Volume 325, number 3, 276–280 FEBS 12639 July 1993

Intracellular protons control the affinity of skeletal muscle ATP-sensitive K^+ channels for potassium-channel-openers

Cyrille Forestier, Yves Depresle and Michel Vivaudou

Laboratoire de Biophysique Moléculaire et Cellulaire (URA CNRS 520), Centre d'Etudes Nucléaires de Grenoble, 85X, 38041 Grenoble, France

Received 5 April 1993; revised version received 27 April 1993

Levcromakalim, a potential antihypertensive agent, is known to activate potassium channels dependent on intracellular ATP (K-ATP channels). In inside-out patches excised from frog skeletal muscle, levcromakalim or a related compound, SR 47063, caused a strong and persistent activation of K-ATP channels at a cytoplasmic pH of 7.1. However, at pH 6, these activators could no longer affect the K-ATP currents. Conversely, in the continuous presence of activator, lowering pH from 7.1 to 6 returned channel activity to its level in pH 6 alone. After wash-out of the activator, recovery from activation took minutes at pH 7.1 but only seconds at pH 6, thus ruling out an effect of protons on the activators in solution. These experiments suggest that K-channel-activators are unable to bind to their receptor when it is protonated, and more generally, they provide evidence at the microscopic level for proton-induced allosteric modulation of drug–receptor interaction

Levcromakalim; SR 47063; K-ATP channel; pH; *Rana esculenta*

1. INTRODUCTION

ATP-sensitive potassium channels are present in numerous types of cells [1] but the reason of their presence often remains enigmatic. K-ATP channels have nonetheless generated unusual interest among pharmacologists as they have been demonstrated to be the target of a number of molecules with potential therapeutic applications. Among those molecules are the K-channel-openers (KCOs) exemplified by cromakalim and its active enantiomer levcromakalim [2]. We have tested levcromakalim on frog skeletal muscle K-ATP channels and found that its action was highly dependent on intracellular pH, since it became ineffective at acidic pH. The same observation was true of another more potent benzopyran derivative, SR 47063 [3]. Our data show that protons accelerate KCOs dissociation from their receptor. These observations, together with the fact that protons, by themselves, can activate skeletal muscle K-ATP channels by shifting their ATP sensitivity [4], suggest that protonation of the K-ATP channel induces drastic changes in its conformation which transform the topology of both the ATP and KCOs sites of fixation. In this respect, the parallelism between ATP and KCOs supports the notion that they occupy a common site on the K-ATP channel complex. A preliminary report of some of the results presented here has been published in abstract form [5].

Correspondence. M. Vivaudou, CENG, DBMS, BMC 85X, 38041 Grenoble, France. Fax: (33) 7688 5487.

Abbreviations. K-ATP channel, ATP-sensitive K^+ channel; KCO, potassium channel opener; DMSO, dimethylsulphoxide, SR 47063, 4-(2-cyanoimino-1,2-dihydropyrid-1-yl)-6-nitro-2,2-dimethyl-2H-1-benzopyran; Levcromakalim, (−)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-1-benzopyran-3-ol.

2. MATERIALS AND METHODS

Using the patch-clamp technique [6] in the excised inside-out mode, currents through K-ATP channels were recorded from sarcolemmal blebs elicited by mechanical cleavage of fibers dissected from the iliofibularis thigh muscle of the adult frog, *Rana esculenta* [7].

The patch pipette contained 150 mM K^+, 136 mM Cl^-, 2 mM Mg^{2+}, and 10 mM PIPES. The cytoplasmic face of the patch was bathed in solutions which all contained 150 mM K^+, 40 mM Cl^-, 1 mM EGTA, 10 mM PIPES, and methanesulfonate$^-$ as the remaining anions. Mg^{2+} (5 mM) was present only in the solution containing 3 mM ATP. The pH values were obtained with addition of KOH or KCl and were found to remain stable during the course of an experiment.

Unless otherwise specified, pH was set to 7.1 and the membrane potential V_m was −50 mV. Experiments were conducted at room temperature (22–24°C). ATP (potassium salt) was purchased from Sigma. Levcromakalim, also known as BRL 38227 or lemakalim (Smith Kline Beecham Pharmaceuticals, UK) and SR 47063 (Sanofi Recherche, France) were added from stock solutions (respectively, 100 mM and 10 mM) in DMSO. DMSO alone was without effects on K-ATP channel activity at all pHs used.

