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Skeletal Muscle ATP-Sensitive K^+ Channels Recorded from Sarcolemmal Blebs of Split Fibers: ATP Inhibition is Reduced by Magnesium and ADP

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Summary. A new, nonenzymatically treated preparation of amphibian sarcolemmal blebs has been used to study the regulation of skeletal muscle ATP-sensitive K^+ [K(ATP)] channels.

When a frog skeletal muscle fiber is split in half in a Ca^{2+}-free relaxing solution, large hemispherical membrane blebs appear spontaneously within minutes without need for Ca^{2+}-induced contraction or enzymatic treatment. These blebs readily formed gigaseals with patch pipettes, and excised inside-out patches were found to contain a variety of K^+ channels. Most prominent were K(ATP) channels similar to those found in the surface membrane of other muscle and nonmuscle cells. These channels were highly selective for K^+, had a conductance of ≈53 pS in 140 mM K^+, and were blocked by internal ATP. The presence of these channels in most patches implies that split-fiber blebs are made up, at least in large part, of sarcolemmal membrane.

In this preparation, K(ATP) channels could be rapidly and reversibly blocked by glibenclamide (0.1–10 μM) in a dose-dependent manner. These channels were sensitive to ATP in the micromolar range in the absence of Mg. This sensitivity was noticeably reduced in the presence of millimolar Mg, most likely because of the ability of Mg^{2+} ions to bind ATP. Our data therefore suggest that free ATP is a much more potent inhibitor of these channels than MgATP. Channel sensitivity to ATP was significantly reduced by ADP in a manner consistent with a competition between ADP, a weak inhibitor, and ATP, a strong inhibitor, for the same inhibitory binding sites.

These observations suggest that the mechanisms of nucleotide regulation of skeletal muscle and pancreatic K(ATP) channels are more analogous than previously thought.

Key Words K(ATP) channel · glibenclamide · sulphonylurea · patch clamp · *Rana esculenta*

Introduction

The presence of a basal membrane and a tight connective tissue matrix makes it particularly challenging to use the patch-clamp technique to study surface membrane ionic channels of noncultured adult skeletal muscle fibers. Single-channel activity in native membranes of such cells have been recorded almost exclusively from sarcolemmal vesicles which appear after prolonged treatment of the muscle with proteolytic enzymes (Standen et al., 1984; Burton, Dörstelmann & Hutter, 1988), or from the surface membrane of enzymatically dispersed fibers (Woll, Lönnendonker & Neumcke, 1989). The difficulties and possible adverse effects associated with such enzymatic treatments (Trube, 1983) led us to search for an alternative nonenzymatic method of preparation.

We have discovered that large sarcolemmal blebs may be elicited by a mechanical procedure which consists in splitting a muscle fiber in half in an appropriate relaxing solution. This technique is rapid, simple, and requires no special manual dexterity.

Using this preparation, a study of the effects of pharmacological and metabolic inhibitors of K(ATP) channels was undertaken. Skeletal muscle K(ATP) channels have not been characterized as extensively as pancreatic or cardiac K(ATP) channels, and their function is still an open question (Quast & Cook, 1989; Ashcroft & Ashcroft, 1990). In order to better apprehend the physiological role of these channels, it is necessary to understand the mechanisms which regulate their activity in normal physiological conditions. As a step toward this goal, this study provides new insights on the role of Mg^{2+} and ADP in the inhibition of channel activity by ATP.

Brief accounts of this work have been published in abstract form (Vivaudou, Arnoult & Villaz, 1989, 1990; Vivaudou et al., 1990).

Materials and Methods

Preparation of Split-Fiber Blebs

Fibers were prepared from the iliofibularis thigh muscle of the adult frog, *Rana esculenta*. The muscle is dissected and placed in a relaxing solution (typically, in mM: 150 K^+, 120 methanesulfo-

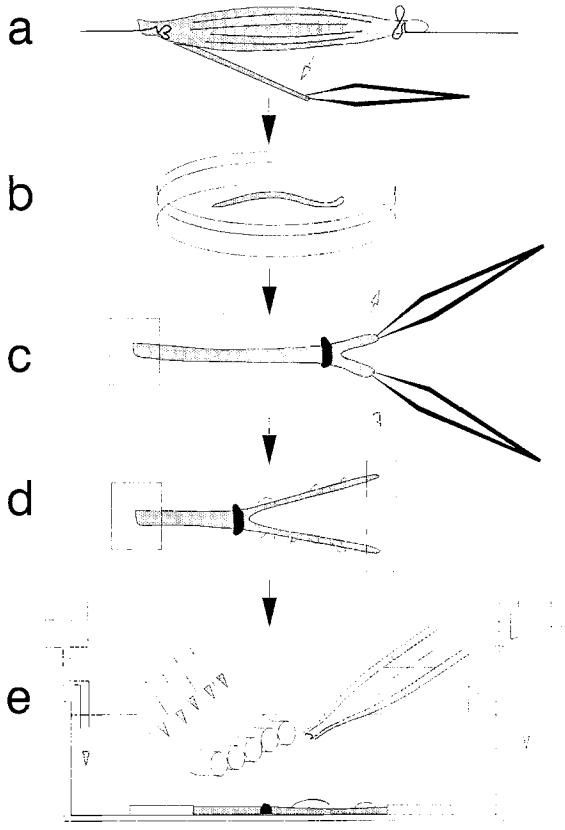

Fig. 1. Successive steps involved in the production of sarcolemmal split-fiber blebs (*see* text)

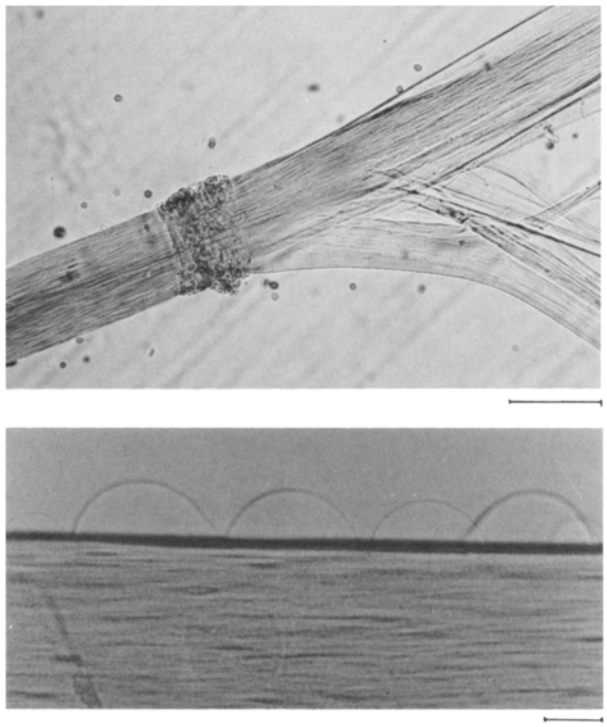

Fig. 2. Photographs of sarcolemmal blebs observed through phase-contrast optics. Top photograph shows a muscle fiber which has been partly split in half by the procedure described in Fig. 1. Several blebs have formed on the external face of the top branch. Bottom photograph shows blebs from another split fiber at a higher magnification. Calibration bars represent 200 μM (top) and 50 μM (bottom)

nate$^-$, 20 Cl^-, 3 MgATP, 1 EGTA, 5 PIPES, and KOH to pH 7.1). A single fiber is isolated (Fig. 1*A*) and placed in a plastic petri dish (Fig. 1*B*) in relaxing solution. Under a microscope, one end of the fiber is cut in half over a short distance with a scalpel. The fiber is then split over half its length by pulling apart the two split ends with tweezers (Fig. 1*C*). This procedure tended to roll up external membranes over the fiber, thereby forming a cuff (Fig. 2). To keep the fiber still, the two split ends and the intact end were fixed to the dish with adhesive tape (Fig. 1*D*). Small hemispherical vesicles would normally form after this procedure on the external faces of the split ends. Fusion of these vesicles over a period of several minutes resulted in blebs of 10 to 100 μm in diameter (Fig. 2).

