Mechanism of action of a K^+ channel activator BRL 38227 on ATP-sensitive K^+ channels in mouse skeletal muscle fibres

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1. Investigations were made into the effects of BRL 38227, a potassium channel activator, on ATP-sensitive potassium channels (K^+_{ATP} channels) in single fibres dissociated from the flexor digitorum brevis muscle of C57BL/6J mice.

2. In cell-attached patches BRL 38227 (100 μM) caused activation of a glibenclamide-sensitive potassium current. Linear slope conductance of the inward current, partial rectification of the outward current and glibenclamide sensitivity indicate that K^+_{ATP} channels are the site of action of BRL 38227.

3. In the absence of ATP at the cytoplasmic side of excised inside-out patches, BRL 38227 caused direct and magnesium-dependent activation of K^+_{ATP} channels. The degree of activation diminished with successive applications of BRL 38227.

4. BRL 38227 also caused activation of K^+_{ATP} channels in the presence of low (< 100 μM) but not high (1·0 mM) ATP, particularly in patches containing large numbers of channels.

5. BRL 38227 and 5 μM MgATP failed to activate channels following complete run-down.

6. Results show that BRL 38227 caused direct activation of K^+_{ATP} in skeletal muscle and that this was mediated through a magnesium-dependent binding site rather than alleviation of inhibition by competitive displacement of ATP from the inhibitory site.

ATP-sensitive potassium channels (K^+_{ATP} channels) form an interesting class of potassium channel whose kinetics, physiological role and pharmacological regulation are of widespread current interest. They have been extensively studied in a variety of cell types including smooth, cardiac and skeletal muscles as well as neurones and pancreatic β-cells (for recent reviews see Davies, Standen & Stanfield, 1991; Nichols & Lederer, 1991; Dunne & Petersen, 1991). They have also been shown to be modulated by a variety of intracellular modulators and pharmacological agents in most of these tissues (see Weston & Hamilton, 1992). Specific targeting of K^+ channels has been the focus of current research aimed at developing clinically usable hyperpolarizing agents. BRL 38227, the active (−) enantiomer of cromakalim (also known as levcromakalim and lemakalim) is currently under investigation as a specific activator of K^+_{ATP} channels.

BRL 38227 produced a glibenclamide-sensitive hypotensive effect in conscious rats (Masuda, Arakawa, Yokoyama, Shigenobu & Tanaka, 1991) and lowered arterial pressure in cats (Hood, McMahon & Kadowitz, 1991). BRL 38227 also inhibited the noradrenaline-evoked increases in intracellular calcium and isometric tension in rabbit mesenteric arteries (Ito, Kajikuri, Itoh & Kuriyama, 1991) and caused *in vitro* relaxation of human bronchi (Black & Barnes, 1990). Electrophysiological studies show BRL 38227 to hyperpolarize the membrane and cause a time-independent outward current in canine colonic cells (Post, Stevens, Sanders & Hume, 1991) and voltage-clamped skeletal muscle fibres (Hong & Chan, 1991). In all these studies glibenclamide block of BRL 38227 effects has been used to imply that K^+_{ATP} channels may be the major site of action. Although cardiac K^+_{ATP} channels have been shown to be activated by BRL 38227 (Shen, Tung, Machulda & Kurachi, 1991) some studies implicated other K^+ channels as possible targets (Carl, Bowen, Gelband, Sanders & Hume, 1992). The present study investigated direct actions of BRL 38227 on K^+_{ATP} channels in mammalian skeletal muscle.

Skeletal muscle K^+_{ATP} channels have been described in amphibian (Spruce, Standen & Stanfield, 1987) and mammalian (Rowe, Wareham & Whittle, 1990)

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sarcolemmal vesicles, as well as dissociated single fibres (Weik & Neumcke, 1989). K^+ channel activators were shown to increase single channel activity of partially inhibited K^+_{ATP} channels in inside-out patches of mouse (Weik & Neumcke, 1990) and frog (Sauviat, Ecault, Faivre & Findlay, 1991) sarcolemma and to cause hyperpolarization through increased K^+ conductance in human (Spuler, Lehmann-Horn & Grafe, 1989) and mouse (Hong & Chan, 1991) skeletal fibres. Such actions may prove useful in myotonic muscle disorders (Quasthoff, Spuler, Spittelmeister, Lehmann-Horn & Grafe, 1990). Investigations into the mechanism of action of potassium channel activators may also provide insights into the nature of the binding sites for channel modulation and help elucidate the molecular mechanisms regulating K^+_{ATP} channel function.

METHODS

Single fibre preparation

Adult C57BL/6J mice (2–3 months old) of either sex, were killed by ether overdose and the flexor digitorum brevis (FDB) muscles ablated from the hindlimbs into prefiltered (0·2 μm; Gelancamp, Gelman Sciences, Ann Arbor, MI, USA) Tyrode buffer (see below for composition). Single fibres were obtained essentially as described previously (Weik & Neumcke, 1989). Muscles were incubated for 1–1·5 h at 37 °C in Tyrode buffer containing 3·0 mg ml^{-1} collagenase (Type I, Sigma, UK) and 1·0 mg ml^{-1} soyabean trypsin inhibitor (Type II-S, Sigma, UK). Upon subsequent transfer to enzyme-free Tyrode buffer, digested muscles were gently triturated to cause dispersion into single fibres. Aliquots of dissociated fibres were transferred to the patch clamp chamber where they adhered spontaneously to the methanol-cleaned glass base. Fibres were maintained at room temperature and used within 2–3 h of preparation.