The intracellular face of the patches was perfused by placing the tip of the patch pipette in the outlet flow of one of 36 polyethylene tubes from which the different solutions continuously flowed by gravity. Tubes were mounted on a revolving barrel linked to a stepping motor interfaced to a PC-compatible computer. This device (rapid-solution-changer RSC-100 commercialized under licence by Bio-Logic, France) was designed to permit rapid switching from one solution to any of the others and automation of perfusion protocols. Signals were acquired and processed as previously described [8]

Published by Elsevier Science Publishers B V

3. RESULTS

Intracellular acidosis is known to activate K-ATP channels in skeletal muscle by reducing their sensitivity to inhibition by ATP [4]. The same mechanism underlies the effects of KCOs in cardiac muscle [9].

Accordingly, in the presence of partially inhibiting doses of ATP (100–300 μM) on the cytoplasmic side of the patch, lowering pH from 7.1 to 6 or applying levcromakalim (100 μM) both caused clear increases in the activity of skeletal muscle blebs K-ATP channels. Activation by low pH was reversible within seconds while activation by levcromakalim persisted for minutes after removal of the activator. When pH was lowered in the presence of levcromakalim, channel activity could either decrease as in Fig. 1A and C (22 out of 44 patches), increase as in Fig. 2 (6 out of 44 patches), or remain constant (16 out of 44 patches). After the initial puzzlement over this apparent lack of coherence, we noticed that in fact in all patches tested the level of K-ATP current in pH 6 was roughly the same with or without levcromakalim, suggesting that acidosis neutralized the action of levcromakalim. In the patch of Fig. 1, pH 6-induced activity was less than levcromakalim-induced activity and consequently in the presence of levcromakalim pH 6 reduced channel activity toward its value in pH 6 alone (Fig. 1A and C). In the patch of Fig. 2, pH 6-induced activity was greater than levcromakalim-induced activity and in the presence of levcromakalim pH 6 increased channel activity toward its value in pH 6 alone (Fig. 2).

The lack of efficacy of levcromakalim at acidic pH was further demonstrated in 38 patches where levcromakalim was found to cause a strong activation of K-ATP channels (i.e. at least 4-fold increase in channel activity) at pH 7.1 but remained ineffective (i.e. less than 10% change in activity) when applied after pH had been reduced to 6 (Fig.1B).

In order to test the specificity of these pH effects, experiments were repeated with another KCO, SR 47063 (50–100 μM), which belongs to the benzopyran family of KCOs like levcromakalim but has been shown to be more potent in relaxing smooth muscle in vivo [3]. As illustrated in Fig. 3, additions of SR 47063 had no effect at pH 6 but resulted in dramatic activation of K-ATP channels at pH 7.1; conversely, in the continuous presence of the activator lowering pH from 7.1 to 6 returned channel activity to the activator-free pH 6 levels. Similar recordings were acquired in 13 patches.