Incubation of the fiber in the high KCl/low Ca relaxing solution was necessary to keep the fiber relaxed since contraction caused disappearance of the blebs, but it was not sufficient to produce blebs (Hilgemann, 1989) since formation of blebs on the intact end of the fiber was never observed.

Electrophysiological Data Acquisition and Analysis

We have used the patch-clamp technique in the excised inside-out configuration (external side of the blebs in contact with the pipette solution) (Hamill et al., 1981). Upon excision, a closed vesicle usually formed at the tip of the patch pipette as evidenced by a lack of response of the patch to bath solution changes. This vesicle was disrupted by exposing it briefly to the air (Hamill et al., 1981). Patch pipettes (5–20 MΩ) were pulled from borosilicate capillary tubes with a Mecanex BB-CH puller and coated with dental wax. Gigaseals could be obtained easily without further heat polishing. Single-channel currents were recorded with a Bio-Logic RK300 amplifier, filtered at a 0.3–3 kHz cut-off, and stored in digitized format on digital audio tapes using a Bio-Logic DTR-1200 recorder. Currents were later sampled (1–10 kHz) with a microcomputer for further analysis with custom software. For inclusion in the figures, the original data was further numerically filtered and undersampled in order to accommodate the limited resolution of the output device. The resulting effective sampling rate (f_s) and filter cut-off frequency (f_c) are indicated in the figure captions.

Unitary currents were determined either by fitting amplitude histograms with Gaussian curves or, most often, by measuring directly the difference between successive current levels with cursors. Both methods yielded similar values. From these measurements, plots of unitary current *vs.* potential (*I-V* curves) could be constructed and conductances (g) were measured by linear least-square fitting of the linear portion of these curves. The correlation factor, r^2, was used to assess goodness of fit.

Dose-response curves for nucleotide inhibition were constructed by successively exposing the patch for a few seconds to a range of nucleotide concentrations and computing the mean

current at each concentration. Mean current serves as a measure of channel activity since it is linearly related to channel open probability when there is a fixed population of identical independent channels. One must be aware that this last statement remains a hypothesis as independence is not proven and channel rundown may alter the number of active channels in the patch.

Dose-response curves were fitted using an interactive computer program to the equation:

$$I = I_{max}/[1 + ([ATP]/K_i)^h],$$

where I = mean current, I_{max} = maximum mean current in absence of ATP, [ATP] = ATP concentration, K_i = half-maximal inhibition dose, and h = Hill coefficient. Goodness of fit was judged by eye.

The usual sign conventions are employed, i.e., in the excised inside-out configuration, currents flowing into the pipette are positive and membrane potential is the difference between bath and pipette potentials.

Experiments were conducted at room temperature. Results are based on data from 54 patches.

Solutions

Bath solution changes were performed either by rapidly perfusing the 2-ml dish with 30 ml of the new solution, or by placing the patch pipette in the mouth of one of 12 polyethylene tubes from which the different solutions continuously flowed (Fig. 1*E*). The flow rate of 0.2 to 0.5 μl/sec had no noticeable influence on the channels under study.

Solutions were designed using ALEX, a program to compute free concentrations of the various ionic species of solutions containing multiple metal and ligands. The Pascal program ALEX, which is available upon request, uses the same methodology as Fabiato's Fortran program SPECS (Fabiato, 1988), but it is more powerful and less prone to errors than SPECS. The apparent stability constants at t = 22°C and pH 7.1 used to calculate free concentrations of nucleotides are those given by SPECS' ancillary program STACONS (Fabiato, 1988), i.e., $K_{Mg.ATP}$ = 11170 M^{-1}, $K_{Mg.ADP}$ = 675.3 M^{-1}, $K_{K.ATP}$ = 4.377 M^{-1}, $K_{Na.ATP}$ = 6.47 M^{-1}, and $K_{Mg.EGTA}$ = 38.35 M^{-1}.

The exact compositions (in mM) of the solutions used are indicated in the figure captions. Unless otherwise specified, pipette and bath solutions (pH 7.1) contained high K^+ (130–150 mM) and low Cl^- (6–40 mM) for optimum recording of K^+ currents.

ATP (potassium and sodium salts), ADP (potassium salt), and glibenclamide were from Sigma. Glibenclamide (0.1–10 μM) was prepared from a 20-mM stock solution in dimethyl sulfoxyde (DMSO).

Results

ATP-Sensitive K^+ Channels in Split-Fiber Blebs

Patches excised from split-fiber blebs contained several classes of K^+-conducting channels of varying conductances. Most prominent among those were channels with a conductance of ≈53 pS in symmetrical 140 mM K^+ solutions and a strong dependence on bath concentrations of MgATP. The properties of these channels placed them without ambiguity within the family of K(ATP) channels.

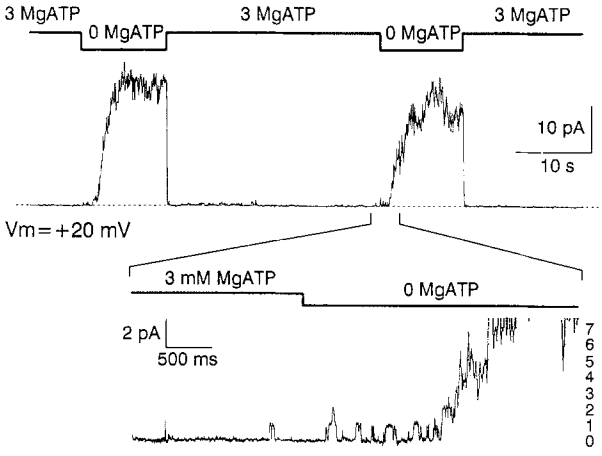

Fig. 3. ATP-inhibited channels were present in large number in inside-out patches excised from split-fiber blebs. Top trace: The presence of more than 30 channels was revealed upon removal of 3 mM ATP. Bottom trace: On a smaller scale, activation of individual channels could be observed just after removal of MgATP. Time courses of activation (relatively slow, latent period) and inhibition (fast) were consistent with previous observations in heart (Qin et al., 1989). Pipette and bath solutions (in mM): 153 K^-, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, 5 PIPES, and 3 MgATP added to bath as indicated. Top trace: f_s = 30 Hz; f_c = 10 Hz. Enlarged area: f_s = 1 kHz; f_c = 100 Hz. Channel conductance was 64.1 pS (r^2 = 99.7%). Patch 93317

ATP-sensitive K^+ channels have been found in the plasma membrane of amphibian and mammalian skeletal muscle fibers (Spruce, Standen & Stanfield, 1985; Burton et al., 1988; Weik & Neumcke, 1989), in the transverse-tubule membrane of mammalian skeletal muscle fibers (Parent & Coronado, 1989), as well as in the plasma membrane of other muscle and nonmuscle cells (Ashcroft & Ashcroft, 1990). Despite tissue-specific differences, a set of several distinctive key features confers K(ATP) channels a unique signature. These features which are shared by split-fiber bleb channels are as follows (Ashcroft & Ashcroft, 1990):

(*i*) K(ATP) channels are rapidly and reversibly inhibited by physiological concentrations of cytosolic Mg and ATP (Fig. 3).