Patch clamp

Patch-clamp experiments were performed either in the cell-attached or inside-out configurations using conventional patch clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were double pulled from borosilicate capillaries (hard glass; GC150F, Clark Electromedical Ltd, Pangbourne, Reading, UK) using a vertical List microelectrode puller (model L/M-3P-A; List-electronic, Darmstadt, Germany). Microelectrode tip resistance was typically 10 MΩ after the pull and increased to 15–20 MΩ after fire-polishing the tip. Seal formation was carried out in Tyrode bathing media (except when stated otherwise) by pressing fire-polished tips of fresh microelectrodes against the fibre and applying slight negative pressure to the inside of the pipette. Seal formation was successful in over 90% of the fibres and seal resistances in excess of 20 GΩ were typical. Cell-attached experiments were carried out using Tyrode solution as the bathing medium.

Solutions and drugs

Bathing solution during incubation, seal formation and cell-attached experiments was Tyrode of the following composition (mM): NaCl, 120; KCl, 5; $MgCl_2.6H_2O$, 1·0; $NaHPO_4$, 0·5; $CaCl_2$, 2·5; Hepes, 10·0; and glucose 11·0, adjusted to pH 7·3 with NaOH. This was replaced with high-KCl solution for inside-out patches. The high-KCl solution contained (mM): KCl, 120; EGTA, 1·0; $MgCl_2$, 1·0 or zero; and Hepes, 10·0 adjusted to pH 7·3 with KOH; this was also used as the pipette (extracellular) solution. The cytoplasmic side of detached patches was then exposed to test substances flowing down a glass capillary tube connected by a manifold to a series of reservoirs containing different solutions. The dead space of 0·05 ml and a flow rate of 1·0 ml min^{-1} allowed rapid exchange of solutions. ATP and other drugs were also prepared in this high-KCl solution and the pH readjusted to 7·3 before application. BRL 38227 (a gift from SmithKline Beecham Pharmaceuticals, Harlow, UK) and glibenclamide were prepared in DMSO as 40 and 100 mM stocks, respectively.

Data acquisition and analysis

Ionic currents were measured using a List patch clamp amplifier (L/M-EPC 7; 10 kHz internal filtering) and recorded on FM videotape using a modified Sony PCM-701ES digital audio processor. Off-line analysis was carried out by replaying data through an 8-pole Bessel filter at 1–3 kHz cut-off frequencies. Segments of data were either played back to a TDM PAR 1000 pen recorder (Tape Services Ltd, UK) or acquired at 5 kHz, into the Fetchex series of pCLAMP programs (pCLAMP 5·5; Axon Instruments, Foster City, CA, USA) using a TL-1 interface linked to an Opus PCV 386 computer. Current amplitudes for full openings were determined by the Fetchan series of programs while open probabilities (P_o) for patches containing multiple channels were obtained using SCAP, a computer program written by Dr M. Hunter of Leeds University, UK. Average P_o for 5 s segments of data were calculated according to the following algorithm:

$$P_o = \Sigma (nt_n)/TN,$$

where n is the number of simultaneously active channels (0 is no channels open, 1 is 1 channel open, etc.), t_n is the length of time the channel is in state n, T is the total time of the recording and N is the maximum number of channels detectable during the experiment. Statistical analysis was carried out using Student's t test for unpaired data.

RESULTS

Characteristics of K^+_{ATP} channels in skeletal muscle

Figure 1*A* illustrates the general protocol employed throughout the present study except where otherwise indicated. In patches containing K^+_{ATP} channels, no activity was usually detectable in the cell-attached configuration even after stepping the pipette potential (V_p) to +57·4 mV (first arrow). Channel activity became apparent after detaching the patch into Tyrode solution (second arrow) while V_p was still held at +57·4 mV. Tyrode solution was exchanged with high-KCl solution by washing the patch chamber with 4–5 volumes of the latter (*). Channel activity, visible under symmetrical KCl conditions, was inhibited by 1·0 mM K_2ATP or Na_2ATP (*B*) in the presence or absence (*D*) of magnesium and thus identified as due to K^+_{ATP} channels. Under symmetrical high-KCl solutions, K^+_{ATP} or the delayed rectifiers, similar to those previously described by Standen, Stanfield & Ward (1985) were the

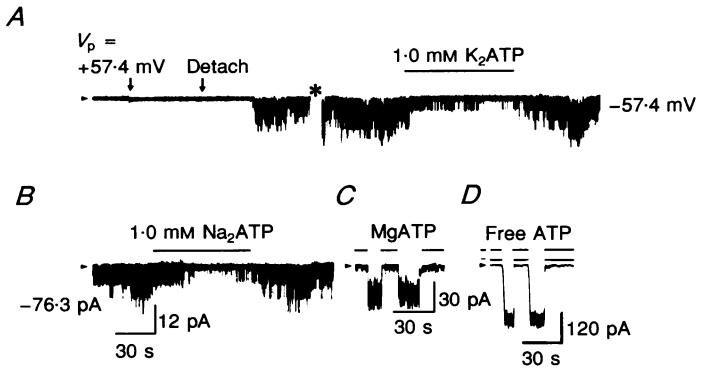

Figure 1. ATP-sensitive potassium channels in mouse skeletal muscle
A, no channel activity was visible in the cell-attached configuration, even when the driving force was increased by stepping V_p to +57·4 mV (first arrow). Channels became visible shortly after detachment (second arrow) into Tyrode bathing solution. The channels were confirmed as being K^+_{ATP}, after exchanging Tyrode solution for high-KCl solution (indicated by the asterisk), by inhibition with 1·0 mM K_2ATP (*A*) or Na_2ATP (*B*). Some patches from fibres had more than 6 or 7 active channels (*C*) while patches from sarcolemmal vesicles frequently had in excess of 30 simultaneously active channels that could be inhibited by 1·0 mM ATP even in magnesium-free high-KCl solution (*D*). Pipette solution was (mM): 120 KCl, 1·0 EGTA, 10·0 Hepes, pH 7·3 and 1·0 (*A–C*) or 0 (*D*) $MgCl_2$.

most frequently observed types of K^+ channels. These were easily distinguished from one another by their characteristic opening behaviour and conductances. Figure 1 also shows that the number of K^+_{ATP} channels varies from patch to patch, and illustrates patches containing several simultaneously active channels (*A–C*). Larger numbers of channels were frequently observed in sarcolemmal vesicles (*D*), as previously described for frog skeletal muscle (Davies *et al.* 1991; Vivaudou, Arnoult & Villaz, 1991). Patches with even higher numbers of K^+_{ATP} channels could be obtained using electrodes with larger tip diameters.