These results could be explained simply if proton in-

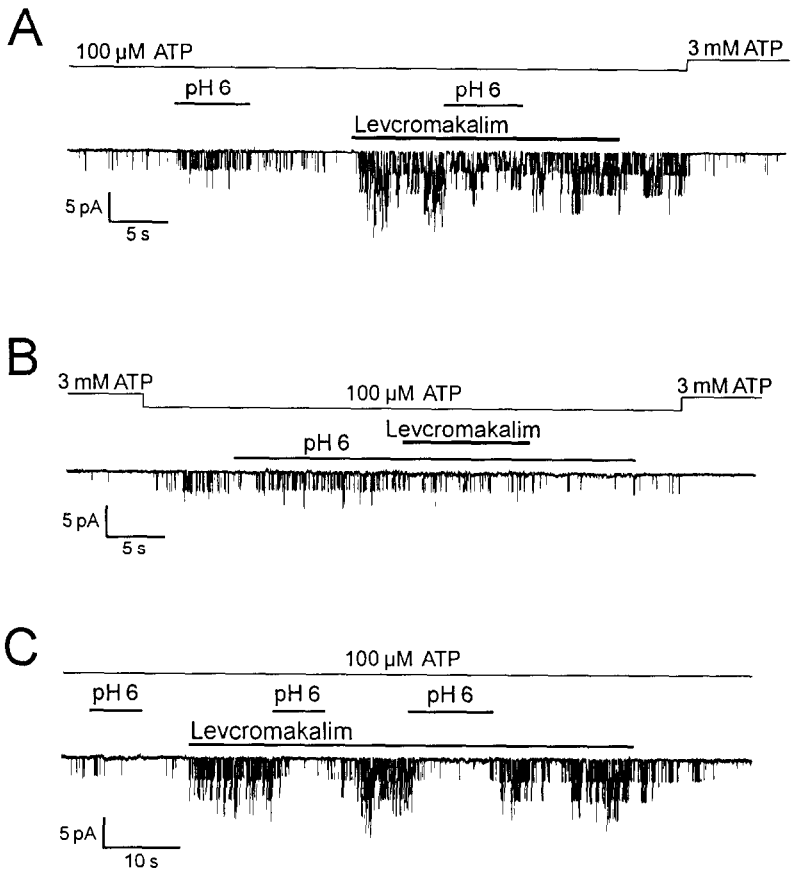

Fig. 1. Lowering intracellular pH from 7.1 to 6 reverses (A and C) and prevents activation of K-ATP channels by 100 μM levcromakalim. Traces were recorded from the same patch in the order presented with a gap of 20 s between A and B and 10 s between B and C. In this and other figures, bath pH was 7.1 except where indicated by the pH 6 bars. Patch 2C1705.

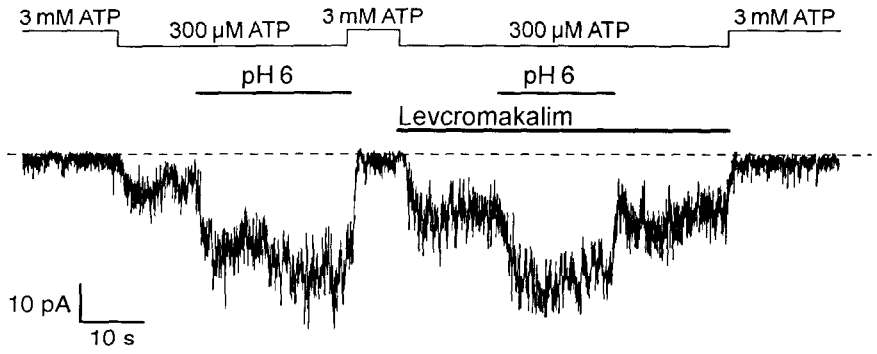

Fig. 2. Acidification from pH 7.1 to 6 elicits the same level of channel activity with and without 100 μM levcromakalim in a case where acidification caused a greater increase in K-ATP current than levcromakalim. Patch 293008.

teraction with KCOs modified their affinity or efficacy. In order to exclude this hypothesis, pH sensitivity of levcromakalim was directly assessed by titration. The titration curve of levcromakalim (1 mM in H_2O) revealed no effective buffering capacity in the range pH 4–10 and therefore no particular acidic or basic character. The opposite would have been surprising as the chemical structure of either levcromakalim or SR 47063 does not point to any obvious site of protonation.

The possibility of a more subtle interaction between KCOs and pH was further ruled out by taking advantage of the poor reversibility of the channel activation by KCOs. After a brief application of either levcromakalim or SR 47063 (100 μM), significant K-ATP current activation can persist for minutes at a neutral pH of 7.1 after wash-out of the activator (Fig. 4), probably reflecting the slow dissociation rate of the drug from the channel. When pH is lowered to 6 for a short period while the drug is no longer in solution but still bound to the channel, the current rapidly and permanently returns to its control level (Fig. 4) suggesting that the interaction of protons with the K-ATP channel triggers a loss of affinity for the activator. The experimental protocol used in Fig. 4 yielded equivalent results in 15 patches (10 patches with levcromakalim and 5 patches with SR 47063) where return to control level took more than 50 s at pH 7.1 but less than 3 s at pH 6. This type of protocol where KCOs and pH 6 are applied in 0 ATP also demonstrates that: (1) although activation by KCOs is seen only in the presence of ATP, KCOs are able to bind to the channel even in the absence of nucleotides; and (2) the effects of acidic pH on KCOs binding

