Selectivity and gating properties of a cAMP-modulated, K^+-selective channel from *Drosophila* larval muscle

Ricardo Delgado, Ramon Latorre, Pedro Labarca*

Centro de Estudios Cientificos de Santiago, Casilla 16443, Santiago 9, Chile
Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

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Abstract The selectivity and gating properties of cAMP-modulated, voltage-independent, K^+-selective channel from *Drosophila* larval muscle were investigated using the patch-clamp technique. In symmetrical 115 mM K^+ the channel displayed a linear current–voltage relation with slope conductance of 43 pS. Under biionic conditions (115 mM K^+ pipette/115 mM X^+ cytoplasmic) the permeability sequence was $K^+ > Rb^+ > NH_4^+ \gg Cs^+, Na^+$. The channel was impermeable to Ca^{2+} ($P_{Ca}/P_K < 0.02$). Under steady-state conditions and regardless [cAMP], open dwell times showed a double exponential distribution. [cAMP] did not affect the time constants of the two components of open times, or their relative amplitudes. Moreover, successive openings were correlated in open time. Closed dwell times were made of at least three exponential components. Fast application of cAMP to the cytoplasmic side of the channel induced a transient increase in open probability that relaxed to a lower value within seconds. This last result suggests that cAMP can activate and desensitize this cAMP-modulated, K^+-selective channel.

Key words: Ion selectivity; Open time correlation; Mechanism of activation; Transient activation

1. Introduction

The first evidence for the presence of cyclic nucleotide modulated K^+ channels was obtained in *Drosophila* larval muscle [1]. Upward modulation by cAMP was sigmoidal, with an apparent dissociation constant of 50 μM, and a Hill coefficient of 2.9. This cAMP-modulated channel proved to be voltage-independent. The above findings were accompanied by the cloning of the *Drosophila ether à go-go (eag)* gene [2]. *Eag* was found to encode a polypeptide sharing sequence similarities with K^+-selective, voltage-dependent channels. Furthermore, the *eag* gene product is also related to cyclic nucleotide-gated cation channels. The similarities between the *eag* and cyclic nucleotide-gated channels include a putative cyclic nucleotide binding domain in the COOH-terminal region [3]. *Eag* homologs have been identified in human and mouse [4]. Expression of *eag* RNA in *Xenopus* oocytes yielded voltage-dependent channels upwardly modulated by cAMP, but not by cGMP [5]. *Eag* channels expressed in *Xenopus* oocytes show the permeability sequence $P_{K+} > P_{Rb+} > P_{Cs+} > P_{NH4+} > P_{Na+}$ and a sizable permeability to Ca^{2+} ($P_{Ca2+}/P_{K+} \sim 0.1$; [5]). Here, it is reported that, unlike the *eag* channel, the cAMP-modulated K^+-selective channel from *Drosophila* larval muscle exhibits the permeability sequence $K^+ > Rb^+ > NH_4^+ \gg Cs^+, Na^+$, and is essentially impermeable to Ca^{2+} ($P_{Ca2+}/P_{K+} < 0.02$). The analysis of single-channel current records allowed us to develop, for the first time, a kinetic scheme for direct cAMP-modulation of a K^+-selective channel. Furthermore, experiments with fast application of cAMP revealed that channel activity soon after stimulation with the agonist is high, decaying to a low, steady-state activity, within seconds. This suggests that cAMP can trigger two events: a fast activation, and a slow, desensitization-like process.

2. Materials and methods

Singel channel currents were recorded in inside-out excised patches from longitudinal ventrolateral muscles of third instar *Drosophila* larvae, essentially as described [1]. Summarily, after dissection, larvae were pinned down to the Sylgard-coated bottom of a plastic chamber (500 μl). The experimental setup was endowed with a perfusion system allowing to exchange the solution bathing the experimental specimen with 10 volumes of desired medium, in 10–30 s. In some cases, fast stimulation of membrane patch with cAMP from a pipette located in its vicinity was achieved using a homemade picospitzer. The sign of the applied voltage is referred to the cytoplasmic face of the membrane (pipette interior is virtual ground). Experiments carried out at room temperature (18–20°C). Single channel records, unfiltered, were stored in digital tape. Data acquisition and analysis of records exhibiting only one channel active at any given time, were performed using PClamp6 software (Axon Instruments Inc.), after lowpass filtering at 500 Hz with an 8-pole Bessel filter (Freq. Devices).

