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Regulation of Calcium-activated Nonselective Cation Channel Activity by Cyclic Nucleotides in the Rat Insulinoma Cell Line, CRI-G1

V. Reale[1]*, C.N. Hales[1], M.L.J. Ashford[2]

[1]Department of Clinical Biochemistry, University of Cambridge, New Addenbrookes Hospital, Hills Road, Cambridge, CB2 2QD
[2]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ

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Abstract. The regulation of a calcium-activated nonselective cation ($Ca\text{-}NS^+$) channel by analogues of cyclic AMP has been investigated in the rat insulinoma cell line, CRI-G1. The activity of the channel is modulated by cyclic AMP in a complex way. In the majority of patches (83%) tested concentrations of cyclic AMP of 10 μM and above cause an inhibition of channel activity which is immediately reversible on washing. In contrast, lower concentrations of cyclic AMP, between 0.1 and 1.0 μM, produce a transient activation of channel activity in most patches (63%) tested. One group of analogues, including N^6-monobutyryl cyclic AMP and N^6, 2′-O-dibutyryl cyclic AMP reduced the activity of the $Ca\text{-}NS^+$ channel at all concentrations tested and 2′-O-Monobutyryl cyclic AMP produced inhibition in all patches tested except one, at all concentrations. A second group produced dual concentration-dependent effects on $Ca\text{-}NS^+$, low concentrations stimulating and high concentrations inhibiting channel activity. 6-Chloropurine cyclic AMP and 8-bromo cyclic AMP produced effects similar to those of cyclic AMP itself. In contrast, 8-[4-chlorophenylthio] cyclic AMP also showed a dual action, but with a high level of activation at all concentrations tested up to 1mM. $Ca\text{-}NS^+$ channel activity was also predominantly activated by low concentrations of S_p-cAMPS. The activating effects of both S_p-cAMPS and cyclic AMP are antagonized by R_p-cAMPS, which by itself only produced a weak inhibition of $Ca\text{-}NS^+$ channel activity even at concentrations of 10 μM and above. The results are discussed in terms of a model in which cyclic AMP, and other cyclic nucleotides, modulate the activity of the $Ca\text{-}NS^+$ channel by binding to two separate sites.

Key words: Rat insulinoma cell line, CRI-G1 — Cyclic nucleotide regulation — Calcium-activated nonselective cation channel — Patch clamp

Introduction

Cyclic nucleotides directly modulate the activity of a wide range of ion channels including the nonselective cation channels present in a range of different tissues (Hockberger & Swandulla, 1987; Swandulla & Partridge, 1990; Kaupp, 1991; Yau, 1994). Cyclic GMP, for example, directly activates the voltage insensitive nonselective cation conductance responsible for the generation of the vertebrate photoreceptor response to light (Fesenko et al., 1985; Haynes & Yau, 1985; Ildefonse et al., 1992; Nizzari et al., 1993). A similar cyclic GMP-activated conductance has recently been described in dissociated cells from the chick pineal gland (Dryer & Henderson, 1991, 1993), whilst cyclic GMP has been reported to close a nonselective cation channel in insect olfactory neurons (Zufall, Hatt & Keil, 1991; Zufall & Hatt, 1991). In addition, although a cyclic nucleotide-gated conductance in olfactory receptor cilia has been shown to be activated by both cyclic AMP and cyclic GMP, it is cyclic AMP that is proposed to be the physiologically important mediator of olfactory responses (Nakamura & Gold, 1987; Frings, Lynch & Lindemann, 1992; Kramer & Siegelbaum, 1992; Kleene, 1993; Zufall & Firestein, 1993). Cyclic AMP can also directly activate a nonselective cation conductance, the i_f current (K_m = 0.2 μM), underlying the generation and modulation of pacemaker activity in cardiac pacemaker cells (DiFrancesco & Tortora, 1991). Also, at high concentrations

* *Present address:* The Babraham Institute Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ

Correspondence to: V. Reale

(above 10^{-5} M) cyclic AMP inhibits a nonselective cation channel in patches excised from the basolateral membrane of the thick ascending limb of Henle's loop in mouse kidney (Paulais & Teulon, 1989).