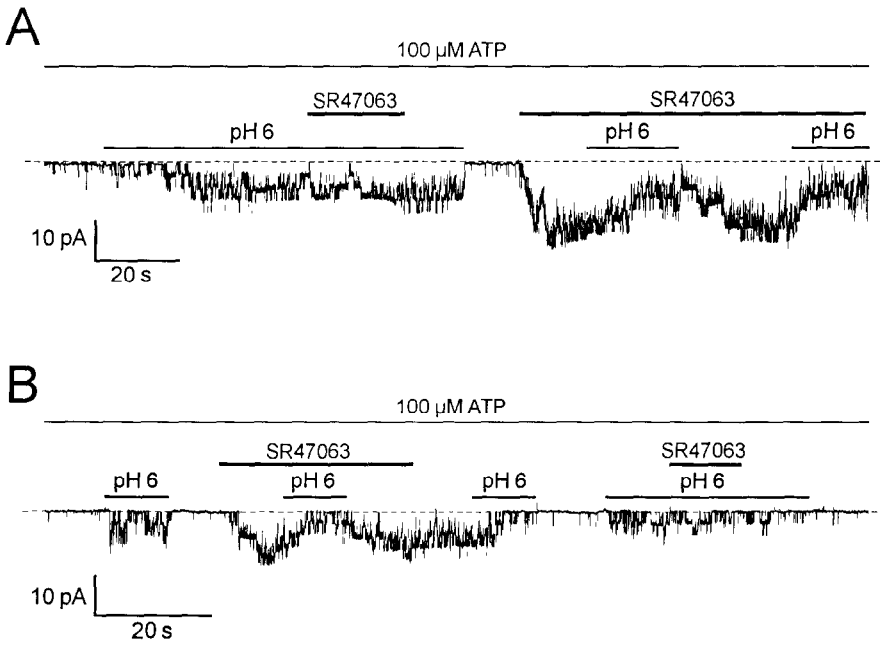

Fig. 3. Lowering pH from 7.1 to 6 blocks activation by 100 μM SR 47063. Trace B was recorded 20 min later than A from the same patch. Patch 320203.

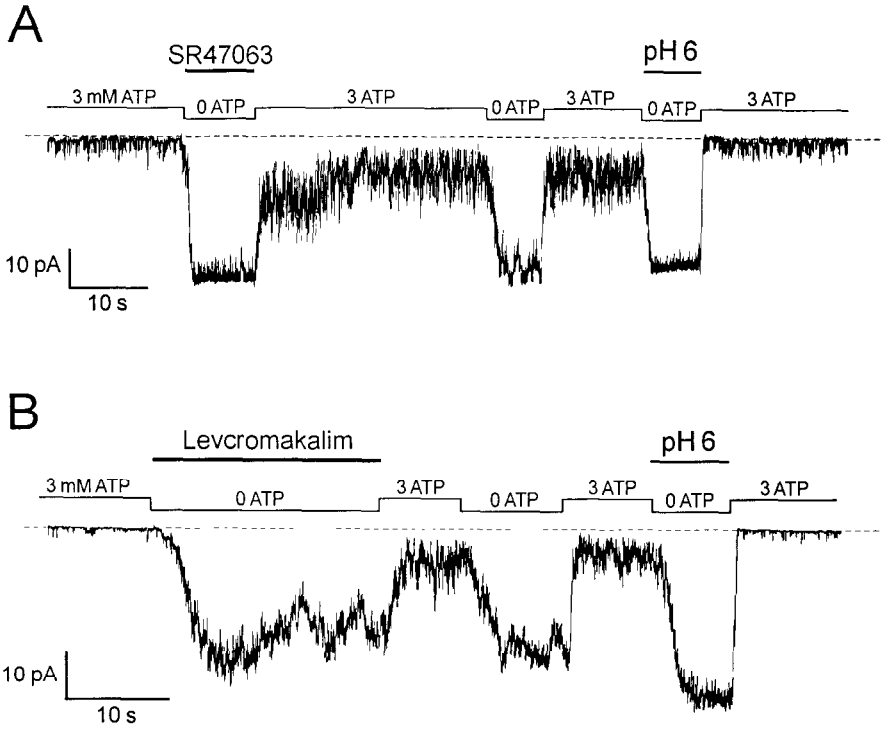

Fig. 4. A brief acidification from pH 7.1 to 6 accelerates the wash-out of the persistent activation which remains after a short application of SR 47063 or levcromakalim (100 μM). (A) V_m = −50 mV. Patch 320102. (B) V_m = −80 mV. Patch 38M3.

do not require ATP as would be the case if variations in the proportion of the ionized forms of ATP ($HATP^{3-}$ and ATP^{4-}) played a role.

Application of acidic solutions was used in many other patches as a practical means of speeding up recovery from KCOs activation (see for example third application of pH 6 in Fig. 3).