3. Results

3.1. Selectivity properties of cAMP-modulated K^+ channels in larval muscle

Fig. 1A shows records of cAMP-modulated K^+ channels monitored under biionic conditions in the presence of 115 mM K^+, 115 mM Rb^+, or 115 mM Na^+ in the cytoplasmic side. In the presence of Rb^+ the current sign reverted at about 10 mV. However, when the external solution contained Na^+, the current approached asymptotically the voltage axis at positive voltages. Fig. 1B shows current–voltage relations built under biionic conditions from single channel current amplitudes for several external monovalent cations. The *I–V* curve obtained under symmetrical K^+ is also shown. For Rb^+ and NH_4^+, reversal potentials were 10.3 ± 4.3 mV, n = 4 and 44.0 ± 0.2 mV, n = 3, respectively. Permeability ratios were estimated using the expression:

$$P_{K+}/P_{X+} = [X^+]/[K^+] \exp A E_{rev}, \quad (1)$$

where P's are permeability coefficients, E_{rev} is the reversal potential, $A = F/RT$. Accordingly, a $P_{K+}/P_{Rb+} = 1.8 \pm 0.6$

*Corresponding author. Centro de Estudios Cientificos de Santiago, Casilla 16443, Santiago 9, Chile. Fax: (56) (2) 23383.

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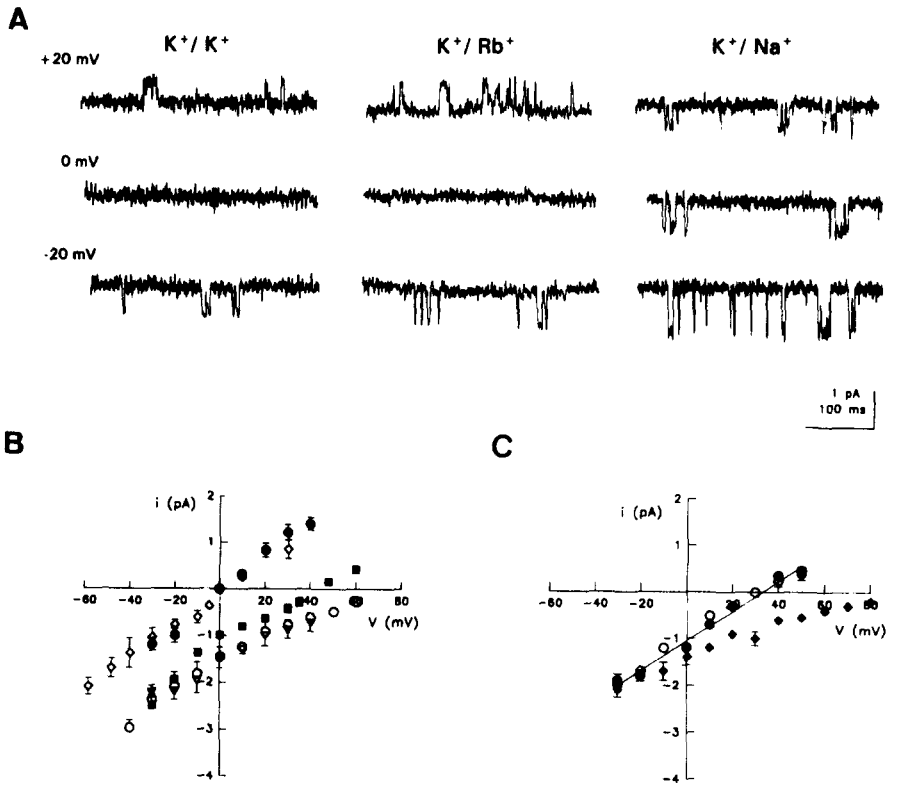