Although cyclic AMP does not appear to be directly involved in the release of insulin from pancreatic β-cells, there is considerable evidence to suggest that it may modulate insulin release, mediated by mechanisms that involve activation of the inositol phosphate pathway, and also change the responsiveness of voltage-sensitive calcium channels in the plasma membrane of the β-cell (De Weille et al., 1989; Zawalich & Zawalich, 1990; Ämmälä, Ashcroft & Rorsman, 1993). In addition, the importance of cyclic AMP in the control of insulin secretion has been emphasized in a number of recent reviews (Prentki & Matschinsky, 1987; Ashcroft & Rorsman, 1991; Zawalich & Rasmussen, 1990). Further, the calcium-activated nonselective cation channel present in the rat insulinoma cell line, CRI-G1, has recently been shown to be modulated by cyclic nucleotides in a complex way (Reale, Hales & Ashford, 1992, 1994*a*). Cyclic AMP predominantly increasing channel activity at low concentrations (0.1–10 μM) and reducing it at higher concentrations (100 μM and 1 mM). Specificity studies indicate that the cyclic nucleotide site mediating inhibition of channel activity exhibits a strong preference for cyclic AMP over cyclic GMP, with cyclic UMP being almost equipotent with cyclic AMP. Cyclic IMP and cyclic CMP were not active at this site. The cyclic nucleotide site mediating activation of the channel showed much less nucleotide specificity than the inhibitory site, with cyclic AMP, cyclic GMP and cyclic IMP being almost equally active. These results raise the possibility that cyclic AMP, and other cyclic nucleotides, can modulate the activity of the Ca-NS^+ channel by binding to two different sites on the channel or a closely associated protein. This possibility is examined further in the present paper by testing a range of cyclic AMP analogues substituted at different positions on the purine ring and ribose sugar, for their ability to modulate the activity of the Ca-NS^+ channel.

Materials and Methods

Cell Culture

Cells of the rat pancreatic islet cell line, CRI-G1, were used in all experiments. These were grown in Dulbecco's modified Eagles medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and passaged at weekly intervals (Carrington et al., 1986). For the patch clamp studies cells of 2–6 days old were used. After this time they become unsuitable for experimentation because of their high density.

Recording and Analysis

In all the experiments the inside-out configuration of isolated membrane patches (Hamill et al., 1981) was used. The recording pipettes used had resistances of 8–10 MΩ. Single channel currents were recorded using a Dagan 8900 patch clamp amplifier, coupled to a 8930 probe (Dagan, Minneapolis, MN). Seal formation was monitored using a digital oscilloscope (Gould 1421) and currents were stored on video cassettes (VHS video cassette recorder, SLV-201, Sony; digital pulse code modulation, PCM-701ES). Recorded data from the experiments were played back into a Gould 3000 chart recorder which filtered the signals at 0.14 KHz. Outward currents (defined as the current flowing from the intra- to extracellular side of the membrane) are indicated as upward deflections of the trace.

Single channel current analysis was determined off-line by use of a program that incorporates a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an Elonex PC286C-100 microcomputer. Data segments between 1–4 min were replayed into the computer at the recorded speed, filtered at 600 Hz (−3dB) using an 8-pole Bessel filter and digitized at a frequency of 3.3 kHz using a Data Translation 2801A interface. The average channel activity ($N_f \cdot P_o$) where N_f is the number of functional channels in the patch and P_o is the open state probability, was determined by measuring the total time spent at each unitary current level and expressed as a percentage of the total time recorded (Kozlowski, Hales & Ashford, 1989; Kozlowski & Ashford, 1990). Experimental values are given as mean ± SEM; *n* denotes the number of results.