(*ii*) K(ATP) channels are highly selective for K^+ ions over other ions. In our case, outward unitary currents were reduced to the level of the noise when all bath K^+ was replaced by either Na^+, Cs^+, or Tris while the magnitude of unitary inward currents was unchanged. Moreover, manipulation of the bath or pipette anionic composition (6 to 106 mM Cl^- completed with MOPS, gluconate, methanesulfo-

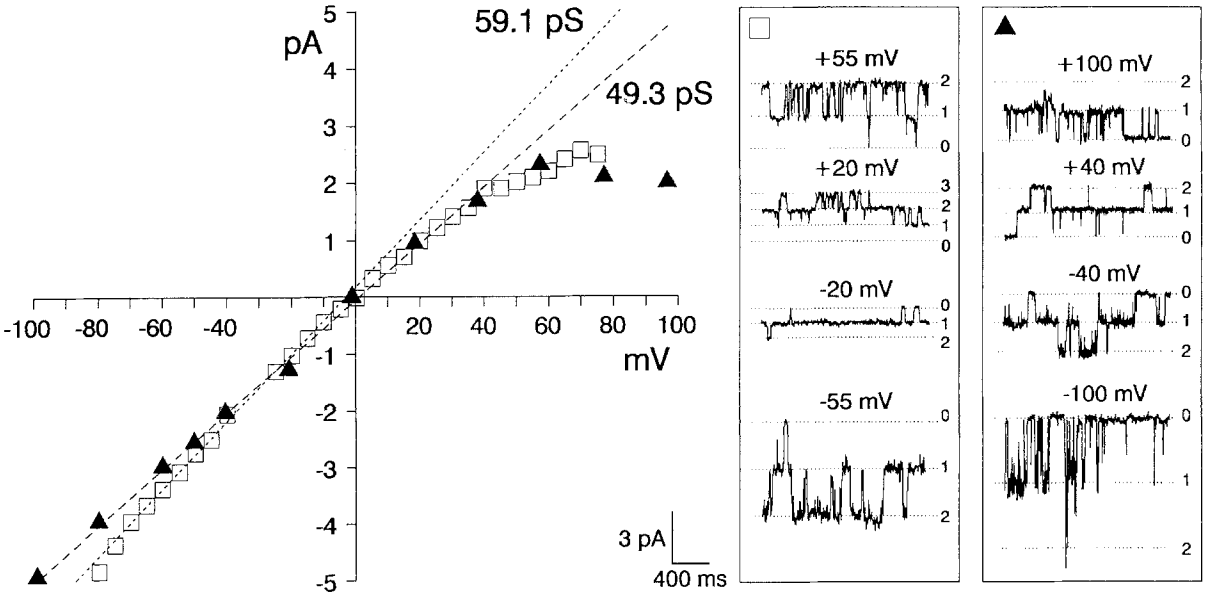

Fig. 4. *I-V* curves (left) and corresponding sample recordings at various potentials (right) for two different patches. Although the pipette solutions were not identical in these experiments, patch-to-patch variability in channel conductance was also observed when experimental conditions were identical. Squares: g = 59.1 pS (r^2 = 99.7%). Pipette solution (in mM): 153 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, and 5 PIPES. Bath solution (in mM): 154 K^+, 3 Mg^{2+}, 6 Na^-, 3 ATP, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, and 5 PIPES. f_s = 500 Hz; f_c = 100 Hz. Patch 94104. Triangles: g = 49.3 pS (r^2 = 99.8%). Pipette solution (in mM): 145 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 3 Mg^{2+}, 0.1 EGTA, and 5 PIPES. Bath solution (in mM): 154 K^+, 3 Mg^{2+}, 6 Na^-, 3 ATP, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, and 5 PIPES. f_s = 500 Hz; f_c = 200 Hz. Patch 9A042

nate, or aspartate) did not visibly affect the recorded currents.

(*iii*) K(ATP) channels have an intermediary conductance of 40 to 80 pS for inward currents in elevated K^+ solutions, and they show a mild inward rectification which is accentuated by intracellular Mg^{2+}. Accordingly, at negative potentials, split-fiber bleb channels had conductances ranging from 45 to 66 pS depending on the patch (Fig. 4) yielding an average of 52.7 ± 7 pS (n = 38). This patch-to-patch variability, which is not uncommon in the literature (e.g., Arena & Kass, 1989; Weik & Neumcke, 1989; Fan, Nakayama & Hiraoka, 1990), could reflect different levels of conductance of K(ATP) channels (Weik, Lönnendonker & Neumcke, 1989). At positive potentials, inward rectification was evident and could be reduced, but not eliminated, by removing Mg^{2+} (Fig. 5).

(*iv*) Activity of K(ATP) channels is not overtly dependent on voltage. In split-fiber blebs, sustained channel activity was observed at all potentials between −100 and +100 mV (Fig. 4). Voltage dependence of gating, while not quantified because of the large number of channels per patch, was not obvious.

(*v*) K(ATP) channels are blocked by sulphonylureas like tolbutamide or glibenclamide. We show

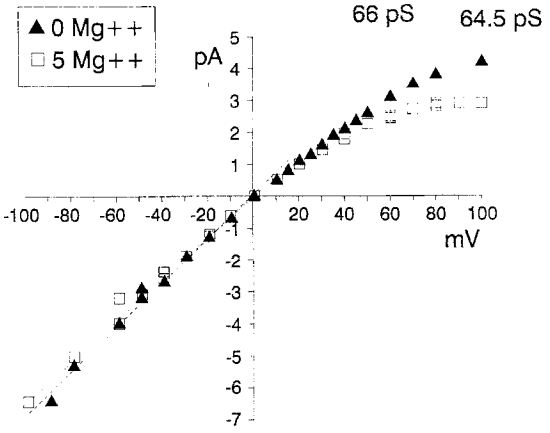

Fig. 5. Inward rectification of ATP-sensitive K^- channels was more pronounced in the presence of cytoplasmic millimolar Mg^{2+} while inward current conductance was unaffected. Squares: g = 64.5 pS (r^2 = 99%). Triangles: g = 66 pS (r^2 = 99.95%). Pipette solution (in mM): 150 K^+, 40 Cl^-, 100 aspartate$^-$, and 5 PIPES. Bath solution (in mM): 150 K^+, 40 Cl^-, 100 aspartate$^-$, 1 EGTA, and 5 PIPES, with 5 Mg^{2+} and 1 ATP (squares) or 0 Mg^{2+} and 0.32 ATP (triangles). Patch 05112

below that this was indeed the case in our preparation.

