6610 Journal of Physiology (1997), 501.3, pp.565–571

Activation of f-channels by cAMP analogues in macropatches from rabbit sino-atrial node myocytes

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1. The action of the two diastereometric phosphorothioate derivatives of cAMP, Rp-cAMPs and Sp-cAMPs, was investigated on hyperpolarization-activated 'pacemaker' current (i_f) recorded in inside-out macropatches from rabbit sino-atrial (SA) node myocytes.
2. When superfused on the intracellular side of f-channels at the concentration of 10 μM, both cAMP derivatives accelerated i_f activation; their action was moderately less pronounced than that due to the same concentration of cAMP.
3. The measurement of the i_f conductance–voltage relation by voltage ramp protocols indicated that both cAMP analogues shift the activation curve of i_f to more positive voltages with no change in maximal (fully activated) conductance.
4. Dose–response relationships of the shift of the i_f activation curve showed that both Rp-cAMPs and Sp-cAMPs act as agonists in the cAMP-dependent direct f-channel activation. Fitting data to the Hill equation resulted in maximal shifts of 9·6 and 9·5 mV, apparent dissociation constants of 0·82 and 5·4 μM, and Hill coefficients of 0·82 and 1·12 for Sp-cAMPs and Rp-cAMPs, respectively.
5. The activating action of Rp-cAMPs, a known antagonist of cAMP in the activation of cAMP-dependent protein kinase, confirms previously established evidence that f-channel activation does not involve phosphorylation. These results also suggest that the cAMP binding site of f-channels may be structurally similar to the cyclic nucleotide binding site of olfactory receptor channels.

The ionic mechanisms underlying diastolic depolarization of the action potential in mammalian cardiac muscle are important in the initiation of pacemaker activity and regulation of heart rate. The hyperpolarization-activated, so-called 'pacemaker' current (i_f) plays a key role in the generation and control of diastolic depolarization and spontaneous rate of sino-atrial (SA) node myocytes (DiFrancesco, 1993). A property of f-channels relevant to the physiological modulation of pacemaker activity is their regulation by neurotransmitters and metabolic stimuli. Unlike L-type Ca^{2+} channels and delayed rectifier K^+ channels, also expressed in SA node cells, f-channels are modulated by cAMP independently of phosphorylation, through a mechanism involving a direct interaction of cAMP with the intracellular side of the channels (DiFrancesco & Tortora, 1991). Although f-channels are voltage gated, they share the property of phosphorylation-independent activation mechanism with other neuronal cyclic nucleotide-gated (CNG) channels, such as the light-activated channels of retinal photoreceptors and the odour-modulated channels of olfactory receptors (see Zagotta, 1996 for review).

CNG channels possess a conserved region in the cytoplasmic C-terminal domain that is highly homologous to cyclic nucleotide binding domains in cGMP- and cAMP-dependent protein kinases (Kaupp *et al.* 1989; Dhallan, Yau, Schrader & Reed, 1990; Goulding, Ngai, Kramer, Colicos, Axel, Siegelbaum & Chess, 1992; Chen, Peng, Dhallan, Ahamed, Reed & Yau, 1993). The properties of the cyclic nucleotide-binding site of CNG channels in the rod and in the olfactory receptors have been recently investigated using the cAMP phosphorothioate derivatives S_P-adenosine cyclic 3′,5′-phosphorothioate (Sp-cAMPs) and R_P-adenosine cyclic 3′,5′-phosphorothioate (Rp-cAMPs) (Kramer & Tibbs, 1996), which act as cAMP agonist and antagonist, respectively, in

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the cAMP-dependent activation of protein kinase A (PKA; Parker Botelho, Rothermel, Coombs & Jastorff, 1988). These studies have shown that whereas Sp-cAMPs activates both CNG channels, Rp-cAMPs acts as a partial agonist on olfactory channels and as a partial antagonist on rod channels. To help determine the nature of the cyclic-nucleotide binding site of f-channels in SA node cells, we have investigated the action of R_P and S_P diastereometric phosphorothioate analogues of cAMP on i_f by perfusing known concentrations of the two substances on the intracellular side of f-channels in inside-out macropatches.