Solutions

The ionic composition of the solution in the pipette was (mM) 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, titrated to pH 7.2 with NaOH. The bathing solution consisted of (mM): 140 KCl, 1 $MgCl_2$, 0.9 $CaCl_2$, 1 EGTA and 10 HEPES, titrated to pH 7.2 with KOH, giving a final free calcium concentration of 1 μM. Because a high $[Ca^{2+}]$ is needed to activate the nonselective cation channel (Ca-NS^+), the bathing solution was replaced with a solution containing (mM): 140 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES titrated to pH 7.2 with KOH on formation of an inside-out patch. The extent of the chelation of calcium and magnesium ions by the nucleotides used was calculated by the "METLIG" program (P. England and R. Denton, University of Bristol) and was found to be negligible in the experiments described for all nucleotides up to a concentration of 1 mM.

Drugs were applied to membrane patches by superfusing the bath, using a gravity feed system, at a rate of approximately 0.5 ml/sec, which allowed a complete solution exchange within 45 sec. All experiments were performed at room temperature, 22–25°C. All reagents used were obtained from the Sigma Chemical (Poole, Dorset, U.K.).

Results

The Effects of Cyclic AMP

The actions of cyclic AMP on Ca-NS^+ channel activity are complex (Reale, Hales & Ashford, 1992, 1994*a*). At concentrations of 10 μM and above cyclic AMP causes an inhibition of channel activity in the majority of patches (83%; n = 18) tested, which is immediately reversible on washing (Fig. 1*A*). In contrast, lower concentrations of cyclic AMP between 0.1 and 1.0 μM produce a transient activation of channel activity in most patches (63%; n = 19) tested. Figure 1*B* shows a continuous recording of channel activity from a patch that

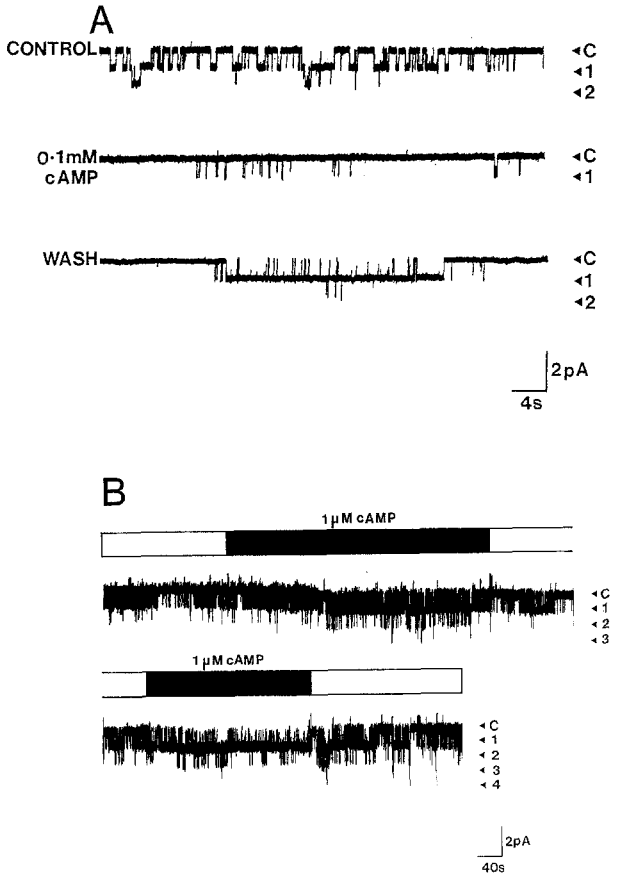


Fig. 1. Single channel records showing the inhibitory effects of 0.1 mM (*A*) and 1 μM (*B*) cyclic AMP on the activity of the Ca-NS$^+$ channel recorded from inside-out patches held at a membrane potential of –45 mV. (*A*) The values of the $N_f \cdot P_o$ are as follows: Control, 0.461; 0.1 mM cyclic AMP, 0.008 and Wash, 0.290 (*B*) a continuous recording of single channel activity of the Ca-NS$^+$ channel. The lower trace is a continuation of the upper trace. Cyclic AMP (1 μM) (black bars) was applied to the cytoplasmic face of the patch for the two periods indicated and was washed from the patch using nucleotide-free solution. The values of $N_f \cdot P_o$ are as follows: Control, 0.339 1μM cyclic AMP, 0.720; Wash, 0.378; 1 μM cyclic AMP, 1.043 and Wash, 0.225. In (*A*) the traces start 170 sec after patch formation. The gap between the top and middle traces was 320 sec, of which the patch spent 256 sec in the presence of 0.1 mM cyclic AMP. The gap between the middle and bottom traces was 440 sec, of which the patch spent 368 sec in the wash. In (*B*) the trace starts 20 sec after patch formation. In this figure, and all subsequent figures, the current levels are indicated on the right (c = closed; 1 = one channel open; 2 = two channels simultaneously open, etc.).