4. DISCUSSION

We have investigated in this work the pH-dependence of the action of two K-channel-openers of the benzopyran family, levcromakalim and SR 47063, on amphibian skeletal muscle K-ATP channels. At a normal cytoplasmic pH of 7.1, KCOs opened K-ATP channels in the presence of inhibiting doses of ATP. Acidification to pH 6 prevented this activation and could reverse it rapidly, suggesting that protons reduce the affinity of the drugs for their binding site. In our experimental system, one can identify 3 possible protonation targets which could mediate these effects: the KCO molecule, the ATP molecule, or the K-ATP channel complex.

The evidence that protons act directly on the channel rather than on soluble constituents comes from the experiments of Fig. 4, where acidic pH was applied in the absence of either KCOs or ATP and found to accelerate the dissociation of the KCOs from the K-ATP channel. In addition, these experiments indicate that, if activation by KCOs requires ATP, binding of KCOs does not. More importantly, they demonstrate that the block by protons of KCO activation involves a change in channel affinity for the KCOs rather than a change in the efficacy of the bound KCOs.

The channel protonation site mediating this effect is likely to be distinct from the KCO binding site, but allosterically linked to it. Indeed if KCOs and protons shared the same site, one would not expect KCO dissociation to be dependent on proton concentration. By the same argument, one would expect the two sites to be localized within the same complex if not the same protein.

In skeletal muscle [4], protons are known to interfere strongly with ATP binding, and they cause channel activation, as we have verified in this work, by relief of inhibition. Thus protons, over the same range of concentrations, reduce channel affinity for ATP just as they reduce affinity for KCOs. This similarity reinforces the notion of a common site for ATP and KCOs already suggested by the apparent competition between KCOs and ATP reported in cardiac muscle [9,10]. Therefore our data would be consistent with a model where KCOs and ATP share a single binding site allosterically coupled with the proton-binding site or sites.

In other tissues, particularly cardiac muscle [11] and β-cells [12], pH has either very weak or opposite effects on the ATP dependence of K-ATP channels. These channels may lack the protonation site of skeletal muscle K-ATP channels. In these tissues it would be desira-

ble to test the pH dependence of KCO activation to obtain further insights in the validity of the proposed model and in the tissue specificity of this model. The experiments that we have performed with benzopyran K-channel-activators could also serve as an assay for other structurally-distinct KCOs [2] to investigate whether KCOs bind to a common target site, as hinted by binding studies [13], and whether they exploit the same mechanism of action.

Acknowledgements Invaluable help in designing the rapid-solution-changer was provided by the late Christian Roche as well as by Dr. Yves Dupont. Levcromakalim and SR 47063 were the kind gifts of Dr. J.M. Evans (Smith Kline Beecham Pharmaceuticals) and Dr. P. Gautier (Sanofi Recherche), respectively This work was supported by CEA (Commissariat à l'Energie Atomique), AFM (Association Francaise contre les Myopathies), and CNRS (Centre National de la Recherche Scientifique)

REFERENCES

[1] Ashcroft, S.J.H. and Ashcroft, F.M. (1990) Cellular Signalling 2, 197–214.
[2] Edwards, G and Weston, A.H. (1990) Trends Pharmacol. Sci. 11, 417–442.
[3] Lacour, L., Canals, F., Galindo, G. and Gautier, P. (1991) Fund. Clin. Pharmacol. 5, 829.
[4] Davies, N W., Standen, N.B. and Stanfield, P.R. (1992) J. Physiol. 445, 549- 568
[5] Forestier, C. and Vivaudou, M.B. (1993) Biophys. J. 64, A316.
[6] Hamill, O.P., Marty, A., Neher, E., Sakman, B. and Sigworth, F.J. (1981) Pfluegers Arch. 391, 85 100
[7] Vivaudou, M.B., Arnoult, C. and Villaz, M. (1991) J. Membrane Biol. 122, 165–175.
[8] Forestier, C and Vivaudou, M. (1993) J. Membrane Biol. (in press).
[9] Thuringer, D. and Escande, D. (1989) Mol Pharmacol. 36, 897–902.
[10] Ripoll, C., Lederer, W.J. and Nichols, C.G. (1990) J. Pharmacol. Exp. Ther. 255, 429–435.
[11] Lederer, W.J. and Nichols, C G. (1989) J. Physiol. 419, 193–211
[12] Misler S., Gillis, K. and Tabcharani, J. (1989) J. Membrane Biol 109, 135–143.
[13] Bray, K M. and Quast, U. (1992) J. Biol. Chem 267, 11689-11692.