METHODS

The methods employed in this study for the isolation and electrophysiology of isolated SA node myocytes of the rabbit have been outlined previously (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986). New Zealand White rabbits (0·8–1·2 kg) were killed by cervical dislocation during deep anaesthesia (tribromoethanol, 200 mg kg^{-1}) and hearts were extracted after exsanguination. Cells were allowed to settle in Petri dishes, and were superfused with normal Tyrode solution containing (mM): NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1; D-glucose, 5·5; and Hepes–NaOH, 5; pH 7·4. In macropatch experiments the temperature was kept at 27–28 °C and the patch-pipette solution contained (mM): NaCl, 70; KCl, 70; $CaCl_2$, 1·8; $MgCl_2$, 1; $BaCl_2$, 1; $MnCl_2$, 2; Hepes–KOH, 5; pH 7·4. The control solution perfusing the intracellular side of the membrane patches contained (mM): potassium aspartate, 130; NaCl, 10; $CaCl_2$, 2; EGTA, 5; and Hepes–KOH, 10; pH 7·2, pCa 7.

Macropatches containing hundreds of f-channels were formed using a large-tipped pipette (0·5–2 MΩ) as previously described (Yatani & Brown, 1990; DiFrancesco & Tortora, 1991). Rp-cAMPs or Sp-cAMPs (Calbiochem) were dissolved in distilled water, divided into aliquots and stored at −20 °C until used. Prior to superfusion of any concentration of either cAMP analogue, patches were routinely superfused with cAMP (10 μM) to check for standard response, indicating that the intracellular side of the patch was normally exposed and accessible to superfusing solutions.

Data analysis

The time course of macropatch i_f under the action of modifying agents was recorded by applying hyperpolarizing steps of 2 s duration at a frequency of 1/6 Hz. At steady state the voltage dependence of i_f can be described by the equation:

$$i_f(V) = g_f(V)(V - V_f) = g_{f,max}y_\infty(V)(V - V_f),$$

where g_f is conductance, $g_{f,max}$ the fully activated conductance, $y_\infty(V)$ the steady-state activation parameter and V_f the reversal potential (DiFrancesco & Noble, 1985). We measured steady-state $I–V$ curves by applying 1 min-long hyperpolarizing voltage ramps with a rate of −110 mV min^{-1} from a holding potential of −35 mV. Conductance–voltage ($g_f(V)$) relations were then obtained from the above equation as ratios between steady-state $I–V$ curves ($i_f(V)$) and $V - V_f$, where V_f was set to −12·24 mV (DiFrancesco & Mangoni, 1994). Conductance curves were fitted to the Boltzmann equation:

$$g_f(V) = g_{f,max}y_\infty(V) = g_{f,max}/(1 + \exp((V - V_{1/2})/p)),$$

where $V_{1/2}$ is the half-maximal voltage of activation and p is the inverse slope factor. This allowed estimation of the shifts of the voltage dependence of conductance (i.e. of the activation parameter y_∞) measured as changes in $V_{1/2}$.

Shifts of the i_f activation curve caused by different doses of Rp-cAMPs or Sp-cAMPs were also determined by a quicker method not requiring measurement of the conductance–voltage relation (Accili & DiFrancesco, 1996). This was based on the observation that neither cAMP analogue modifies the steady-state (maximal) i_f conductance (see Fig. 2 below). Shifts were obtained by applying hyperpolarizing steps from −35 mV to near the mid-point of the i_f activation curve and adjusting the holding potential (−35 mV in the control solution) until the Rp-cAMPs- or Sp-cAMPs-induced change in i_f was compensated and the control i_f size fully restored. Since the compensation involved a change of the test voltage (from V to $V + s_m$, where s_m is the measured displacement of the holding potential in millivolts), a correction was introduced to obtain the shift of the activation curve (s, millivolts), according to the relation:

$$s = s_m(1 + (y_\infty/(dy_\infty/dV))/(V - V_f)).$$

In this calculation we used $y_\infty = 0·5$ and $dy_\infty/dV = 0·10811$ mV^{-1} (see Accili & DiFrancesco, 1996 for details).

When comparing two sets of data, statistical analysis was performed using Student's paired t test. Values of $P < 0·05$ were considered significant. Statistical data are given as means ± S.E.M.