was exposed to two separate pulses of 1 μM cyclic AMP. The initial application of cyclic AMP produces an increase in channel activity which lasts as long as the patch is exposed to the nucleotide. After channel activity has recovered to control levels, a second pulse of cyclic AMP again increases channel activity in the same patch.

The Effects of Cyclic AMP Analogues with Substitutions on the Adenine Ring and on the Ribose Sugar

The sensitivity of the Ca-NS$^+$ channel to cyclic AMP analogues was assessed using single channel recording from inside-out membrane patches held at a membrane potential of –45 mV in the presence of 0.2 mM Ca^+. Each analogue was tested at four different concentrations, 0.1 and 1 μM, and at 0.1 and 1 mM, to make a comparison with the activating and inhibiting actions of cyclic AMP respectively. Every analogue was tested on at least four different membrane patches for each concentration used. The structures of the analogues tested are shown in Fig. 2 and the results obtained are compared to those obtained with similar concentrations of cyclic AMP in the Table.

One group of analogues consistently reduced the activity of the Ca-NS$^+$ channel at all concentrations tested. In compounds such as N^6-monobutyryladenosine 3′,5′-cyclic monophosphate (N^6-monobutyryl cyclic AMP) and N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (N^6,2′-O-dibutyryl cyclic AMP), substitutions are made on the amino group attached to the 6-position of the purine ring. In the case of the latter analogue, a second butyryl group is attached via an ether linkage to the 2′-position of the ribose sugar of cyclic AMP. Figure 3 shows an example of the effect of N^6-monobutyryl cyclic AMP. Exposure of the patch to 0.1 μM N^6-monobutyryl cyclic AMP produced a reduction in channel activity to 33% of the control level, with a reduction of the number of functional channels in the patch from three to one. The effect on channel activity was reversible. Similar effects were observed in patches exposed to N^6,2′-O-dibutyryl cyclic AMP. The Table summarizes the effects of these analogues on the Ca-NS$^+$ channel. Both analogues consistently reduced the activity of the Ca-NS$^+$ channel in all patches tested (n = 42). These analogues did not cause an increase in channel activity at any of the concentrations tested. It also shows that both of the N^6-substituted analogues were more potent than cyclic AMP itself in reducing the activity of the Ca-NS$^+$ channel and that N^6-monobutyryl cyclic AMP was consistently more potent than N^6,2′-O-dibutyryl cyclic AMP at all concentrations tested. 2′-O-monobutyryladenosine 3′,5′cyclic monophosphate (2′-O-monobutyryl cyclic AMP) is produced by the addition of a butyryl group, via an ether linkage to the 2′-position on the ribose sugar of cyclic AMP. The results obtained with this analogue are also summarized in the Table and show that it is again more effective than cyclic AMP itself in causing a decrease in Ca-NS$^+$ channel activity. However, unlike cyclic AMP, it consistently caused a decrease in channel activity, except for one patch out of the twenty-one