We believe that these observations provide

definite evidence that ATP-sensitive K^+ channels are present in split-fiber blebs. To our knowledge, channels of this type are localized exclusively to the surface membrane of cells and have no known equivalent in internal membranes such as those of the sarcoplasmic reticulum or mitochondria. Our contention is therefore that split-fiber blebs are composed of sarcolemmal membrane, i.e., plasma membrane or transverse-tubule membrane, and that the internal face of the blebs is the cytoplasmic face. Such a conclusion is further supported by the occasional recordings of a small ≈20-pS K^+ channel, which could be the inward-rectifier channel (Burton et al., 1988; Arena & Kass, 1989), and a large ≈280-pS K^+ channel activated by micromolar bath Ca^{2+}, which could be the large Ca^{2+}-activated K^+ channel (Latorre et al., 1989).

Skeletal Muscle ATP-Sensitive K^+ Channels Are Blocked by Glibenclamide

Sulphonylureas such as tolbutamide and glibenclamide are used therapeutically as antidiabetic drugs which promote insulin secretion. Their mode of action was recently uncovered: they were found to block single K(ATP) channels in pancreatic cells (Sturgess et al., 1985; Schmid-Antomarchi et al., 1987), as well as in cardiac (Belles, Hescheler & Trube, 1987; Fosset et al., 1988) and smooth muscle cells (Standen et al., 1989). In skeletal muscle, tolbutamide has been said to block K(ATP) channels at the single-channel level (Woll et al., 1989).

We also find that glibenclamide can block K(ATP) channels from skeletal muscle (Fig. 6). Glibenclamide had an effect in 16 out of 17 patches where it was tested. In all cases, the lowest dose tried (1 μM and 100 nM, eight patches each) clearly reduced channel activity to less than 50% of control. Higher doses reduced activity further (Fig. 6). Therefore, the concentration required to produce half-maximal inhibition is probably less than 100 nM in our preparation. This value is well within the range of values obtained in pancreatic cells in the whole-cell recording configuration (Ashcroft & Ashcroft, 1990).

In our preparation, single-channel block by glibenclamide was rapidly and fully reversible (Fig. 6). In this respect, skeletal muscle cells appear different from β-cells where inhibition by glibenclamide is poorly reversible (Sturgess et al., 1985; Gillis et al., 1989) and from cardiac cells where glibenclamide block has not been shown to be reversible (Fosset et al., 1988; Arena & Kass, 1989), and inhibition by the less potent tolbutamide is only partly reversible (Belles et al., 1987). Glibenclamide has also been reported to produce a reversible inhibition of single-channel currents in one patch in arterial smooth muscle (Standen et al., 1989).

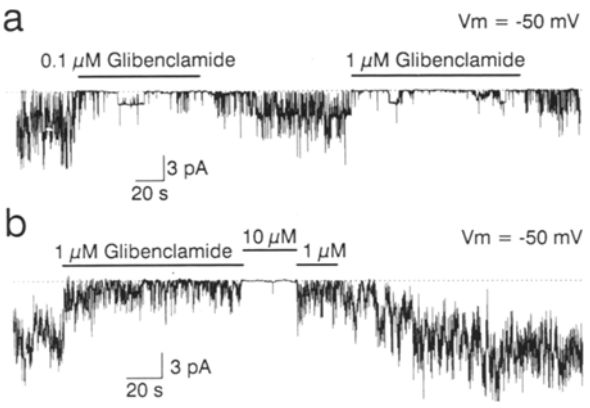

Fig. 6. Glibenclamide reversibly blocked ATP-sensitive K^+ channels. Block was similar in the presence (*a*) and absence (*b*) of cytoplasmic ATP. (*a*) Pipette solution (in mM): 153 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, and 5 PIPES. Bath solution (in mM): 154 K^+, 3 Mg^{2+}, 6 Na^+, 3 ATP, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, and 5 PIPES. f_s = 50 Hz; f_c = 30 Hz. Channel conductance was 55.6 pS (r^2 = 99.6%). Patch 93315. Note that this patch contained such a large number of channels that even in the presence of 3 mM MgATP the remaining activity was significant. (*b*) Pipette solution (in mM): 150 K^-, 40 Cl^-, 100 aspartate$^-$, and 5 PIPES. Bath solution: 150 mM K^+, 5 mM Mg^{2+}, 3.2 μM ATP, 40 mM Cl^-, 100 mM aspartate$^-$, 1 mM EGTA, and 5 mM PIPES. f_s = 20 Hz; f_c = 6 Hz. *I-V* curve shown in Fig. 5. Patch 05112

Inhibition of Channel Activity by ATP Is Reduced by Magnesium

ATP-sensitive K^+ channels are present at a high density in skeletal muscle (Spruce et al., 1985). Accordingly, split-fiber bleb patches contained large numbers of channels (typically, 10 to 100). It was therefore possible to obtain from only a few seconds of data an accurate value of the mean patch current. (Mean current being proportional to channel open probability was used as a measure of channel activity.) Consequently, dose-response curves for nucleotide inhibition could be constructed in a time short enough to minimize errors due to slow drifts in patch resistance, channel properties, or number of active channels ("run-down").

Channel run-down was slower than in other preparations (e.g., Parent & Coronado, 1989) and was often barely noticeable over periods of minutes. Nonetheless, channel activity declined steadily during an experiment and disappeared after 1 or 2 hr. On occasions, unusually rapid run-down could be triggered by removal of all ATP and then partly reversed by exposure to MgATP as illustrated in

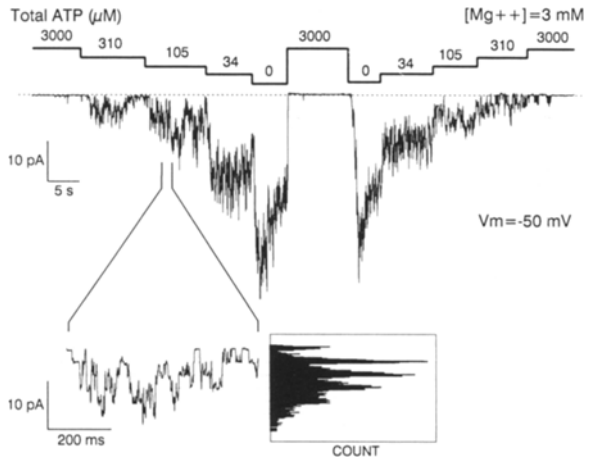

Fig. 7. Typical experimental protocol showing the dependency of channel activity upon ATP in the presence of 3 mM Mg^{2+}. In this patch, unusual rapid run-down of activity was observed in the total absence of ATP and was partially reversed by application of millimolar ATP as seen when [ATP] was changed from 0 to 3 mM and then back to 0. On an expanded time scale (bottom), the current signal is seen to result from the activity of multiple channels of similar conductance as confirmed by the equally spaced peaks on an amplitude histogram. Pipette solution (in mM): 145 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 3 Mg^{2+}, 0.1 EGTA, and 5 PIPES. Bath solution (in mM): 150 K^+, 3 Mg^{2+}, 6 Na^-, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, 5 PIPES, and ATP added as specified. Top trace: f_s = 50 Hz; f_c = 20 Hz. Enlarged area: f_s = 2 kHz; f_c = 300 Hz. Patch 9A315