Fig. 1. Selectivity properties of a cAMP-modulated, K^+-selective channel. Experiments were performed in inside-out excised patches. (A) Single-channel currents under biionic conditions. Left record: single-channel currents in symmetrical, 115 mM KCl, 10 mM HEPES, pH 7.2, at the indicated voltages; center: currents in 115 mM KCl in pipette/115 mM RbCl, external; right: single-channel currents in 115 mM KCl, 10 mM HEPES, pH 7.2 pipette/115 NaCl, 10 mM HEPES, pH 7.2 out. (B) Current–voltage curves under biionic conditions. Symbols are as follows: filled circles, K^+/K^+; diamonds, K^+/Rb^+; filled squares, K^+/NH_4^+; open circles K^+/Na^+; inverted triangles, K^+/Cs^+. (C) Studies of Ca^{2+} permeability properties of cAMP-modulated, K^+ channel. Open circles: Current–voltage relation under conditions in which the pipette solution was made of (in mM) 110 K acetate, 5 KCl, 10 HEPES, pH 7.2; external solution (in mM): 20 KCl, 95 Choline Cl, 10 HEPES, pH 7.2. Filled circles: Pipette solution: 110 mM K acetate, 5 KCl, 10 HEPES, pH 7.2; external solution: 20 KCl, 20 CaCl2, 35 Choline Cl, 10 HEPES, pH 7.2. Filled diamonds: current–voltage relation with K^+ in pipette/Ca^{2+} in external solution. Pipette solution: 115 mM KCl, 10 mM HEPES, pH 7.2; external: 60 mM CaCl2, 50 μM cAMP, 10 mM HEPES, pH 7.2.

($n = 3$), and a $P_K/P_{NH4} = 5.5 \pm 0.1$ was obtained. For Cs^+ and Na^+ no reversal of currents could be observed up to 60 mV applied voltage (Fig. 1B) and a lower limit for $P_{K+}/P_{Cs+,Na+}$ should be >10. Therefore, the selectivity sequence for this cAMP-modulated K^+ channel is: $K^+ > Rb^+ > NH_4^+ \gg Cs^+, Na^+$.

The ability of Ca^{2+} to permeate the cAMP-modulated K^+ channel from *Drosophila* larval muscle was tested. Fig. 1C offers the *I–V* curve obtained in 115 mM KCl pipette/20 mM KCl out (filled circles), and in 20 mM KCl + 20 mM Ca^2 in the external solution (115 mM K^+ in pipette, open circles). Under both experimental conditions *I–V* curves yielded a slope conductance of 39 pS and a reversal potential of ~35 mV. After correcting for activity coefficients, this potential is that predicted by the Nernst equation for a K^+ electrode. Under biionic conditions, (115 mM K^+ pipette/60 mM Ca^{2+} cytoplasmic side) no reversal of single-channel currents, up to 80 mV applied voltage was obtained (Fig. 1C, diamonds). An upper limit for the P_{Ca2+}/P_{K+} ratio was obtained from the relation

$$P'_{Ca2+}/P_{K+} = [K^+]_0/4[Ca^{2+}]_i \exp 2FE_{rev}/RT, \quad (2)$$

with $P'_{Ca2+} = P_{Ca2+}/1 + \exp FE_{rev}/RT$ [6]. Since, $E_{rev} > 80$ mV, $P_{Ca2+}/P_{K+} < 0.02$ for this cAMP-modulated K^+ channel.

3.2. Analysis of channel open dwell times

Fig. 2A, upper panel, shows records of single-channel currents obtained under control conditions. The lower panel shows the distribution of channel open dwell times derived from this experiment. Two exponential components gave an adequate fit to the distribution of open dwell times. Time constants of the fast (τ_F) and slow (τ_S) component of the distribution were 1.2 ms and 4.6 ms, respectively. Relative amplitude of the fast component was 0.72. Fig. 3B, upper panel, shows the experimental record derived from the same patch as in A, after exposing its cytoplasmic face to 80 μM cAMP. Notice the increase in channel activity after exposure to the agonist. The middle panel presents the distribution of open times in the presence of cAMP obtained from the experimental data shown in the upper panel. The distribution was fitted with two exponential functions with $\tau_F = 1.2$ ms, $\tau_S = 4$ ms. Amplitude of the fast component was 0.82. Table 1 shows average values of time constants and relative amplitudes of the two components of open times derived from several experiments. Neither channel open time durations nor the relative contribution of both components of open times were sensitive to [cAMP]. In turn, the analysis showed the presence of at least three components of channel closed times (Fig. 2B, lower panel). Single-channel

