabain (30 min) a response to isoprenaline was still observed.

It was found that reduction of Na_o^+ in low K_o^+ did not change the size of the hyperpolarization, which makes a strong involvement of P_{Na} also unlikely. The interaction with Ba^{2+} is the second argument against the Na^+-K^+ pump as the primary source for hyperpolarization. Without ouabain, Ba^{2+} alone can block the hyperpolarization. To our knowledge, inhibition of the Na^+-K^+ pump by Ba^{2+} is not documented in the literature.

On the grounds of these differential effects of ouabain (ineffective) and Ba^{2+} (effective) we conclude that in mouse lumbrical muscle, increased Na^+-K^+-pump activity has no or only a minor contribution to the observed hyperpolarization induced by isoprenaline and that an increase in P_K is more likely.

The response to isoprenaline is related to an increase in P_K

Though the chloride conductance, g_{Cl}, in skeletal muscle fibre membranes is approximately 3 times that of potassium, g_K (Hodgkin & Horowicz, 1959) it is not a candidate because it is generally accepted that in the steady state the chloride equilibrium potential adjusts itself within a few mV to V_m. Because we compared in our experiments the potentials that reached steady state, it is unlikely that the chloride gradient is strongly involved in the hyperpolarization of V_m.

In solutions in which K_o^+ was lowered to concentrations larger than 1.4 mM we found that isoprenaline induced a hyperpolarization (ΔV_m) related to K_o^+ (see Figure 3 and Ling *et al.* (1984) and Kuba & Nohmi (1987)). If in first approximation the intracellular potassium concentration (K_o^+) is taken as a constant (140 mM), the hyperpolarization is an increasing function of the driving force for potassium $V_m - E_K$ (see Figure 5, E_K is the equilibrium potential for potassium: $RT/F\{\ln K_o^+ - \ln K^+{}_i\}$). The hyperpolarizations in Figure 5 can be fitted to the equation $\Delta V_m = -0.84 - 0.35(V_m - E_K)$ mV ($r = 0.99$). The choice of $K^+{}_i$ determined the ΔV_m-axis intercept (here −0.84 mV) and does not influence the correlation coefficient r. A more detailed description of the dependence of K_i^+ on V_m and K_o^+ (Siegenbeek van Heukelom, 1994) does not qualitatively change the conclusion that in the range 2 to 20 mM K_o^+ isoprenaline induced an increased potassium conductance.

Figure 6 is a schematic description for the interpretation of our results. Only for the explanation we identify the K^+-conductance change induced by isoprenaline as separate: $g_{K,Iso}$. However, we do not exclude any explanation, that identifies this increased conductance differently, such as modification of existing potassium conductances, sub-state conductances

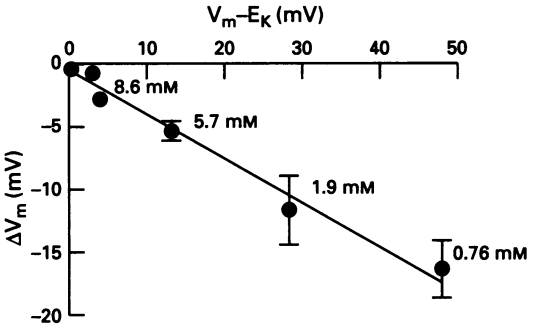

Figure 5 Type 2 hyperpolarizations induced by isoprenaline as a function of the driving force for potassium: $V_m - E_K$, with E_K as the equilibrium potential for potassium. The data have been fitted with the straight line: $\Delta V_m = -0.84 - 0.35(V_m - E_K)$ mV ($r = 0.99$). E_K was calculated taking the intracellular potassium concentration constant: 140 mM.

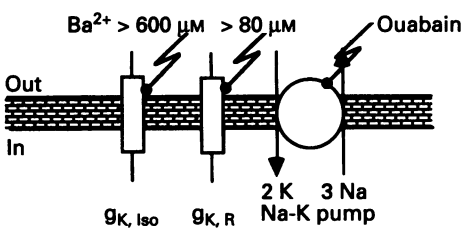

Figure 6 Schematic representation of the membrane components involved in the explanation of the results. Components not discussed, are not shown. $g_{K,Iso}$ is the additional potassium conductance induced by isoprenaline, that is inhibited by high concentrations of Ba^{2+}, the inward potassium rectifier (g_{KR}) is inhibited by 80 μM Ba^{2+} or more and the Na^+-K^+-pump by ouabain.

(Matsuda, 1988; Matsuda *et al.*, 1989) or other interpretations. From our measurements it follows that this conductance is not (strongly) dependent on V_m and K_o^+, less sensitive to Ba^{2+} than the I_{KR}, and not directly associated with the Na^+-K^+ pump. All results can be understood, taking into account the relative importance of $g_{K,Iso}$ in the overall potassium conductance $g_{K,tot} = g_{KR} + g_{K,Iso} + ...$ (the term '...' stands for all other potassium conductances that might also be present, but are not important for the explanation). When K_o^+ drops, the conductance of the inward rectifier g_{KR} also decreases (for review see Hille, 1992) and the relative contribution of $g_{K,Iso}$ becomes more important and observable. This is independent of the fact whether IKR is 'switched -off' or not (see Introduction, Hille, 1992 and Siegenbeek van Heukelom, 1994). The few experiments that we called type 3, we identify as measurements in which, due to the additional effect of $g_{K,Iso}$ the g_{KR} was able to switch from 'off' to 'on'. At higher concentrations Ba^{2+} also blocks $g_{K,Iso}$, which can be interpreted as a lower sensitivity of this potassium conductance for Ba^{2+}.

In line with electrophysiological measurements in other muscle preparations, our results and conclusions suggest that in muscle fibres a variety of responses to isoprenaline may occur as has been reported for the heart (see introduction). Though our study concentrated on the membrane potential only, such differences in reaction might be related to the different responses of muscle groups in the whole organism (Williams & Barnes, 1989).

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