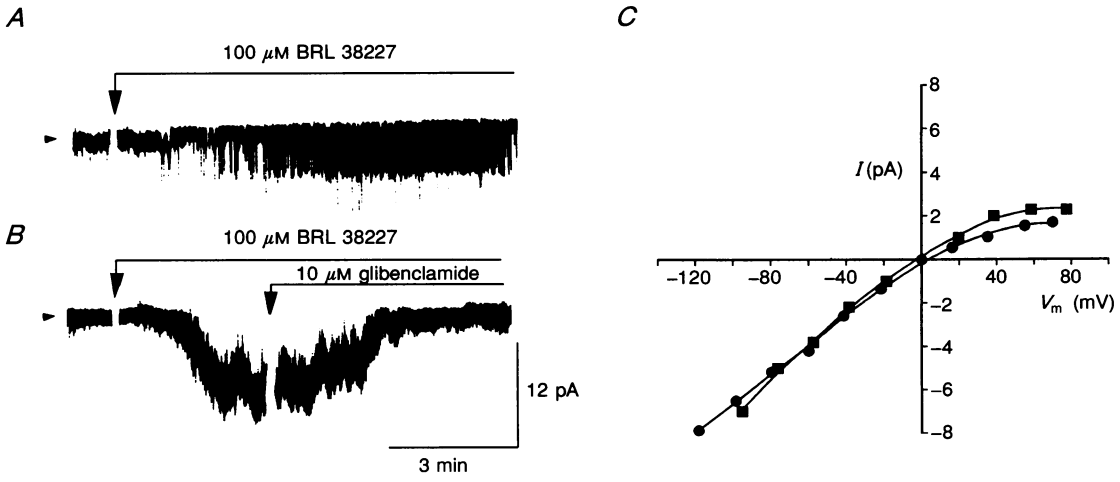

Figure 2. Effect of BRL 38227 on potassium currents in cell-attached patches from intact single fibres from mouse FDB muscle
BRL 38227 was added directly to the bathing Tyrode solution to produce 100 μM final bath concentration (*A* and *B*). At zero pipette potentials and with high-KCl pipette solutions BRL 38227 activated inward potassium currents (*A*) that could be inhibited by 10 μM glibenclamide (*B*). *C*, comparison of *I–V* relations of the BRL 38227-activated potassium current in the cell-attached configuration (●) to *I–V* curves obtained in the inside-out configuration under symmetrical high-KCl solutions (■) without BRL 38227. The *I–V* curve in the cell-attached configuration shows single determinations for each point from 2 separate experiments, while $n = 4 \pm$ S.E.M. for the *I–V* curve in the inside-out configuration; S.E.M. were smaller than the symbols. The patch potential (V_m) in the cell-attached configuration was taken as the difference between cell potential (the reversal potential under these conditions) and the pipette potential at each voltage step.

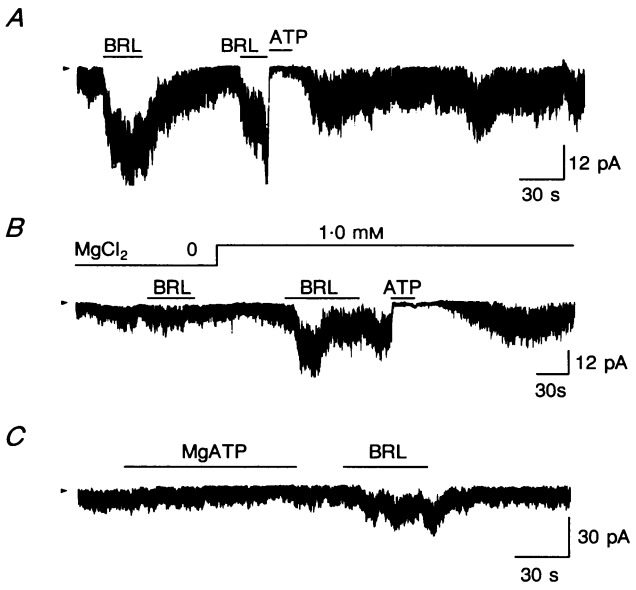

Figure 3. Effects of BRL 38227 on K^+_{ATP} in inside-out patches from mouse skeletal muscle
BRL 38227 (100 μM) applied to the cytoplasmic side of the patch caused direct and reversible activation of K^+_{ATP} in the absence of ATP (*A*). BRL 38227-evoked currents were Mg^{2+} dependent (*B*) and inhibited by 1·0 mM ATP (*A* and *B*). Effects of 5 μM MgATP and BRL 38227 on a separate patch under similar conditions (*C*). Calibrations were as shown for each experiment. Pipette solution (mM): 120 KCl, 1·0 EGTA, 1·0 or zero $MgCl_2$ and 10·0 Hepes, pH 7·3. Bathing solutions were similar but with or without 1·0 mM $MgCl_2$, as indicated by the bold line above the tracing (*B*). Tracing in *B* is typical of 6 such experiments.