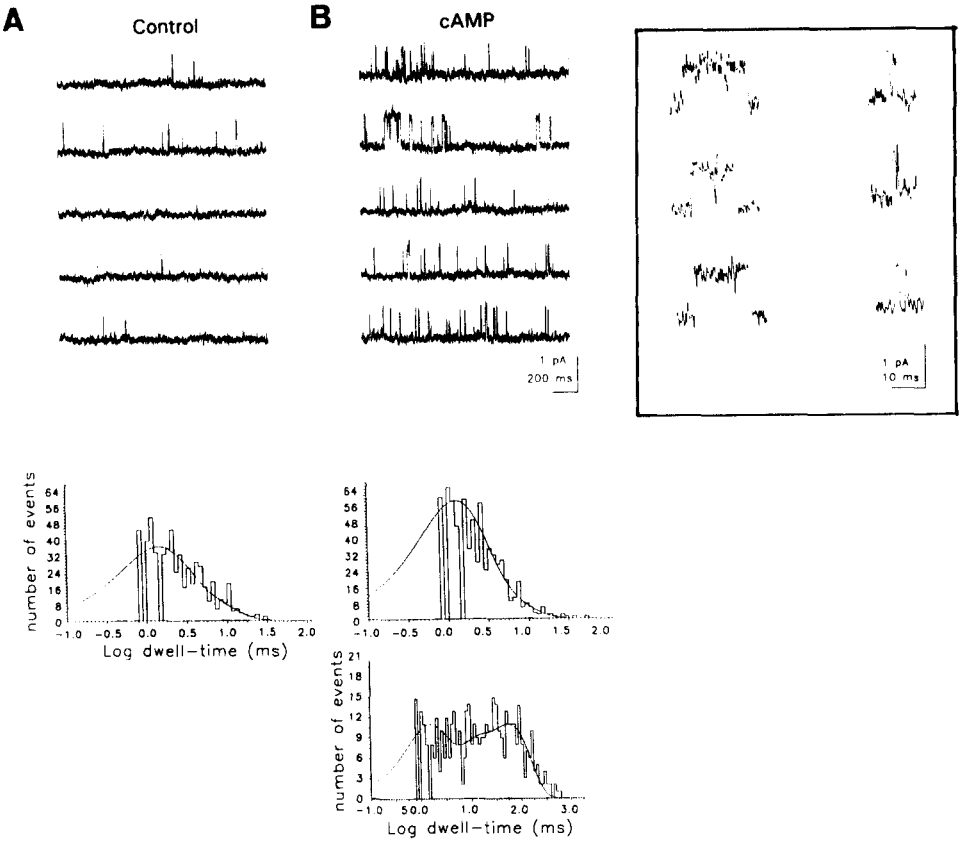

Fig. 2. Analysis of single channel current records. Single-channel currents were recorded under conditions in which the pipette contained 110 mM K acetate, 5 mM KCl, 5 mM HEPES, pH 7.2, at zero-applied voltage. The external solution was made of 115 NaCl, 5 mM HEPES, pH 7.2. When necessary the external solution was supplemented with the appropriate [cAMP]. (A) Upper panel: single channel currents obtained under control conditions, in the absence of cAMP, at zero-applied voltage. Lower panel: open time distribution derived from data shown in upper panel. Two exponentials were fitted to distribution, with time constants τ_F = 1.2 and τ_S = 4.6 ms. Relative amplitude of fast component = 0.72. (B) Upper panel: single channel currents recorded from same inside out patch as in A, at zero-applied voltage, after perfusion with a solution endowed with 80 μM cAMP. Lower panel: open time distribution of data shown in upper panel, fitted with two exponential components. Fit yielded τ_F = 1.2, τ_S = 4.0 ms. Relative contribution of fast component = 0.82. Lower panel: distribution of closed times for experiment shown in upper panel, fitted with three exponential components. τ_1 = 1.2 ms; τ_2 = 7.4 ms; τ_3 = 60 ms. Inset: open time correlation in successive openings in record shown in B. The left panel shows pairs of successive openings classified as 'long', as explained in legend to Table 2; left panel document pairs of openings classified as 'short' openings.