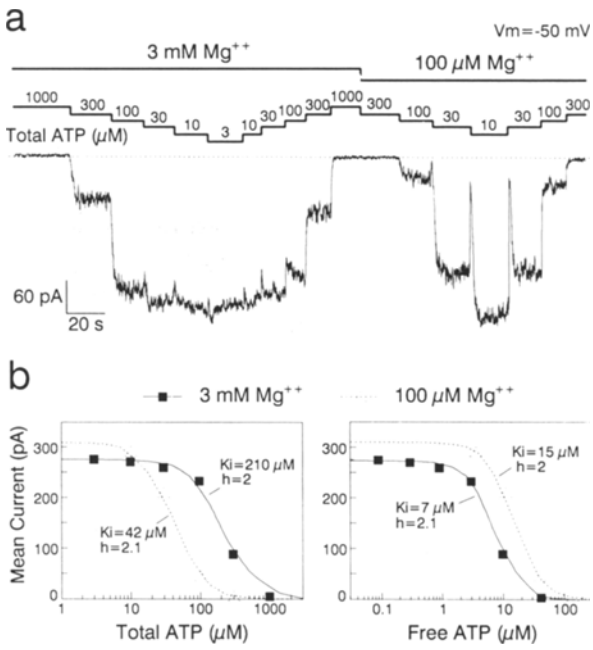

Fig. 8. (*a*) Currents recorded in a patch containing more than 100 active channels at various concentrations of total added ATP in the presence of high (3 mM) and low (100 μM) Mg^{2+}. f_s = 18 Hz; f_c = 10 Hz. (*b*) Corresponding dose-response plots of the mean current at each ATP concentration *versus* total ATP (left) and *versus* computed free ATP (right), in the presence of 3 mM Mg^{2+} (squares) and 100 μM Mg^{2+} (triangles). Pipette solution (in mM): 145 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 3 Mg^{2+}, 0.1 EGTA, and 5 PIPES. Bath solution (in mM): 150 K^+, 10 Na^+, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, 5 PIPES, and Mg^{2+} and ATP added as specified. Channel conductance was 60.6 pS (r^2 = 99.3%). Patch 04126

Fig. 7. Such data is consistent with the hypothesis that channel run-down could involve a dephosphorylation step (Findlay & Dunne, 1986; Findlay, 1987*b*).

Figure 7 provides an example of the dependence of channel activity on cytoplasmic ATP. In the continued presence of 3 mM Mg^{2+}, half-maximal inhibition was achieved in that patch with about 30 μM added ATP. This value was fairly variable from patch to patch like in cardiac cells (Qin, Takano & Noma, 1989). In 10 patches with 3 mM Mg^{2+} in the bath, the ATP concentration producing half-maximal block (K_i) was 96 ± 80 μM. In four other patches with 5 mM Mg^{2+}, K_i was 550 ± 274 μM. This increase in K_i with bath Mg^{2+} in different patches was further confirmed by experiments where dose-response curves were obtained at different Mg^{2+} concentrations in the same patch (Figs. 8 and 9). In those conditions, ATP was clearly more effective in the presence of lower concentrations of Mg^{2+}. As [Mg^{2+}] was lowered from 3 mM to 100 μM (Fig. 8), K_i decreased from 203 ± 21 to 77 ± 40 μM (three patches). As [Mg^{2+}] was lowered from 5 mM to 0 (Fig. 9), K_i decreased from 550 ± 274 to 54 ± 52 μM (four patches).

Mg^{2+} by itself affects K(ATP) channels. It is known to reduce unitary outward currents (Fig. 5; Horie, Irisawa & Noma, 1987; Findlay, 1987*a*,*b*); it is also known to inhibit channel activity (Findlay, 1987*a*,*b*; Ashcroft & Kakei, 1989). Neither effects can explain our observations. First, channel activity was tested at −50 mV where currents are inward and not subject to Mg^{2+} rectification (Fig. 5). Second, in the presence of ATP we see that Mg^{2+} causes an augmentation, and not an inhibition, of channel activity. Hence, a possible role of Mg^{2+} as an intrinsic inhibitor (not tested) could only lead to an underestimation of the present effect.

In pancreatic cells, where similar data has been reported (Dunne, Illot & Petersen, 1987; Dunne et al., 1988; Ashcroft & Kakei, 1989), it has been advanced that the complex MgATP is much less effective at blocking K(ATP) channels than free ATP (i.e., all other forms of ATP which are mainly ATP^{4-} and $ATPH^{3-}$). Accordingly, the potentiating role of Mg^{2+} would reside in its ability to reduce free ATP. Can this explanation be true for skeletal muscle as well? If free ATP is the only blocker, channel activity when plotted against free ATP instead of total

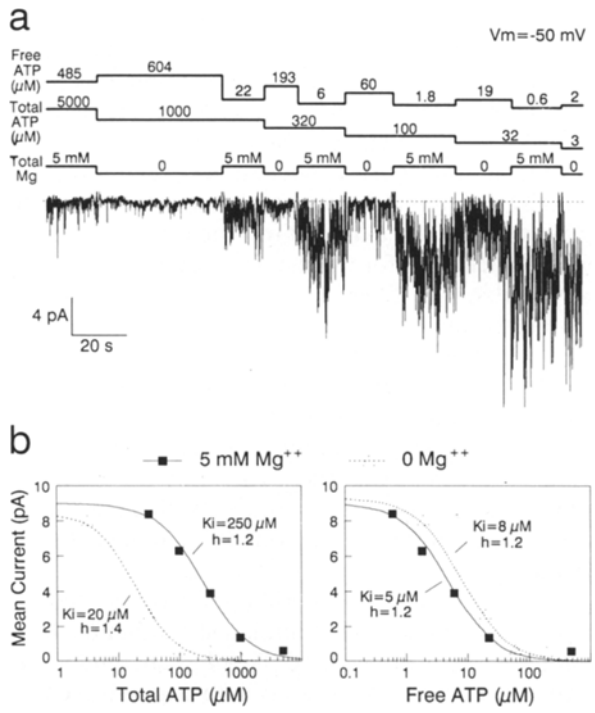

Fig. 9. (*a*) Stimulation of channel activity by Mg^{2+} at various concentrations of ATP. f_s = 50 Hz; f_c = 20 Hz. (*b*) Corresponding dose-response plots of the mean current at each ATP concentration *versus* total ATP (left) and *versus* computed free ATP (right), in the absence of Mg^{2+} (triangles) and in 5 mM Mg^{2+} (squares). Pipette solution (in mM): 150 K^+, 40 Cl^-, 100 aspartate$^-$, and 5 PIPES. Bath solution (in mM): 150 K^+, 40 Cl^-, 100 aspartate$^-$, 1 EGTA, 5 PIPES, and Mg^{2+} and ATP added as specified. Channel conductance was 62.3 pS (r^2 = 99.9%). Patch 0601B