RESULTS

In Fig. 1*A*, i_f was activated by 2 s hyperpolarizing steps to −95 mV in one macropatch. Perfusion of the intracellular side of the patch with 10 μM cAMP accelerated the current activation time course, with only a moderate increase in steady-state amplitude, in agreement with previous evidence (DiFrancesco & Mangoni, 1994), indicating that cAMP shifts the single channel open probability curve to more positive voltages, without altering the single-channel conductance. In whole-cell measurements, this is equivalent to shifting the i_f activation curve without altering the fully activated $I–V$ relation (DiFrancesco *et al.* 1986). Following complete washout, sequential perfusion of the same macropatch with the phosphorothioate cAMP derivatives Rp-cAMPs (10 μM, Fig. 1*B*) and Sp-cAMPs (10 μM, Fig. 1*C*) induced a qualitatively similar action, characterized by a slightly less pronounced acceleration of the activation time course and increase of current at 2 s.

These observations are consistent with the view that both cAMP derivatives activate i_f, like cAMP, by a shift of the current activation curve. To confirm this hypothesis, conductance–voltage $g_f(V)$ relations were determined by use of slow ramp protocols (see Methods), and the actions of Rp-cAMPs (10 μM) and Sp-cAMPs (10 μM) were compared with that of cAMP on i_f in the same patches. As shown in the examples in Fig. 2, both the cAMP analogues reversibly shifted the voltage dependence of the conductance–voltage curve to more positive voltages, without altering the i_f fully activated conductance. The voltage dependence of the conductance curve shifted by 6·5 mV (from −120 to −113·5 mV) after exposure to Rp-cAMPs (Fig. 2*A*) and 8·5 mV (from −127 to −118·5 mV) after exposure to Sp-cAMPs (Fig. 2*B*); in both cases the shifts were smaller

than those induced in the same patches by a similar concentration (10 μM) of cAMP (10 and 12·5 mV, respectively).

In a set of experiments such as those in Fig. 2, the maximal (fully activated) conductance did not change significantly with either analogue ($P > 0.05$); when compared with control conditions, $g_{f,max}$ varied by +5·1 ± 4·8% with Rp-cAMPs ($n = 4$) and by +1·3 ± 1·5% with Sp-cAMPs ($n = 7$). In the same patches the half-activation voltage ($V_{½}$) shifted in the positive direction by 6·22 ± 0·28 mV in the presence of Rp-cAMPs ($n = 4$) and by 8·28 ± 0·24 mV in the presence Sp-cAMPs ($n = 7$).

To construct the dose–response relationships of the shift of the i_f activation curve against the concentration of Rp-

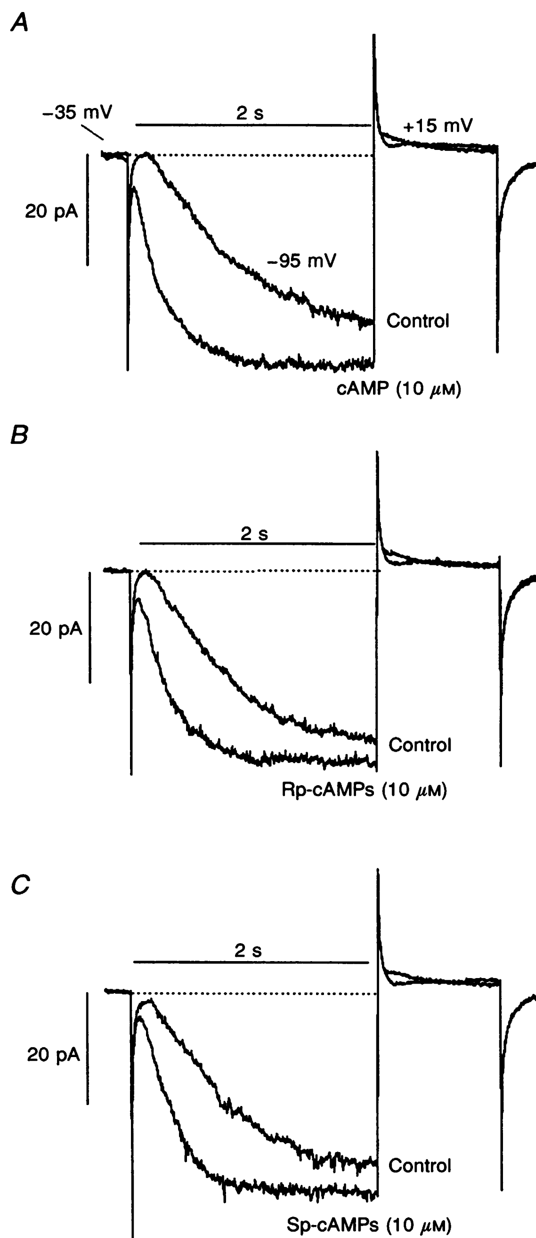

Figure 1. Action of cAMP and its analogues Rp-cAMPs and Sp-cAMPs on i_f activation in an inside-out macropatch