Effects of BRL 38227 in cell-attached patches

In the cell-attached mode, the patch potential is the difference between the membrane and pipette potentials ($V_m - V_p$) so that at zero V_p the driving force for current amplitudes is determined solely by V_m. Any variations in the latter will therefore be reflected in the current amplitude. With physiological bathing solutions, V_m is usually depolarized with respect to the potassium equilibrium potential (E_K) thereby providing a driving force for outward K^+ currents. However, when the patch pipette is filled with high-KCl solution, the membrane area under the patch is exposed to approximately the same KCl concentrations on either side (assuming resting $[K^+]_i$ to be near 120 mM). Under these conditions, E_K across the patch (but not the cell) is close to zero, so that negative V_m values provide a driving force for inward K^+ currents across the patch while K^+ moves outwards across the rest of the cell. The opening of K^+_{ATP} channels by 100 μM BRL 38227 did indeed activate an inward K^+ current (Fig. 2*A*) that was blocked by 10 μM glibenclamide (Fig. 2*B*). A current amplitude of −5 pA in Fig. 2*A* suggests a V_m of approximately −60 mV, whereas smaller current amplitudes (Fig. 2*B*) imply that some fibres may be partially depolarized.

Figure 2*C* shows current–voltage (*I–V*) relations of K^+_{ATP} channels in excised inside-out patches (■) and of BRL 38227-activated currents in the cell-attached configuration (●). The *I–V* curve in the inside-out configuration was linear between −80 and 0 mV, with a slope conductance of 68 pS in symmetrical 120 mM KCl. Partial rectification of the outward current was clearly evident even in the absence of 1·0 mM Mg^{2+} and has previously been shown to be exacerbated by cytoplasmic Mg^{2+} or Na^+ (Woll, Lönnendonker & Neumcke, 1989). At potentials between 0 and −90 mV, the slope conductance measured from the *I–V* relationship of the BRL 38227-evoked inward currents was 69 pS, providing further evidence that the BRL 38227-activated currents were indeed carried through K^+_{ATP} channels. The outward current in the cell attached configuration also showed partial rectification at potentials more positive than 0 mV, probably due to blocking of the pore by cytoplasmic mono- and divalent cations.

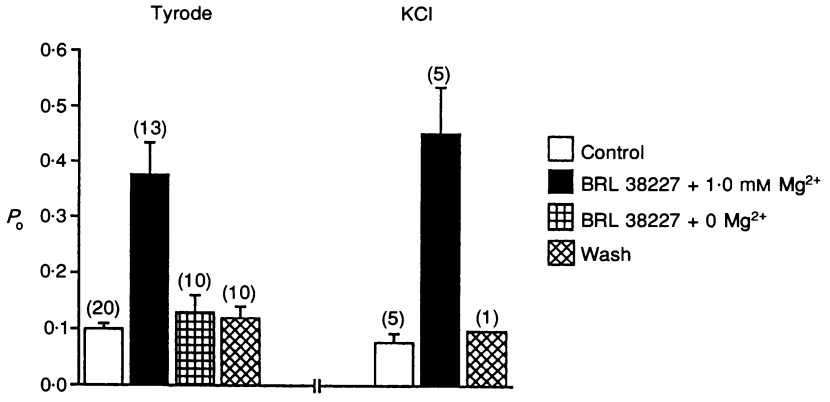

Figure 4. Activation of K^+_{ATP} by 100 μM BRL 38227
Patches were excised either into Ca^{2+}-free high-KCl bathing solutions or Ca^{2+}-containing Tyrode solution and then transferred to the high-KCl superfusing solution before application of BRL 38227. The figure shows means ± S.E.M. channel P_o from separate experiments. Numbers in parentheses indicate the number of determinations.

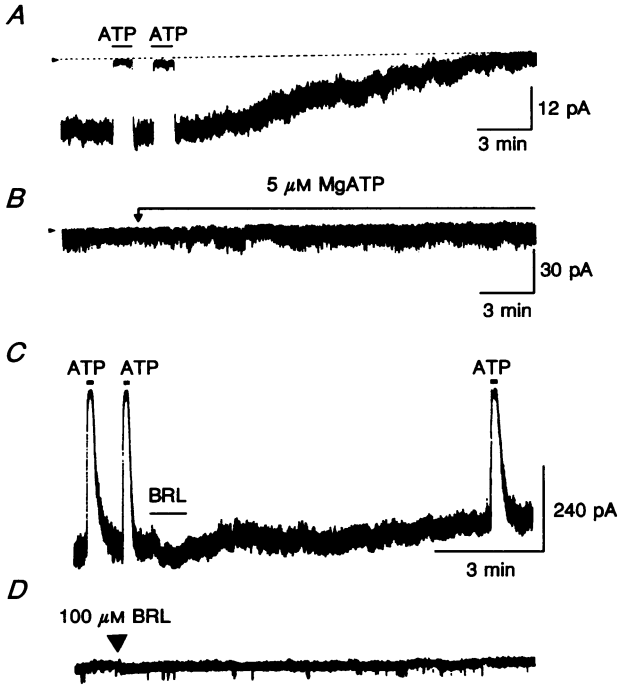

Figure 5. Run-down and the effects of MgATP and BRL 38227 on K^+_{ATP} in mouse skeletal muscle
A patch containing multiple ATP-sensitive potassium channels was superfused with high KCl and allowed to run down (*A*). Attempts to reactivate K^+_{ATP} with 5 μM $MgCl_2$ (*B*; $n = 6$) or BRL 38227 (*D*; $n = 6$) after almost complete run down were unsuccessful. Tracing in *C* is from an experiment where K^+_{ATP}, in a particularly large patch containing approximately 140 active channels, showed little run-down over a 30 min period even in the presence of 1·0 mM magnesium. Application of 100 μM BRL 38227 to such very active channels produced little or no further enhancement of activity. Membrane potential was −38·5 mV for all experiments in this figure. Calibration bars shown in *C* represent 30 s and 30 pA for the tracing in *D*.