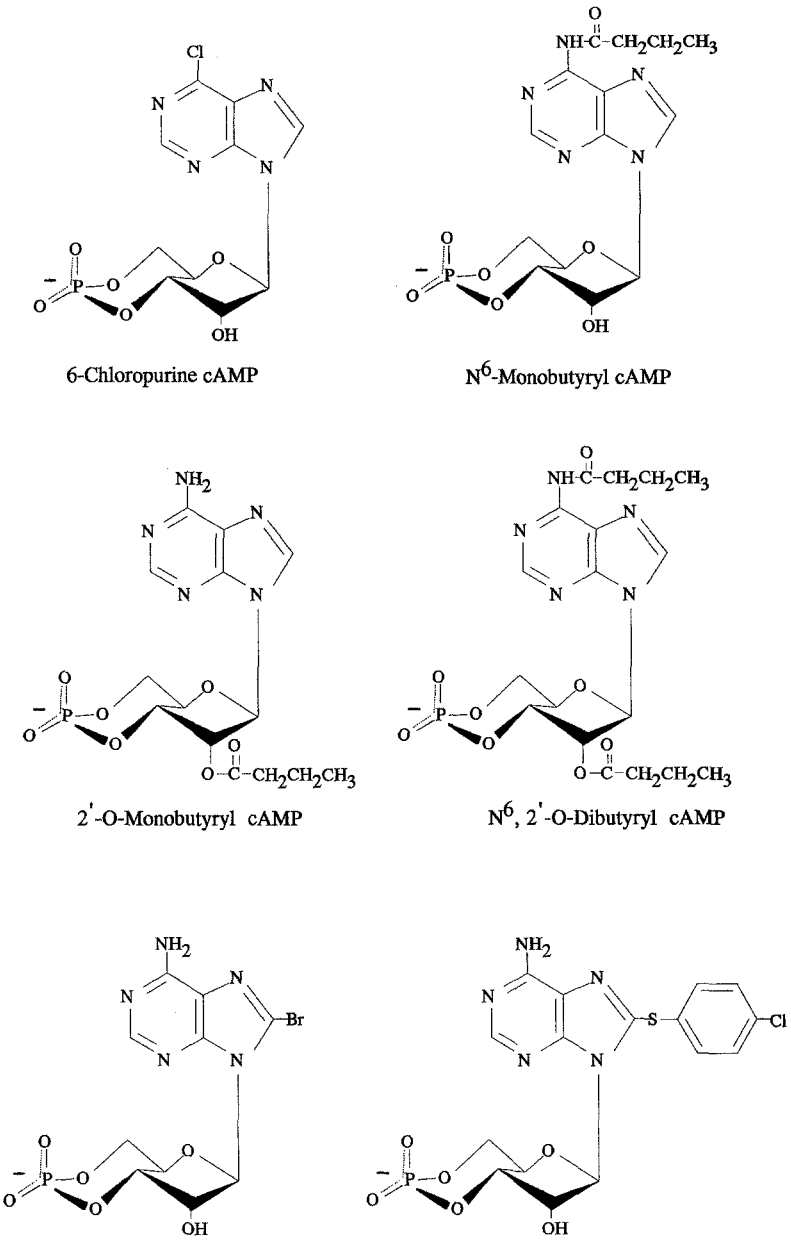


Fig. 2. Structures of cyclic AMP analogues.

tested. For this analogue concentrations <0.1 μM were not tested.

A possible explanation for the apparent lack of activation of Ca-NS$^+$ channel activity by the N^6-substituted analogues could be that because of their increased potencies relative to cyclic AMP, then activations may be induced at concentrations <0.1 μM. To examine this possibility both N^6-analogues were tested at lower concentrations (0.0001, 0.001 and 0.01 μM; n = 3 at each concentration) (*data not shown*). No significant changes in channel activity were produced by either of the analogues in any of the patches tested at these concentrations. This suggests that these analogues are likely to act only on the cyclic nucleotide site producing an inhibition of channel activity.

A second group of analogues produced dual, concentration-dependent, effects on Ca-NS$^+$ channel activity. 6-Chloropurine riboside 3′,5′-cyclic monophosphate (6-chloropurine cyclic AMP) has a chloride group replacing the amino group on the 6-position of the adenine ring. This analogue produced dual effects on Ca-NS$^+$ channel activity (Table). In the presence of 1mM of the analogue, channel activity was reduced in all patches tested, while at the other concentrations tested, some patches exhibited a reduction in activity and others showed an increase in activity. Similar effects were ob-