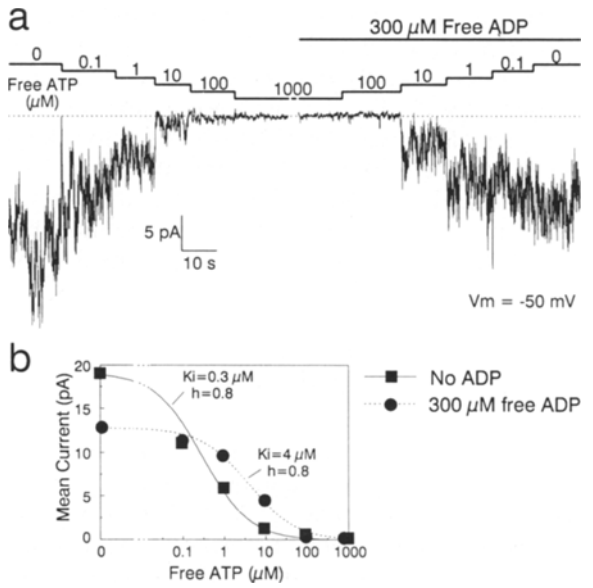

Fig. 10. (*a*) Currents recorded at various concentrations of free ATP before and after addition of ADP. Total ATP and ADP necessary for the indicated free concentrations were computed as described in Materials and Methods. Gap in the record corresponds to ≈20 sec of data. f_s = 30 Hz; f_c = 10 Hz. (*b*) Corresponding dose-response plots of the mean current at each ATP concentration *versus* free ATP in the absence of ADP (squares) and in 300 μM free ADP (circles). Pipette solution (in mM): 145 K^+, 20 Cl^-, 120 methanesulfonate$^-$, 3 Mg^{2+}, 0.1 EGTA, and 5 PIPES. Bath solution (in mM): 150 K^+, 10 Na^+, 20 Cl^-, 120 methanesulfonate$^-$, 1 EGTA, 5 PIPES, and ATP and ADP as needed for specified concentrations of free ATP and free ADP. Patch 02025

ATP (Ashcroft & Kakei, 1989) would show no dependence on Mg^{2+}. Such plots are shown in Figs. 8 and 9; they demonstrate a clear reduction in Mg^{2+} dependence, but not quite its elimination. Channel activity at a given concentration of free ATP was slightly but consistently lower in the presence of millimolar Mg^{2+}. This shift could mean that MgATP can block the channel but less potently than free ATP. However, it could also be due to the inhibitory action of Mg^{2+} ions as discussed above. One can also argue that the shift was caused by poor estimation of free ATP. For instance, if the value used for the stability constant $K_{Mg,ATP}$ was higher than the actual *true* value, a Mg^{2+}-dependent shift would result because free ATP would be underestimated in the presence of Mg^{2+}.

The observed effect of Mg^{2+} was independent of the actual value of K_i which could vary by one order of magnitude or more from one patch to another. It appeared also independent of the degree of run-down, the number of active channels, or the protocol employed to acquire the dose-response data (*compare* Figs. 8 and 9).

ADP Reduces the Sensitivity to ATP

Reports have indicated that ADP could antagonize the effect of ATP in pancreatic (Dunne & Petersen, 1986; Kakei et al., 1986; Misler et al., 1986) and cardiac cells (Findlay, 1988*b*; Lederer & Nichols, 1989). We find this to be true for skeletal muscle as well. In the experiment of Fig. 10, addition of 0.3 mM free ADP caused a reduction in the currents recorded at low concentrations of ATP. At higher concentrations of ATP, ADP caused an increase in current. Thus, ADP shifted the ATP dose-response curve, increasing K_i by an order of magnitude from 0.3 to 4 μM. In another patch, K_i was increased from 3 to 20 μM by 0.3 mM free ADP, while in two other patches, K_i was increased from 11 to 500 μM and from 1 to 45 μM by 2 mM total ADP (in the presence of 5 mM Mg^{2+}, the concentration of free ADP equaled roughly 0.6 mM for free ATP levels below

100 μM.) In those experiments, dose-response data was obtained both before and after application of ADP in order to verify that the decrease in channel activity at low ATP levels was not a consequence of channel run-down. As ADP is known to inhibit K(ATP) channels, though less potently than ATP (Spruce, Standen & Stanfield, 1987), our observations would be consistent with a competition between ADP and ATP for the channel nucleotide binding sites.

Discussion

The work presented here may be divided into two parts: one part is the development of a new preparation for patch-clamp studies of adult skeletal muscle, i.e., split-fiber blebs. The other is the further characterization of skeletal muscle ATP-sensitive K^+ channels. Clearly, these two parts overlap since the channel results both rely on and prove the adequacy of the preparation.

Split-Fiber Blebs

The split-fiber blebs technique is based on the observation that hemispherical membrane blebs appear spontaneously at the surface of a single muscle fiber which has been split in half in a relaxing solution. The prevalence in these blebs of K^+ channels, especially K(ATP) channels, which have so far been found only in the surface membrane of cells from muscle and other tissues, constitutes, if not absolute proof, rather strong evidence that blebs are extrusions of the sarcolemma. Whether blebs are composed of plasma membrane or transverse-tubule membrane or both is not known. However, the presence in the blebs of significant amount of transverse-tubule membrane would seem unlikely since transverse tubules are mechanically anchored to the sarcoplasmic reticulum by the junctional foot proteins. Extrusion of transverse tubules would entail disruption of the junctional complex. It is unlikely that this would be achieved by our procedure as these structures can resist comparatively much harsher treatments at the hands of biochemists, such as grinding, top speed blending, and hours of ultrafast centrifugation (Mitchell, Palade & Fleischer, 1983). Another possibility still to be investigated could be that blebs are formed not by surface membrane but by subsurface caveolae (Ishikawa, Sawada & Yamada, 1983) remaining after removal of the outer surface membrane.

In many respects, split-fiber blebs resemble sarcolemmal spheres obtained by enzymatic treatment (Standen et al., 1984). However, our technique replaces the time-consuming and possibly damaging enzyme treatment with a rapid and simple mechanical procedure. It should be noted that, while this technique was developed, Stein and Palade (1989) reported another purely mechanical method of producing sarcolemmal spheres which involved stretching a fiber to the breaking point. The two approaches bear similarity but for the fact that, unlike sarcolemmal spheres, split-fiber blebs remain firmly attached to the fiber and are a less mobile target for a patch pipette. In that respect, split-fiber blebs are morphologically like *Sarcoballs* which are blebs of sarcoplasmic reticulum origin produced by Ca^{2+}-induced contraction of single skinned muscle fibers (Stein & Palade, 1988). Yet, the two types of blebs are undoubtedly distinct since, unlike split-fiber blebs, *Sarcoballs* do not have obvious K^+ channels (Stein & Palade, 1988).

The split-fiber blebs technique is well-suited for patch-clamp studies of amphibian skeletal muscle as demonstrated by our study of K(ATP) channels. The technique is applicable as well to human skeletal muscle obtained by biopsies (Quasthoff et al., 1990) for which enzymatic treatment gives poor result (S. Quasthoff, *personal communication*). Blebs have also been observed on split muscle fibers from pig and rat (S. Quasthoff, *personal communication*). Thus, the method might be of general use for electrophysiological studies of skeletal muscle from amphibian or mammalian origin.