records were further analyzed to investigate a possible correlation in open times in successive openings [7]. For this, adjacent openings, separated by brief closing gaps (<2 ms) were monitored (Fig. 2, inset). Examples of this analysis are offered in Table 2. Open times were defined as 'fast' (F) or 'slow' (S) as explained in legend to Table 2. Pairs of openings, separated by short closing gaps, were classified as short-short (S-S), mixed (M), and long-long (L-L). In two of the experiments analyzed, the frequency of pairs obtained departed from those predicted by the binomial distribution at a 0.01 level of significance. In a third case, departure was at 0.1 level of significance. Moreover, as shown in Table 2, in all three cases, there was an excess of L-L pairs and S-S pairs, respect to those expected, and too few mixed pairs. These results indicate the presence of a correlation in open time in successive openings [7,8]. Considering that: (i) amplitudes and time constants of the two components of the distribution of open times were independent of [cAMP]; (ii) the presence of at least three components of closed times; and (iii) open time correlation in successive openings a minimal kinetic model for cAMP-modulation is proposed below:

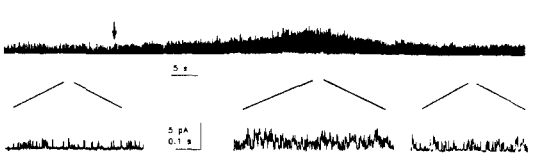

Fig. 3. Transient activation by cAMP of K^+ channels in *Drosophila* larval muscle. Currents were recorded at −20 mV in inside out patches. Other conditions as in Fig. 2. The arrow signals the moment in which a pulse of cAMP, lasting the whole recording period, was initiated with the picospitzer, from a pipette, located in the vicinity of the patch, containing 100 μM cAMP.

$$\begin{array}{ccc} C_0 + n\ \text{cAMP} \rightleftarrows C_1 & \rightleftarrows & C_2 \\ \uparrow\downarrow & & \uparrow\downarrow \\ O_F & & O_S \end{array} \qquad (3)$$

In the scheme, C_0 represents the unliganded, closed conformation, C_1 and C_2 are closed-liganded states with n cAMP mole-

Table 1
Analysis of open time distribution in cAMP-modulated K^+-selective channels

	A_1	τ_1	A_2	τ_2
Control	0.71 ± 0.1	1.1 ± 0.1	0.29 ± 0.1	4.9 ± 1.3
40 μM cAMP	0.75 ± 0.06	1.0 ± 0.2	0.25 ± 0.06	4.9 ± 1.0
80 μM cAMP	0.73 ± 0.08	1.4 ± 0.6	0.27 ± 0.08	3.8 ± 0.4

Single channel currents were recorded, from same excised patches, at zero mV applied voltage, under control conditions and in the presence of 40 and 80 μM cAMP. Open time distributions were fitted with two exponential components. In Table, A_1 and τ_1 are relative amplitude and time constant of fast component of the distribution, respectively; A_2 and τ_2 represent amplitude and time constant of slow component. Averages values, derived from four different studies ± S.E.M. are shown.

cules bound, and O_F and O_S represent fast and slow components of the distribution of open times. In the model, agonist binding leads to the formation of the C_1, bound conformation, at the expense of unbound C_0 state. From C_1, C_2, O_F, and O_S states are reached, via spontaneous thermal transitions.

3.3. *Upward modulation by cAMP is transient*

In the steady state, several seconds after exposure to cAMP, the channel undergoes a robust increase in open probability, 6–10-fold above the basal level. In spite of this, the steady-state open probability, even at high (≥80 μM) cAMP, amounts to only 6–10% [1]. However, it was noticed that during perfusion of the experimental chamber with cAMP channel activity could be significantly higher than in steady state. In view of this observation, a picospitzer was used to achieve fast application of the agonist on the excised membrane patch. The results show that, channel activity increases to a maximum value few seconds after application of a cAMP pulse. However, channel activity decays within several seconds, to a low, steady-state activity, even if the cAMP stimulus was still present (Fig. 3). In Fig. 3, the arrow signals the initiation of the cAMP pulse. After cAMP was applied, channel activity reached a maximum, during which up to two channel opened simultaneously, to decay to a lower, steady-state activity. This, in spite of the maintenance of the stimulus during the whole period shown. The maximum fraction of time the channel spent in the open state after exposure to cAMP was ~40%, decaying to ~5% in the steady state. On the other hand, the fraction of time the channel spent open in the control, before cAMP exposure was 0.5–1%. Similar results were obtained in five additional patches. Thus, soon after stimulation with cAMP activation can be as high as 40–80-fold above the basal level.