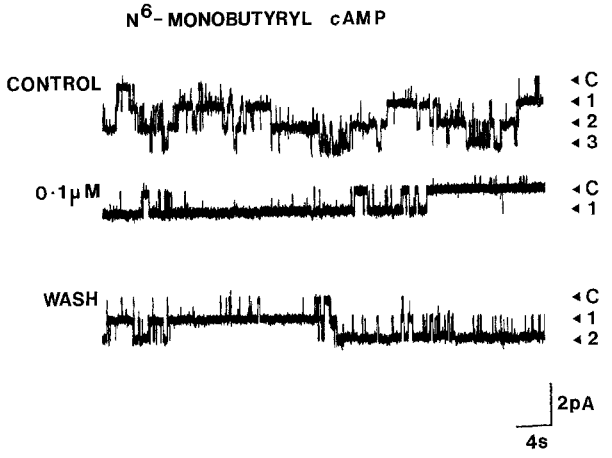


Fig. 3. Single channel records from a patch exposed to 0.1 μM N^6-Monobutyryl cyclic AMP. Channel currents were recorded from excised inside-out patches held at a membrane potential of −45 mV in the presence of 0.2 mM Ca^{2+}. $N_f \cdot P_o$ values are Control, 1.5; 0.1 μM N^6-monobutyryl cyclic AMP, 0.5; Wash, 1.2. The traces start 34 sec after patch formation. The gap between the top and middle traces was 212 sec, of which the patch spent 146 sec in the presence of 0.1 μM N^6-Monobutyryl cyclic AMP. The gap between the middle and bottom traces was 268 sec, of which the patch spent 188 sec in the wash.

served for 8-Bromo-adenosine 3′5′-cyclic monophosphate (8-bromo cyclic AMP) where a bromide group is added to the 8-position of the adenine ring. Low concentrations of this analogue exhibited an increase in channel activity and high concentrations a decrease (*see* Table).

In 8-[4-chlorophenylthio] adenosine 3′,5′-cyclic monophosphate (8-[4-chlorophenylthio] cyclic AMP) a large additional side group is attached to the 8-position of the adenine ring. Figure 4 shows the effects of two different concentrations of 8-[4-chlorophenylthio] cyclic AMP tested in separate patches. Exposure of one of the patches to 1 μM of the analogue increased the activity of the Ca-NS^+ channel to 238% of the control and increased the number of functional channels observed from two to three (Fig. 4*A*). The effect was reversible on washing the patch with nucleotide-free solution. Figure 4*B* shows a continuous recording of Ca-NS^+ channel activity in which a patch was exposed to 1mM 8-[4-chlorophenylthio] cyclic AMP. Exposure of the patch to the nucleotide analogue increased channel activity to 289.5% of control levels and the effect was again reversible when the analogue was washed from the patch with nucleotide-free solution. The results summarized in the Table show that this was the only analogue tested that produced very large increases (greater than those observed for cyclic AMP and other analogues) in the activity of the Ca-NS^+ channel in patches where activation occurred. Similar to cyclic AMP this analogue produced both increases and decreases in channel activity at 0.1 μM, 1 μM and 0.1 mM, but unlike cyclic AMP and other analogues, it also produced dual effects at a concentration of 1mM.

In the above studies with cyclic AMP, and with many of the analogues tested, the activity of the Ca-NS^+ channel frequently showed a transient refreshment. This was observed during the period when the patch was washed with nucleotide-free solution after exposure to a concentration of nucleotide that reduced channel activity. Refreshment was not observed in all patches and when present, its degree was very variable from one patch to another, for a given concentration of an individual nucleotide. This effect has not been investigated in detail in this study.