Glibenclamide Block

ATP-sensitive K^+ channels are found at a high density in split-fiber blebs. They are highly selective for K^+ ions, have a conductance to inward current of $\approx$53 pS in elevated K^+, display a pronounced inward rectification which is partly dependent on internal Mg^{2+}, and are not conspicuously voltage dependent. Therefore, biophysically, those channels are not different from K(ATP) channels already described in skeletal muscle and other tissues (Ashcroft & Ashcroft, 1990).

The main focus of this work was the effects of pharmacological and metabolic inhibitors on K(ATP) channels. On this point, skeletal muscle K(ATP) channels have not been as extensively studied as their counterparts in the pancreas and the heart (Ashcroft & Ashcroft, 1990).

At the single-channel level, we find that submicromolar doses of the sulphonylurea glibenclamide blocks K(ATP) channels. Block is achieved within seconds of drug application and relieved completely within minutes after removal of the drug. While com-

parable glibenclamide sensitivity is seen in other tissues (Ashcroft & Ashcroft, 1990), the clear reversibility of the action of glibenclamide is in contrast with the extremely poor reversibility seen in other tissues such as pancreas (Sturgess et al., 1985; Gillis et al., 1989) and heart (Fosset et al., 1988; Arena & Kass, 1989).

Inhibition by ATP

Regarding the metabolic regulation of channel activity, we provide evidence that channel activity is controlled mainly by free ATP, i.e., ATP not complexed with Mg^{2+}. To support this conclusion, experiments were performed in a number of patches to assess the effect of varying Mg^{2+} on the sensitivity of the channels to ATP. Mg^{2+} caused a rightward shift of plots of channel activity *versus* total ATP, but when channel activity was plotted against free ATP, Mg^{2+} caused only a slight leftward shift, consistent with a much stronger inhibition by free ATP than MgATP. Effects of Mg^{2+} ions unrelated to their ability to bind ATP could be ruled out: outward current attenuation or reduction of channel activity by free Mg^{2+} could not account for these observations as they would have produced opposite effects; reactivation of run-down channels by MgATP was avoided by using different short experimental protocols in patches where run-down was slow.

Free ATP appears also to be a more effective blocker than MgATP in enzymatically dissociated mouse skeletal muscle (Weik & Neumcke, 1989). Nonetheless, it is surprising to see such an effect of Mg^{2+} in our preparation since others, working with sarcolemmal spheres of enzymatically treated frog skeletal muscle fibers, have reported just the opposite finding, i.e., that Mg^{2+} does not affect channel inhibition by ATP (Spruce et al., 1987; Davies, 1990). Since neither supporting data nor experimental protocols were presented, the causes for this discrepancy can only be guessed. One explanation could be that channel properties are influenced by the technique of preparation, and in particular, by the use of proteolytic enzymes. Another explanation would be more likely: we and other (Qin et al., 1989) have found that the K_i for ATP inhibition is rather variable from patch to patch. These intrinsic variations can actually be larger than those caused by Mg^{2+} and could consequently hide the effects of Mg^{2+} if conclusions are drawn from a gathering of data points obtained in different patches. In our case, this problem was avoided because dose-response curves for ATP inhibition were measured at different Mg^{2+} concentrations in the same patch.

Role of ADP

ADP had a dual effect. In the presence of low doses of ATP, channel activity was reduced by ADP (0.3–0.6 mM free ADP). However, at higher doses of ATP, ADP stimulated channel activity. Overall, plots of channel activity *versus* free ATP were scaled down and shifted to the right by ADP as if ADP and ATP competed for the same binding sites. This reduction in ATP sensitivity by ADP in skeletal muscle has also been described in the heart (Findlay, 1988*b*; Lederer & Nichols, 1989) and the pancreas (Dunne & Petersen, 1986; Kakei et al., 1986; Misler et al., 1986). We have not yet studied the complex role of Mg^{2+} in the interaction ATP-ADP (Findlay, 1988*b*), and therefore, we do not know the relative inhibitory and stimulatory efficacies of free ADP and MgADP.

Comparison with Other Preparations

This work suggests that skeletal muscle K(ATP) channels studied in sarcolemmal blebs of split fibers are regulated by free ATP rather than MgATP. This finding which is in contrast with previous reports would imply that, on the basis of ATP regulation of K(ATP) channels, skeletal muscle fibers behave like pancreatic cells and unlike cardiac cells where MgATP is a better inhibitor than free ATP (Findlay, 1988*a*). Another similarity between skeletal muscle and pancreas is the reduction by ADP of the sensitivity of the channel to ATP. Thus, the same mechanisms appear to control K(ATP) channels in the two tissues. One may then speculate that this resemblance could extend to channel physiological function. In pancreatic β-cells, the resting potential is controlled by K(ATP) channels which serve to couple excitability to metabolic state. If in resting skeletal muscle K(ATP) channels are probably inactive since sulphonylureas alter neither membrane potential (Spuler, Lehmann-Horn & Grafe, 1989) nor contraction force (Grafe et al., 1990), in exercising skeletal muscle, these channels could open in response to diminishing free ATP and increasing ADP and likewise have an influence on fiber excitability.

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References

Arena, J.P., Kass, R.S. 1989. Activation of ATP-sensitive K channels in heart cells by pinacidil: Dependence on ATP. *Am. J. Physiol.* **257**:H2092–H2096

Ashcroft, F.M., Kakei, M. 1989. ATP-sensitive K^- channels in pancreatic B-cells: Modulation by ATP and Mg^{2+} ions. *J. Physiol.* **416:**349–367

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

Belles, B., Hescheler, J., Trube, G. 1987. Changes of membrane currents in cardiac cells induced by long whole-cell recordings and tolbutamide. *Pfluegers Arch.* **409:**582–588

Burton, F., Dörstelmann, U., Hutter, O.F. 1988. Single-channel activity in sarcolemmal vesicles from human and other mammalian muscles. *Muscle Nerve* **11:**1029–1038

Davies, N.W. 1990. Modulation of ATP-sensitive K^+ channels in skeletal muscle by intracellular protons. *Nature* **343:**375–377

Dunne, M.J., Illot, M.C., Petersen, O.H. 1987. Interaction of diazoxyde, tolbutamide and ATP^{4-} on nucleotide-dependent K^+ channels in an insulin-secreting cell line. *J. Membrane Biol.* **99:**215–224

Dunne, M.J., Petersen, O.H. 1986. Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett.* **208:**59–62

Dunne, M.J., West-Jordan, J.A., Abraham, R.J., Edwards, R.H.T., Petersen, O.H. 1988. The gating of nucleotide-sensitive K^+ channels in insulin-secreting cells can be modulated by changes in the ratio ATP^{4-}/ADP^{3-} and by nonhydrolyzable derivatives of both ATP and ADP. *J. Membrane Biol.* **104:**165–172

Fabiato, A. 1988. Computer programs for calculating total from specified or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *In:* Methods in Enzymology. Vol. 157. pp. 378–417. S. Fleischer and B. Fleischer, editors. Academic, San Diego

Fan, Z., Nakayama, K., Hiraoka, M. 1990. Pinacidil activates the ATP-sensitive K^+ channel in inside-out and cell-attached patch membranes of guinea-pig ventricular myocytes. *Pfluegers Arch.* **415:**387–394