Effects of BRL 38227 on K^+_{ATP} channels in excised inside-out patches

In the inside-out configuration, BRL 38227 caused direct and reversible activation of K^+_{ATP} channels (Fig. 3*A*). In patches where BRL 38227-activated K^+ currents were sustained after removal of the drug, ATP (1·0 mM) caused immediate and reversible inhibition, confirming the activated component to be K^+_{ATP} channels (Fig. 3*A* and *B*). Furthermore, BRL 38227-evoked activation was Mg^{2+} dependent (Fig. 3*B*). These were surprising and novel results for two reasons. First, an earlier study has demonstrated that K^+_{ATP} needed to be partially inhibited by ATP before cromakalim (BRL 38447) exerted its effect (Weik & Neumcke, 1990). Such inhibition was clearly not required in experiments during the present study. Secondly, Mg^{2+} dependence of activation by BRL 38227 is particularly interesting in view of the evidence pointing to the existence of a Mg^{2+}-dependent activation site for nucleotide diphosphates in pancreatic (see Dunne & Petersen, 1991), cardiac muscle (Tung & Kurachi, 1991) and skeletal muscle cells (Allard & Lazdunski, 1992). Figure 3*C* shows a separate experiment where application of 5 μM MgATP failed to activate K^+_{ATP} that could be activated by 100 μM BRL 38227 in the same patch. This result has important implications for the nature of the binding site for BRL 38227 and phosphorylation of channel proteins by MgATP (see Discussion).

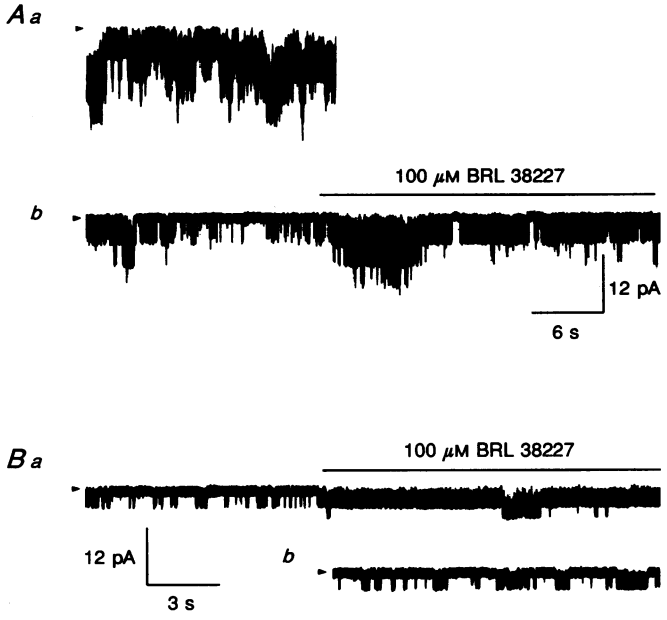

Figure 6. Effects of BRL 38227 on K^+_{ATP} from mouse skeletal muscle after partial run-down
A, original channel activity after detachment into Ca^{2+}-free high-KCl solution (*a*; $P_o = 0·42$) and activity 8 min after excision (start of tracing *b*; $P_o = 0·03$). Application of BRL 38227 (100 μM) increased P_o to 0·25, apparently through an increase in the number of active channels and an increase in both the frequency and duration of individual bursts of openings. *B*, a separate experiment with a patch containing only 2 active channels. BRL 38227 caused a clear increase in P_o (from 0·10 to 0·59) that appeared to be mediated by an increase in the duration of individual bursts of openings. *Bb*, the activity returning to control at 2 min after the removal of BRL 38227.

Quantitative analysis of data shows that 100 μM BRL 38227 increased P_o from $0{\cdot}10 \pm 0{\cdot}01$ ($n = 20$) to $0{\cdot}38 \pm 0{\cdot}06$ ($n = 13$) at the peak of the response ($P < 0{\cdot}001$). P_o then declined to $0{\cdot}12 \pm 0{\cdot}02$ ($n = 10$) 3–4 min after removal of the drug (Fig. 4). BRL 38227 did not cause such large increases in channel activity in the absence of cytoplasmic Mg^{2+}. Furthermore, BRL 38227-evoked effects appeared to be due to an increase in the number of active channels in the patch, thereby raising the question: why are some K^+_{ATP} channels inactive or silent in the patch? K^+_{ATP} channels in cardiac muscle have been shown to be inactivated by the application of Ca^{2+} to the cytoplasmic side of the patch (Findlay, 1988). It is therefore possible that some channels were inactivated upon excising the patch to Ca^{2+}-containing Tyrode solution. However, while inactivation by Ca^{2+} can also occur in frog (Krippeit-Drews & Lönnendonker, 1992) and mouse skeletal muscles (Hussain & Wareham, 1993), Fig. 4 shows that BRL 38227 could also activate K^+_{ATP} channels to a comparable degree even when patch excision was carried out into Ca^{2+}-free high-KCl bathing solutions. Thus, inactivation by Ca^{2+} may not be responsible for the silent channels that were activated by BRL 38227 in Figs 3 and 4.

Effects of MgATP and BRL 38227 after run-down of K^+_{ATP} channels

Activity due to K^+_{ATP} channels is also known to run down with time after excising the patch to the inside-out configuration. This is usually attributed to dephosphorylation of channel proteins because low concentrations of MgATP, but not free ATP or the non-hydrolysable analogues of ATP (AMP-PNP or PCP), can reactivate pancreatic (Findlay & Dunne, 1986; Ohno-Shosaku, Zünkler & Trube, 1987) and cardiac K^+_{ATP} after run-down (Takano, Qin & Noma, 1990). Although there is considerable variability in the time course of run-down of K^+_{ATP} channels in skeletal muscle, the process is often much slower than that reported for cardiac and pancreatic cells (see Fig. 5*A* and *C* and Spruce *et al.* 1987 for frog skeletal K^+_{ATP} channels). Application of 5 μM MgATP or 100 μM BRL 38227 to channels that had been allowed to run down fully for over 30–40 min did not reactivate skeletal K^+_{ATP} channels (Fig. 5*B* and *D*, respectively). BRL 38227 also failed to activate K^+_{ATP} channels in patches that displayed no run-down at all (Fig. 5*C*).