The i_f current was activated on hyperpolarization to −95 mV from a holding potential of −35 mV with a frequency of 1/6 Hz in one macropatch sequentially exposed to cAMP (*A*), Rp-cAMPs (*B*) and Sp-cAMPs (*C*) at the concentrations indicated. A depolarization to +15 mV followed the hyperpolarizing step to deactivate the current. Each exposure to a cyclic nucleotide-containing solution was preceded by full wash-out of preceding test solution and return to control.

cAMPs and of Sp-cAMPs, we measured shifts using an alternative, simpler method based on the correction of current changes by manual compensation of the holding potential (see Methods). The dose–response relation for Sp-cAMPs ($n = 53$ patches, ▲) and that for Rp-cAMPs ($n = 38$ patches, ■) are plotted in Fig. 3 and compared with the cAMP dose–response curve previously reported by DiFrancesco & Tortora (1991).

Fitting experimental data points to the Hill equation (see Fig. 3 legend) yielded apparent dissociation constants (K') of 0·825 and 5·512 μM, maximal shifts (s_{max}) of 9·61 and 9·77 mV, and Hill coefficients (n) of 0·819 and 0·951 for Sp-cAMPs and Rp-cAMPs, respectively. These values can be compared with those reported for the cAMP dose–response relationship by DiFrancesco & Tortora (1991) (dotted line: $K' = 0{\cdot}211$ μM, $s_{max} = 11$ mV, $n = 0{\cdot}850$). To verify that the comparison between the present data and the cAMP data is significant, we plotted the mean shift obtained in our control experiments (see Methods) with 10 μM cAMP (○ in Fig. 3). This was 10·21 ± 0·23 mV, which compares well with the value on the cAMP curve (10·6 mV).

These data indicate that both analogues behave, like cAMP, as agonists in f-channel activation, although with a lower affinity. The presence of an i_f-activating effect of both Sp-cAMPs and Rp-cAMPs, compounds known to act as agonist and antagonist, respectively, of cAMP-mediated PKA activation, rules against the possibility that activation of i_f occurs by a PKA-mediated phosphorylation mechanism. Interestingly, qualitatively similar differences in efficiency have been reported for the activation of the cGMP-activated