4. Discussion

The presence in animal cells of a new type of K^+-selective channels, directly modulated by cyclic nucleotides is well documented now [1–5,10–13]. The first functional evidence for direct cyclic nucleotide modulation of a K^+ channel was reported by Delgado et al. [1] in *Drosophila* larval muscle. This finding was accompanied by the cloning of the *eag* gene by Warmke et al. [2]. The *eag* gene was found to code for a protein exhibiting remarkable structural similarities to other K^+-selective channels. The gene also showed similarities with cyclic nucleotide-gated cation selective channels, having a putative cyclic nucleotide binding domain in the carboxyl terminus [3]. Expression of functional *eag* channels in *Xenopus* oocytes was achieved by Bruggemann et al. [5] yielding channels that were voltage-dependent and upwardly modulated by cAMP.

The *eag* channel was reported to follow the monovalent cation selectivity sequence $P_K > P_{Rb} > P_{Cs} > P_{NH4} > P_{Na}$. At difference with the selectivity sequence of the *eag* product, the K^+ channel from *Drosophila* larval muscle displayed the sequence $P_K > P_{Rb} > P_{NH4} \gg P_{Cs}, P_{Na}$. In addition, *eag* channels expressed in *Xenopus* oocytes were reported to exhibit a sizeable permeabilty to Ca^{2+} ($P_{K+}/P_{Ca2+} \sim 0.1$; [5]). The present work indicates that Ca^{2+} permeates very poorly, if at all, the cAMP-modulated K^+ channel from *Drosophila* larval muscle ($P_{Ca2+}/P_{K+} < 0.02$). Recently, a voltage-independent, K^+-selective channel modulated by cAMP, but not cGMP, from plasma membranes of sea urchin sperm was characterized. The channel was impermeable to Ca^{2+} and blocked by Ba^{2+} [10]. Work by Gomez and Nassi [9,12] revealed that Ca^{2+} and Mg^{2+} blocked cGMP-activated K^+ channels in ciliated photoreceptor cells from bay scallop. Thus, because of its high Ca^{2+} permeability, the *eag* K^+ channel is unique among known cyclic nucleotide-modulated K^+-channels.

The kinetic analysis of channel open times was addressed to gain information on the mechanisms by which cyclic nucleotide binding to K^+ channels causes an increase in open probability (P_o). Open time distributions were accounted for in terms of two exponential components. The time constants of the two components of open dwell times were refractory to [cAMP]. Furthermore, the relative contribution of each component of open times was also insensitive to agonist concentration. This, although, in the steady state and at 80 μM, cAMP increased P_o 6–10-fold [1]. It was observed also that successive openings, separated by brief closing gaps, were correlated in open time.

Table 2
Analysis of open time time correlation in successive openings

	Observed	Expected
Expt. 1.		
F-F	34	29
M	15	26
S-S	11	6
$\chi^2 = 9.7$ $(0.01<P)$		
Expt. 2.		
F-F	37	32
M	13	23
S-S	9	4
$\chi^2 = 11.4$ $(0.01<P)$		
Expt. 3.		
F-F	25	22
M	22	29
S-S	13	10
$\chi^2 = 3.7$ $(0.1<P)$		

Pair of openings, separated by brief closing gaps (<2 ms) were considered for the analysis. Openings were classified as 'fast' if they had durations shorter than t', or as 'slow' if they were longer than t'. t' defined as the time at which both components of the distribution of open times become equal: $A_F/\tau_F (\exp - t'/\tau_F) = A_S/\tau_S (\exp - t'/\tau_S)$ Jackson et al., 1983). Here, A_F and A_S are amplitudes, and τ_F and τ_S are time constants of fast and slow components of the distribution of open times, respectively. Expected frequencies of fast-fast, mixed and slow-slow pairs were estimated from the binomial distribution.

In turn, our preliminary analysis showed that closed times could be accounted for in terms of at least three exponential components. Based on the above information, a minimal kinetic scheme was chosen, which can be used now as a working model in further, more exhaustive studies of the mechanism underlying cAMP modulation of this K^+ channel. Indeed, this minimal kinetic model, derived from data taken under steady state, many seconds after addition of the agonist, must be extended. This, because experiments in which cAMP was applied fast, using a picospitzer, indicated that channel activity was high early after stimulation, decaying to a lower, steady-state activity, within seconds. Thus, cAMP increases the probability of channel opening but induces also a slower process which resembles the 'desensitization' of neurotransmitter-gated channels, described first by Katz and Thesleff [14] in motor end-plates.

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