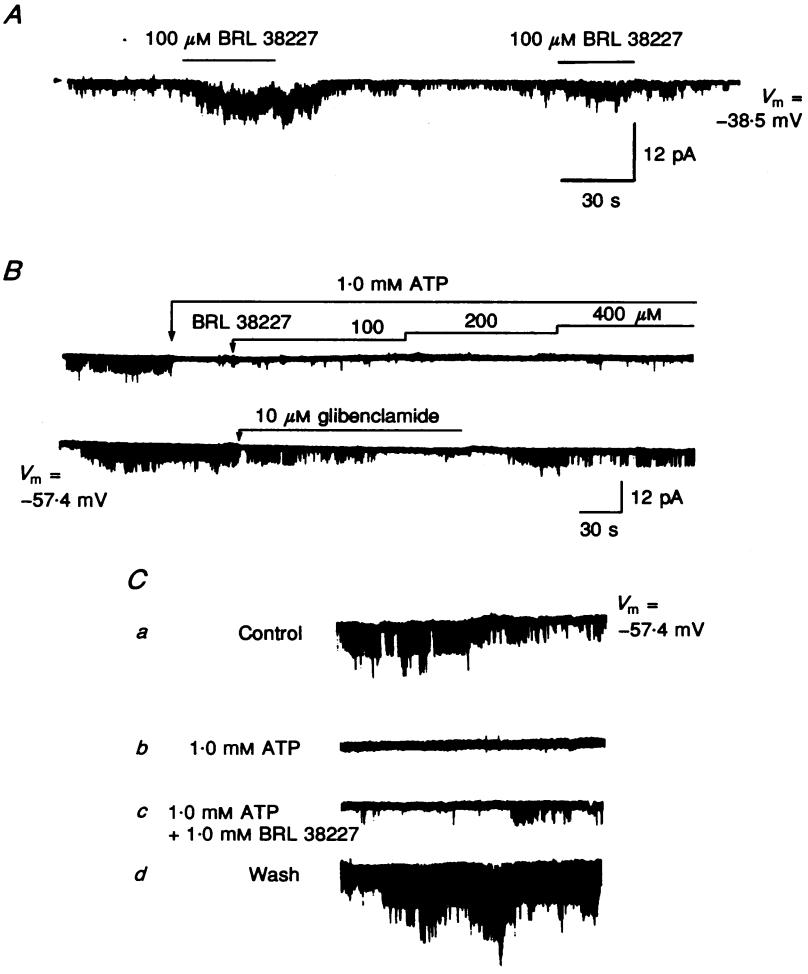

Figure 7. Effects of BRL 38227 on skeletal muscle K^+_{ATP} in the absence and presence of 1·0 mM ATP
A, tracing from one of 5 such experiments, showing diminishing absolute responses of K^+_{ATP} to successive applications of 100 μM BRL 38227 in the absence of ATP. The second application was made after the channel activity from the first challenge returned to or below control levels. *B*, BRL 38227 (100–400 μM) failed to activate K^+_{ATP} inhibited by 1·0 mM ATP (continuous tracing from one of 6 such experiments). Channel activity was consistently higher than pre-inhibition levels following simultaneous wash-out of the drug and ATP. Glibenclamide (10 μM) caused reversible inhibition of K^+_{ATP} in the same patch. *C*, increasing BRL 38227 to 1·0 mM did not increase channel activity inhibited by 1·0 mM ATP (*Cc*). Activity following wash-out (*Cd*) was again higher than that seen prior to inhibition by 1·0 mM ATP (*Ca*).

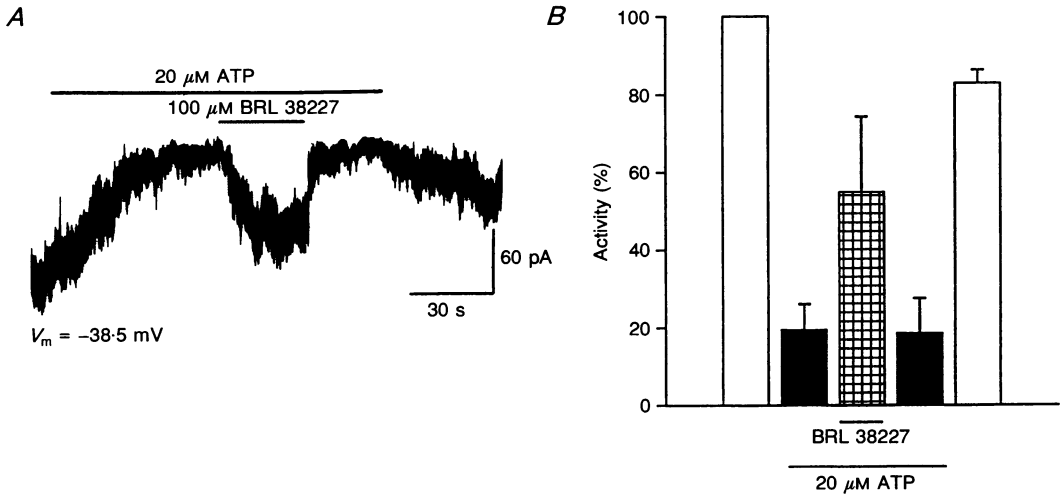

Figure 8. Effects of BRL 38227 on skeletal K^+_{ATP} inhibited by low concentrations of ATP
A, original chart recording displaying the degree of inhibition of K^+_{ATP} by 20 μM ATP and then reactivation of the inhibited channels by BRL 38227. Note that in the presence of ATP, channel activation by BRL 38227 was reversed immediately upon removal of the drug. This is in contrast to the gradual return of channel activity to control levels in the absence of ATP (previous figures) and indicates that the inhibitory effect of ATP is probably more dominant than the activation by lower concentration of BRL 38227 as the drug dissociates from its binding site. *B*, histograms showing the mean (± S.E.M) effects of 20 μM ATP and 100 μM BRL 38227 in the presence of 20 μM ATP from 5 separate determinations. Channel activity is normalized to the control activity (100%) in the absence of ATP.