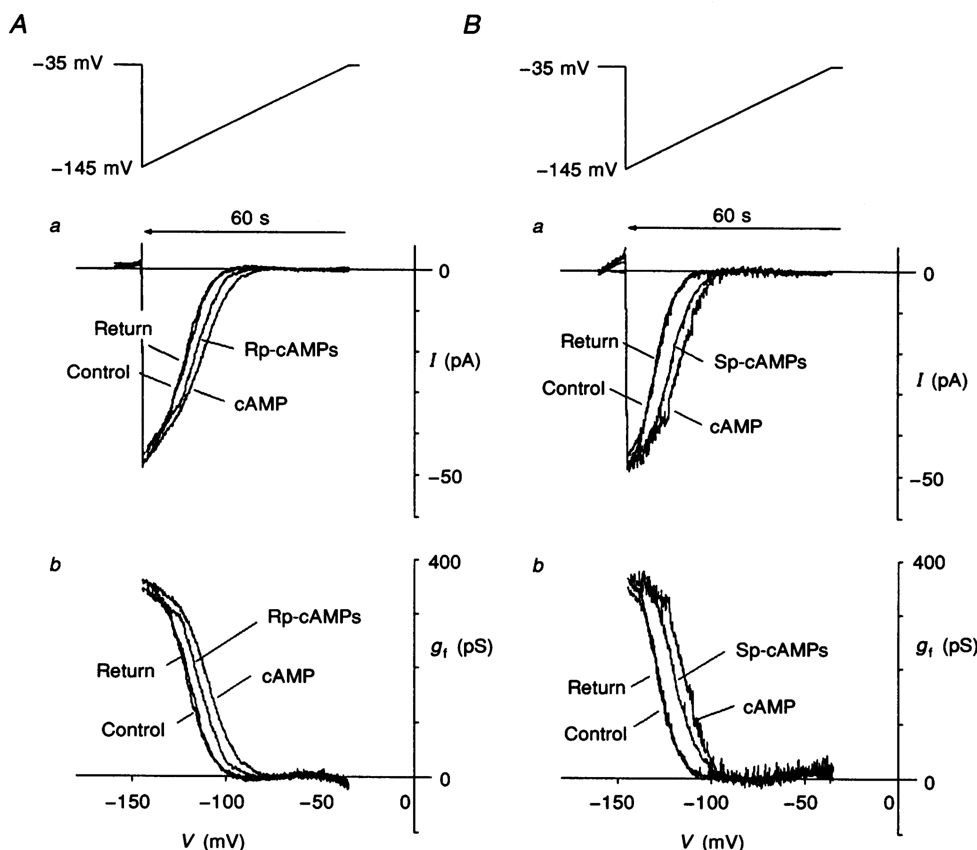

Figure 2. Action of Rp-cAMPs and Sp-cAMPs on the conductance–voltage, $g_f(V)$, relation

Hyperpolarizing ramps from −35 to −145 mV were applied to inside-out macropatches at a rate of −110 mV min^{-1} (top insets). *Aa*, steady-state i_f–V relations in a control solution, in the presence of 10 μM cAMP or of 10 μM Rp-cAMPs and after return to control, as indicated. The curve in the reference solution preceding exposure to Rp-cAMPs, essentially overlapping control and return curves, was also obtained and was omitted for clarity. Notice that time runs backward for comparison with curves in *b*. *Ab*, $g_f(V)$ curves obtained as detailed in Methods. Fitting curves to the Boltzmann equation yielded the following values in control, Rp-cAMPs, cAMP and after return to control, respectively. $g_{f,max}$: 345·0, 346·0, 349·9 and 344·8 pS; $V_{½}$: −120·0, −113·5, −110·0 and −120·3 mV; and p: 6·27, 6·17, 7·58 and 5·88 mV. *B*, same as in *A*, in another patch using Sp-cAMPs (10 μM). Boltzmann fitting yielded the following values in control, Sp-cAMPs, cAMP and after return to control, respectively. $g_{f,max}$: 350·2, 360·5, 313·6 and 357·8 pS; $V_{½}$: −127·0, −118·5, −114·5 and −127·0; p: 4·80, 5·66, 5·73 and 4·94 mV.

CNG channel of olfactory neurons by Sp-cAMPs and Rp-cAMPs (Kramer & Tibbs, 1996).

The presence of an activating action of the cAMP analogues suggests the existance of a common binding site for cyclic nucleotides on the channel protein. As a way to confirm the competition between cAMP analogues and cAMP for the same site, we checked if activation of f-channels by saturating concentrations of cAMP occludes the action of either of the two analogues. The results of an 'occlusion' experiment are shown in Fig. 4.

Here, cAMP (100 μM, an over-saturating dose for i_f activation) and Rp-cAMPs (30 μM) were applied either alone or in combination (Fig. 4*A*). Whereas both nucleotides, as expected, activated i_f when applied and washed off in sequence, Rp-cAMPs was without effect when applied in the presence of cAMP. In a similar protocol using Sp-cAMPs (30 μM), the analogue was active when applied alone, but not when applied during perfusion with cAMP (100 μM). The same results were obtained in three patches challenged with 100 μM cAMP and 30 μM Rp-cAMPs and in three patches challenged with 100 μM cAMP and 30 μM Sp-cAMPs. These data corroborate the evidence for the presence of a common binding site for cyclic nucleotides.

DISCUSSION

We have found that Sp-cAMPs and Rp-cAMPs, two diastereoisomers of adenosine 3',5'-(cyclic)phosphorothioate known to activate and inhibit, respectively, PKA, act as full cAMP agonists in the cAMP-dependent f-channel activation process. Although with a lower apparent affinity than cAMP, these cAMP analogues share with cAMP the same type of f-channel activating action, characterized by a direct interaction with the intracellular side of the channel, resulting in a shift of the current activation curve to more positive voltages with no change in maximal conductance.