Findlay, I. 1987*a*. ATP-sensitive K^+ channels in rat ventricular myocytes are blocked and inactivated by internal divalent cations. *Pfluegers Arch.* **410:**313–320

Findlay, I. 1987*b*. The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J. Physiol.* **391:**611–629

Findlay, I. 1988*a*. ATP^{4-} and ATP · Mg inhibit the ATP-sensitive K^+ channel of rat ventricular myocytes. *Pfluegers Arch.* **412:**37–41

Findlay, I. 1988*b*. Effects of ADP upon the ATP-sensitive K^+ channel in rat ventricular myocytes. *J. Membrane Biol.* **101:**83–92

Findlay, I., Dunne, M.J. 1986. ATP maintains ATP-inhibited K^+ channels in an operational state. *Pfluegers Arch.* **407:**238–240

Fosset, M., de Weille, J., Green, R.D., Schmid-Antomarchi, H., Lazdunski, M. 1988. Antidiabetic sulfonylureas control action potential properties in heart cells via high-affinity receptors that are linked to ATP-dependent K^+ channels. *J. Biol. Chem.* **263:**7933–7936

Gillis, K.D., Gee, W.M., Hammoud, A., McDaniel, M.L., Falke, L.C., Misler, S. 1989. Effects of sulfonamides on a metabolite-regulated ATP_i-sensitive K^+ channel in rat pancreatic B-cells. *Am. J. Physiol.* **257:**C1119–C1127

Grafe, P., Quasthoff, S., Strupp, M., Lehmann-Horn, F. 1990. Enhancement of K^+ conductance improves in vitro the contraction force of skeletal muscle in hypokalemic periodic paralysis. *Muscle Nerve* **13:**451–457

Hamill, O.P., Marty, A., Neher, E., Sakman, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hilgemann, D.W. 1989. Giant excised cardiac sarcolemmal membrane patches: Sodium and sodium-calcium exchange currents. *Pfluegers Arch.* **415:**247–249

Horie, M., Irisawa, H., Noma, A. 1987. Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. *J. Physiol.* **387:**251–272

Ishikawa, H., Sawada, H., Yamada, E. 1983. Surface and internal morphology of skeletal muscle. *In:* Handbook of Physiology. Sect. 10: Skeletal Muscle. L.H. Peachey, editor. pp. 1–21. Williams & Wilkins, Baltimore

Kakei, M., Kelly, R.P., Ashcroft, S.J.H., Ashcroft, F.M. 1986. The ATP-sensitivity of K^+ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett.* **208:**63–66

Latorre, R., Oberhauser, A., Labarca, P., Alvarez, O. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* **51:**385–399

Lederer, J.W., Nichols, C.G. 1989. Nucleotide regulation of the activity of rat heart ATP-sensitive K^- channels in isolated membrane patches. *J. Physiol.* **419:**193–211

Misler, S., Falke, L.C., Gillis, K., McDaniel, M.L. 1986. A metabolite-regulated potassium channel in rat pancreatic B cells. *Proc. Natl. Acad. Sci. USA* **83:**7119–7123

Mitchell, R.D., Palade, P., Fleischer, S. 1983. Purification of morphologically intact triad structures from skeletal muscle. *J. Cell. Biol.* **96:**1008–1016

Parent, L., Coronado, R. 1989. Reconstitution of the ATP-sensitive potassium channel of skeletal muscle. *J. Gen. Physiol.* **94:**445–463

Qin, D., Takano, M., Noma, A. 1989. Kinetics of ATP-sensitive K^- channel revealed with oil-gate concentration jump method. *Am. J. Physiol.* **257:**H1264–H1633

Quast, U., Cook, N.S. 1989. Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.* **10:**431–435

Quasthoff, S., Franke, C., Hatt, H., Richter-Turtur, M. 1990. Two different types of potassium channels in human skeletal muscle activated by potassium channel openers. *Neurosci. Lett.* **119:**191–194

Schmid-Antomarchi, H., de Weille, J., Fosset, M., Lazdunski, M. 1987. The antidiabetic sulfonylurea glibenclamide is a potent blocker of the ATP-modulated K^+ channel in insulin secreting cells. *Biochem. Biophys. Res. Commun.* **146:**21–25

Spruce, A.E., Standen, N.B., Stanfield, P.R. 1985. Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* **316:**736–738

Spruce, A.E., Standen, N.B., Stanfield, P.R. 1987. Studies of the unitary properties of adenosine-5′-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.* **382:**213–236

Spuler, A., Lehmann-Horn, F., Grafe, P. 1989. Cromakalim (BRL 34915) restores in vitro the membrane potential of depolarized human skeletal muscle fibres. *Naunyn Schmiedebergs Arch. Pharmacol.* **339:**327–331

Standen, N.B., Quayle, J.M., Davies, N.W., Brayden, J.E., Huang, Y., Nelson, M.T. 1989. Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* **245:**177–180

Standen, N.B., Stanfield, P.R., Ward, T.A., Wilson, S.W. 1984. A new preparation for recording single-channel currents from skeletal muscle. *Proc. R. Soc. London B.* **221:**455–464

Stein, P., Palade, P. 1988. *Sarcoballs:* Direct access to sarcoplas-

mic reticulum Ca^{2+} channels in skinned frog muscle fibers. *Biophys. J.* **54:**357–363

Stein, P., Palade, P. 1989. Patch clamp of sarcolemmal spheres from stretched skeletal muscle fibers. *Am. J. Physiol.* **256:**C434–C440

Sturgess, N.C., Ashford, M.L.J., Cook, D.L., Hales, C.N. 1985. The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* **8453:**474–475

Trube, G. 1983. Enzymatic dispersion of heart and other tissues. *In:* Single-Channel Recordings. B. Sakmann and E. Neher, editors. pp. 69–76. Plenum, New York

Vivaudou, M.B., Arnoult, C., Villaz, M. 1989. Patch-clamp recordings of K^+ channels from membrane blebs ('*Sarcoballs*') from skinned frog skeletal muscle fibers. *Biophys. J.* **55:**483*a*

Vivaudou, M.B., Arnoult, C., Villaz, M. 1990. Skeletal muscle ATP-sensitive K^+ channels recorded from sarcolemmal vesicles obtained by a new non-enzymatic method. *Biophys. J.* **57:**512*a*

Vivaudou, M.B., Robert, M., Arnoult, C., Villaz, M. 1990. Skeletal muscle ATP-sensitive K^+ channels recorded from sarcolemmal vesicles of split fibers: Regulation by internal ATP and ADP. *J. Neurol. Sci.* **98(Suppl.):**221

Weik, R., Lönnendonker, U., Neumcke, B. 1989. Low-conductance states of K^+ channels in adult mouse skeletal muscle. *Biochim. Biophys. Acta* **983:**127–134

Weik, R., Neumcke, B. 1989. ATP-sensitive potassium channels in adult mouse skeletal muscle: Characterization of the ATP-binding site. *J. Membrane Biol.* **110:**217–226

Woll, K.H., Lönnendonker, U., Neumcke, B. 1989. ATP-sensitive potassium channels in adult mouse skeletal muscle: Different modes of blockage by internal cations, ATP and tolbutamide. *Pfluegers Arch.* **414:**622–628

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