Experiments shown in Fig. 6 were carried out to see if BRL 38227 could activate K^+_{ATP} channels after partial run-down. The patch was excised into Ca^{2+}-free high-KCl bathing solution and activity continuously monitored after excision. P_o upon detachment of the patch was 0·42 (Fig. 6*A a*) and declined to 0·03 (Fig. 6*A b*) 8 min after excision. Application of 100 μM BRL 38227 caused an increase in the P_o to 0·25. The increase in channel activity was achieved through both a recruitment of inactive channels in the patch and an increase in the frequency and duration of individual bursts of openings. Thus, BRL 38227 could activate channel activity after partial run-down but not after complete run-down or when there was no run-down at all. That BRL 38227-evoked activation involved an increase in the frequency and duration of individual bursts is shown more clearly in tracings from a separate experiment (Fig. 6*Ba* and *Bb*) where only two active channels were present in the patch. The increase in P_o, from 0·10 to 0·59, was achieved through an increase in the frequency and duration of individual bursts. Repeated applications of BRL 38227 to some fresh patches containing active K^+_{ATP} channels caused progressively smaller absolute responses (Fig. 7*A*), until channel activity became fully run down and no further activation was possible. These results indicate that BRL 38227 may only activate K^+_{ATP} channels while they are in a certain *activatable* conformation.

Effects of BRL 38227 in the presence of ATP

Many studies have previously demonstrated that K^+_{ATP} channels need to be partially inhibited by low concentrations of ATP before some potassium channel activators exert their effect (Arena & Kass, 1989; Weik & Neumcke, 1990) and suggest that competitive displacement from the inhibitory site of ATP may be the mechanism of action of such compounds (Thuringer & Escande, 1989; Sauviat *et al.* 1991). In the present study BRL 38227 (100–400 μM) failed to activate K^+_{ATP} channels inhibited by 1·0 mM ATP (Fig. 7*Ba*). Glibenclamide (10 μM) could also reversibly inhibit K^+_{ATP} channels in excised patches (Fig. 7*Bb*). Increasing the concentration of BRL 38227 further to 1·0 mM still failed to cause activation comparable to that seen in the absence of ATP (Fig. 7*Ca–d*). Figure 8*B* also shows (more clearly than Fig. 7*B*) that channel activity after the removal of both BRL 38227 and ATP was consistently higher than the control activity prior to inhibition by ATP. This may be due to a faster wash-off of ATP than BRL 38227, enabling the latter to exert its activation effect on the uninhibited channels. In contrast, K^+_{ATP} channels inhibited by 20 (and 100) μM ATP could be activated by 100 μM BRL 38227 (Fig. 8). Such activation was particularly apparent in patches containing large numbers of channels. These data are consistent with results from previous studies cited above. However, they

are interpreted differently to explain the mechanism of action of potassium channel openers.

DISCUSSION

Physiological and pharmacological modulation of K^+_{ATP} channels has been studied in a variety of cell types including pancreatic β-cells, cardiac myocytes, neurones and skeletal muscle fibres (see Weston & Hamilton, 1992). Inhibition by free ATP and the non-hydrolysable analogues of ATP serve to indicate that K^+_{ATP} channel inhibition occurs after binding of ATP to a Mg^{2+}-independent site without concomitant phosphorylation. ADP, AMP and other adenine derivatives can also inhibit K^+_{ATP} channels but less effectively (Spruce *et al.* 1987). Activation by low concentrations of ADP and other nucleotide diphosphates suggest there is a relatively non-specific activation site (Tung & Kurachi, 1991). Hence, at least two binding sites for a large variety of intracellular regulators and pharmacological modulators have been proposed for the regulation of K^+_{ATP} channels. An independent third site for glibenclamide probably also exists. However, complete characterization and the physiological significance of such regulatory molecules remains obscure. Investigations into the mechanism of action of BRL 38227 were carried out in the present study to elucidate characteristics of such regulatory sites and gain further insights into the mechanisms controlling channel function in skeletal muscle.

K^+_{ATP} channels in cell-attached patches

In the present study, 100 μM BRL 38227 caused activation of a K^+ current in cell-attached patches in single fibres from mouse skeletal muscle. The direction of current flow could be manipulated by alterations in the driving force set by the pipette potential. Glibenclamide sensitivity, partial rectification and the linear slope conductance were strong indicators that K^+_{ATP} channels were the site of action for BRL 38227 (Fig. 2). This was further substantiated by direct activation of K^+_{ATP} channels in excised inside-out patches (Fig. 3). The ability of BRL 38227 to activate tonically inhibited K^+_{ATP} channels in the cell-attached configuration but not in the presence of 1·0 mM ATP in excised patches suggests that K^+_{ATP} channels may not be regulated by bulk or total ATP within the cell but rather by specifically coupled compartments of low ATP. Alternatively, other antagonistic factors such as ADP may blunt the inhibitory actions of ATP in the intact cell and enable channels to be opened by BRL 38227. In either case, unless our single fibres are already extremely anoxic and therefore depleted of ATP, these data suggest that channel inhibition in the intact cell may be by surmountable ATP concentrations close to threshold so that even small changes in intracellular regulatory factors such as pH (Davies, Standen & Stanfield, 1992) may be sufficient to cause channel activation following metabolic exhaustion. The ability to record currents through K^+_{ATP} channels in the cell-attached configuration in intact single fibres should also prove useful in determining whether the increased K^+ conductance upon muscle exhaustion (Castle & Haylett, 1987) is mediated via K^+_{ATP} channels. In addition, single fibres may also allow other experimental perturbations to manipulate cellular metabolism during cell-attached recordings, and may therefore be a useful preparation for directly exploring the physiological function of K^+_{ATP} channels in the intact cell.