In several tissues, Rp-cAMPs antagonizes cAMP binding to regulatory subunits of cAMP-dependent protein kinase (PKA) and inhibits release of the catalytic subunits (Rothermel, Stec, Baraniak, Jastorff & Parker Bothelo, 1983; Parker Bothelo *et al.* 1988). The activating action of Rp-cAMPs reported here on f-channels confirms previously established evidence that f-channel activation is not mediated by phosphorylation (DiFrancesco & Tortora, 1991). Moreover, the finding that near-saturating concentrations (30 μM) of either Rp-cAMPs or Sp-cAMPs are ineffective on i_f previously fully activated by cAMP indicates that all nucleotides bind to a common binding site on the intra-

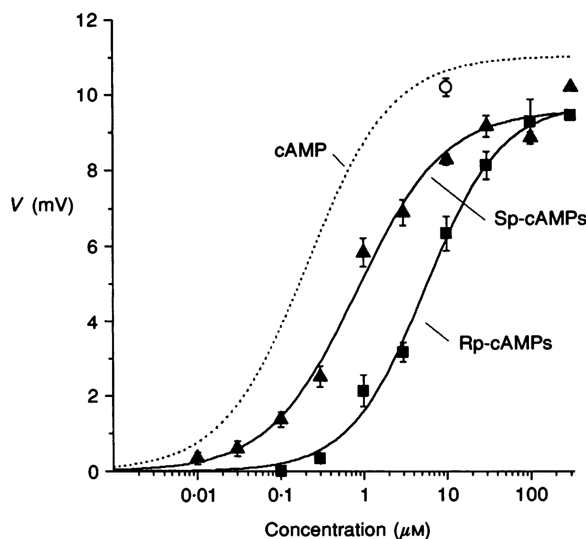

Figure 3. Dose–response relationships of the shift of the i_f activation curve as a function of Sp-cAMPs and Rp-cAMPs concentrations

Shifts were measured as described in Methods. The number of patches tested at the various concentrations of Sp-cAMPs and Rp-cAMPs, respectively, were: 7 and 0 (0·01 μM); 4 and 0 (0·03 μM); 7 and 4 (0·1 μM); 6 and 6 (0·3 μM); 9 and 5 (1 μM); 5 and 6 (3 μM); 7 and 8 (10 μM); 4 and 6 (30 μM); 3 and 2 (100 μM); 1 and 1 (300 μM). Data points were fitted to the Hill equation: $s = s_{max}/(1 + K'/A^n)$, where s_{max} is maximal shift, A is agonist concentration, K' is the apparent dissociation constant and n is the Hill coefficient. The fit yielded apparent dissociation constants of 0·825 and 5·512 μM and Hill coefficients of 0·819 and 0·951 for Sp-cAMPs and Rp-cAMPs, respectively. Also plotted for comparison is the cAMP dose–response relationship (dotted line) obtained in the same conditions by DiFrancesco & Tortora (1991). ○ represents the mean value of the shift obtained with 10 μM cAMP (10·21 $\pm$ 0·23 mV, $n = 39$ patches).

cellular aspect of the channel protein, or of a protein closely associated to the channel itself.

The direct activation by cyclic nucleotides is one of the properties that f-channels share with cyclic nucleotide-gated channels such as those in photoreceptors and olfactory receptor neurons. These also include lack of cation specificity and a low single-channel conductance in physiological conditions (Kaupp, 1991).

Hyperpolarization-activated f-channels, however, differ substantially from CNG channels in several respects, including the voltage dependence of channel activation, the lack of permeability to Ca^{2+} and the block by external Cs^{+}