Mechanism of activation of skeletal K^+_{ATP} channels by BRL 38227

Activation by BRL 38227 was Mg^{2+} dependent but was unlikely to have been mediated via phosphorylation because BRL 38227 could activate K^+_{ATP} channels that could not be activated by 5 μM MgATP in the same patch. Activation of skeletal K^+_{ATP} channels by 5 μM MgATP was very rarely observed except when ATP had been left standing at room temperature for several hours. This suggests that ATP hydrolysis may generate ADP that can stimulate the channels. ADP (1–100 μM) has recently been shown to increase activity of skeletal K^+_{ATP} in mouse flexor digitorum brevis fibres (Forestier & Vivaudou, 1993). Moreover, activation was only seen in approximately 50% of the patches. These results may also explain the variability seen in our experiments following attempts to reactivate the channels with MgATP. The lack of phosphorylation of K^+_{ATP} channels in skeletal muscle may be due to wash-out of additional regulatory molecules such as endogenous G-proteins and kinases that may be associated with the channels.

Activation of cardiac K^+_{ATP} channels by diazoxide was also found to be Mg^{2+} dependent but only in the presence of 100 μM MgATP, suggesting that phosphorylation may be involved (Kozlowski, Hales & Ashford, 1989). However, in the same study, cromakalim (10–100 μM) failed to activate cardiac K^+_{ATP} channels in the presence or absence of ATP, whereas numerous other reports have documented the direct actions of cromakalim or BRL 38227 in cardiac muscle (Escande, Thuringer, Leguern & Cavero, 1988; Shen *et al.* 1991). In the present study, BRL 38227 caused activation even in the absence of exogenously applied ATP. Direct phosphorylation is therefore unlikely to be part of the mechanism of action for BRL 38227 in skeletal muscle. The presence of endogenous ATP within the patch is an unlikely source of the phosphorylating potential because most patches could be activated repeatedly, albeit to a progressively smaller degree, over long periods of time (10–20 min) during which continuous superfusion of the patch would wash out any superficial membraneous ATP. Leakage of ATP from damaged fibres in the patch chamber was also considered unlikely to provide micromolar concentrations of ATP in the bath because only small aliquots of fibres, containing approximately 6–10 single fibres, were used during each experiment.

Moreover, continuous and direct superfusion of the patch with ATP-free buffers would prevent such contaminants in the bathing solution from inhibiting the channels. The presence of contaminant ATP in the superfusion system was also ruled out by demonstrating that K^+_{ATP} channels could be activated by BRL 38227 in experiments where no ATP at all had been introduced into the system.

Diminishing responses to repeated applications of BRL 38227 may be due to a gradual increase in the proportion of non-activatable channels achieved through run-down. A number of previous reports have indicated that partially (but not completely) run-down cardiac K^+_{ATP} channels can be activated by cromakalim or BRL 38227 (Escande *et al.* 1988; Takano & Noma, 1990; Shen *et al.* 1991). A phosphorylated state of channel proteins, maintained by ATP (Findlay & Dunne, 1986), may provide a preferred conformation for activation by K^+ channel openers. In the present study, BRL 38227 could not activate K^+_{ATP} channels after complete run-down even after prolonged exposure to 1–10 μM MgATP alone, suggesting that an activatable state of the channels could not be achieved through phosphorylation.

Previous studies showing that K^+ channel openers could activate K^+_{ATP} channels inhibited by low but not high concentrations of ATP (Arena & Kass, 1989) have often been interpreted as suggesting that K^+ channel openers may act via competitive displacement of ATP from the inhibitory site. Although such displacement may well occur, competitive displacement was not the major mechanism of action for BRL 38227 in the present study because partial inhibition by low ATP was not a prerequisite for channel activation by BRL 38227. This view is also held by Fan, Nakayama & Hiraoka (1990) who demonstrated that a rightward shift in the ATP inhibition curve caused by pinacidil was limited only to certain concentrations of pinacidil. Such antagonism was described as pseudo-competitive because pinacidil was thought to bind to a site other than the inhibitory site and modulate the affinity of receptors for ATP rather than compete for the same site.

Our results are consistent with the view that K^+_{ATP} channels need to be closed before BRL 38227 and possibly other channel openers will exert their effect. This can explain why very active K^+_{ATP} channels, in patches that do not show run-down (Fig. 5*C*), are not susceptible to further activation. The closed state of the channel that is sensitive to modulation by BRL 38227 can be achieved in a number of different ways including partial run-down, inhibition by low concentrations of ATP and perhaps even through inactivation by cytoplasmic calcium. Detailed kinetic analysis of amphibian skeletal K^+_{ATP} channels demonstrated the existence of two open and four closed time constants (Davies *et al.* 1991). It may be that only in some of these closed states is the channel in a conformation from which it can be reopened. Thus when longer closed times are observed after prolonged run-down, the channel proteins may attain an irreversible conformation with a closed channel pore that can not be reopened by BRL 38227. This would explain why channels could not be activated after complete run-down.

In conclusion, the present study provides evidence to suggest that activation of skeletal K^+_{ATP} channels by BRL 38227 is mediated through a magnesium-dependent binding site or activation process and not through competitive displacement of ATP. Association of BRL 38227 to this Mg^{2+}-dependent binding site appeared to increase channel activity by stimulating inactive channels in the patch and by increasing the frequency and duration of individual bursts of openings. BRL 38227 may therefore modulate the intrinsic gating mechanisms of K^+_{ATP} channels and prove to be a useful tool for elucidating the molecular interactions between different regulatory mechanisms controlling channel function.

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