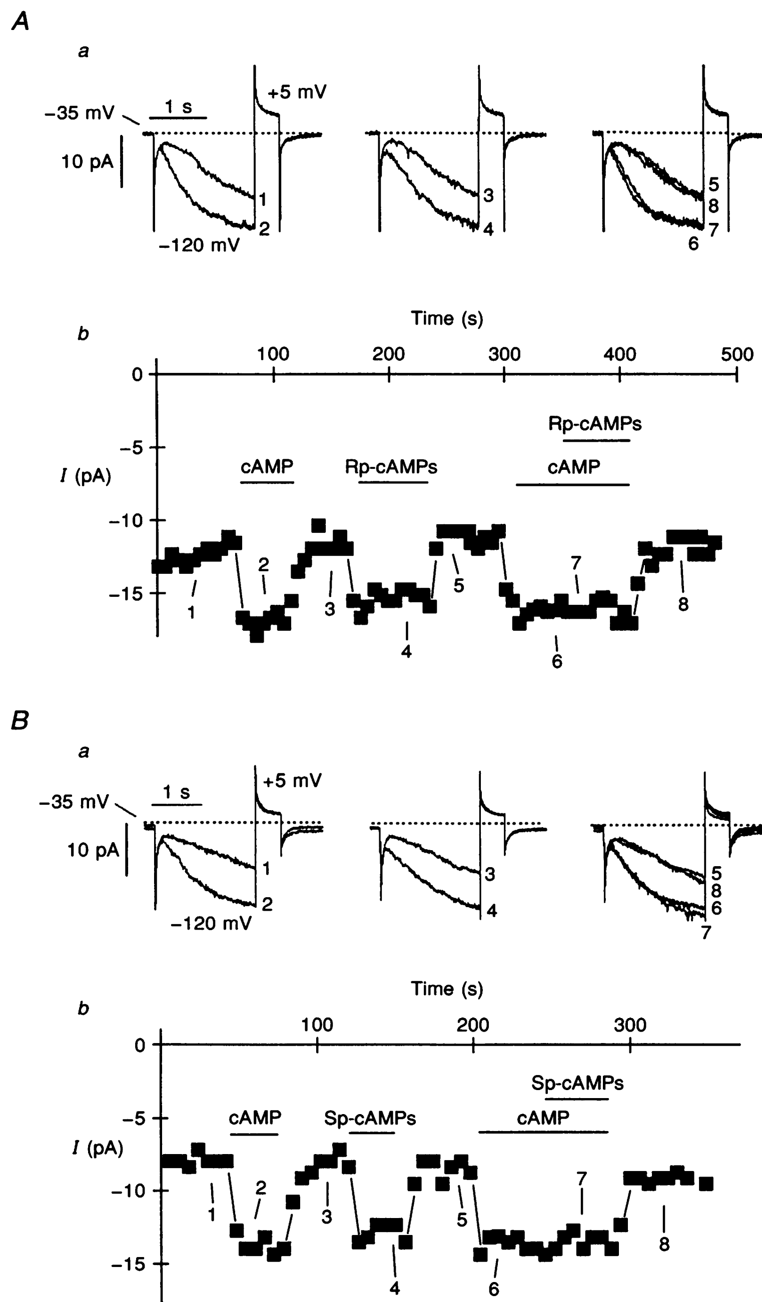

Figure 4. Occlusion of the i_f activating action of Rp-cAMPs and Sp-cAMPs by cAMP

A, the effects of Rp-cAMPs (30 μM) and cAMP (100 μM, a saturating concentration) either alone or in combination are shown on i_f recorded in an inside-out macropatch. *Aa*, i_f records during hyperpolarizing steps to the voltages shown taken at various times as indicated in *Ab*. *Ab*, time course of i_f amplitude at −120 mV during perfusion of the different solutions (bars). *B*, same as in *A*, for a different macropatch challenged with cAMP (100 μM) and Sp-cAMPs (30 μM) either alone or in combination.

ions (DiFrancesco, 1993). The finding reported here that f-channels are activated by the R_P- and S_P-phosphorothioate analogues of cAMP provides further evidence for a structural similarity between f- and CNG channels.

Recently, Kramer & Tibbs (1996) have reported that the phosphorothioate derivatives have different effects on the CNG channels in olfactory receptors and photoreceptors. In particular, Rp-cAMPs behaves as an agonist in the activation of olfactory CNG channels and as an antagonist in the activation of photoreceptor channels. Our results suggest therefore that the cAMP binding site of f-channels is functionally similar to the cyclic nucleotide binding site of olfactory receptor channels.

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Acknowledgements

This work was supported by the Consiglio Nazionale delle Ricerche (CT95.02241.04 to D.D.). P.B. was supported by a fellowship from the OTAN.

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Received 12 February 1997; accepted 26 March 1997.