The Effects of Diastereoisomers of Cyclic Adenosine-3′,5′Monophosphothioate

Cyclic adenosine-3′,5′-monophosphothioate is a cyclic AMP analogue in which one of the two exocyclic oxygen atoms in the cyclic phosphate moiety, is replaced by sulphur. Equatorial thio-substitution leads to the R-isomer (R_p-cAMPS), while axial modification yields the S-isomer (S_p-cAMPS) (Fig. 5). The suffix "$_p$" indicates that the R/S nomenclature refers to phosphorus. In previous studies, it has been shown that S_p-cAMPS acts like an agonist and R_p-cAMPS as an antagonist of cyclic AMP-dependent protein kinase (Rothermel et al., 1984; Dostmann et al., 1990). However, in contrast R_p-cAMPS is an agonist and S_p-cAMPS an antagonist of the catabolite gene activation protein of *Escherichia coli* (Scholuebbers et al., 1984). Thus, to characterize further the specificity of the cyclic AMP interactions with the binding sites on the Ca-NS^+ channel, the effects of the R_p and S_p thio-isomers of cyclic AMP have been assessed on channel activity.

Figure 6 shows the effects of two different concentrations of S_p-cyclic AMP on Ca-NS^+ channel activity assessed in separate patches. Exposure of a patch to 0.1 μM of the analogue (Fig. 6*A*) increased channel activity substantially to 541% of the control and increased the number of functional channels observed from two to five. Removal of the nucleotide, by washing the patch in nucleotide-free solution returned the channel activity to control levels (*not shown*). Subsequent application of 1 μM of the analogue, to the same patch, further increased the activity of the channel to 653% of the control level. This effect was again fully reversible on washing the nucleotide from the patch (*not shown*). Exposure of the second patch to 10 μM of the analogue (Fig. 6*B*) increased channel activity in the patch to 707.6% of the control level, and also increased the number of functional channels observed from two to three. The effects of the analogue were again reversible. The Table summarizes the results obtained with the S_p-cAMPS isomer and shows that it produced similar effects to cyclic AMP itself on Ca-NS^+ channel activity, in that low concentrations (0.1 and 1 μM) increased channel activity, in four

Table 1. The effects of cyclic AMP analogues on Ca-NS$^+$ channel activity

Nucleotide	$N_f \cdot P_o$(test)/$N_f \cdot P_o$(control)								
	Effect +/–	0.1 μM	n	1 μM	n	0.1 mM	n	1 mM	n
Cyclic AMP	+	1.71 ± 0.42	6	1.65 ± 0.3	6	1.36 ± 0.23	3	–	
	–	0.75 ± 0.11	5	0.99,0.68	2	0.29 ± 0.08	7	0.22 ± 0.13	8
N^6-2′-O-dibutyryl	+	–		–		–		–	
cyclic AMP	–	0.57 ± 0.08	4	0.51 ± 0.08	5	0.47 ± 0.16	6	0.09 ± 0.04	8
N^6-monobutyryl	+	–		–		–		–	
cyclic AMP	–	0.14 ± 0.08	5	0.32 ± 0.13	6	0.06 ± 0.04	6	0	3
2′-O-monobutyryl	+	6.78	1	–		–		–	
cyclic AMP	–	0.14 ± 0.14	6	0.42 ± 0.06	6	0.23 ± 0.16	4	0.16 ± 0.16	4
6-Chloropurine	+	2.31 ± 0.88	3	1.13	1	3.08 ± 1.11	3	–	
cyclic AMP	–	0.79	1	0.47 ± 0.23	3	0	1	0.30 ± 0.17	4
8-Bromo cyclic AMP	+	1.18,3.30	2	1.89 ± 0.40	4	–		–	
	–	0.58,0.52	2	–		0.36 ± 0.13	8	0.50 ± 0.27	4
8-(4-Chlorophenylthio)	+	5.30 ± 2.11	3	2.55 ± 0.98	4	4.73 ± 1.71	3	1.89 ± 0.45	4
cyclic AMP	–	0.76,0.95	2	0.42 ± 0.27	3	0.70 ± 0.06	3	0.76,0.04	2
(S_p)-cAMPS	+	4.27 ± 1.51	3	3.36 ± 1.45	5	–		–	
	–	0.63	1	0	1	0.26 ± 0.23	4	–	

Results are expressed as the mean relative change in $N_f \cdot P_o$ ± SEM. + indicates patches showing an increase in activity and – indicates patches showing a decrease in activity. n = number of patches exhibiting increases or decreases in activity at a given concentration of the analogues. – = not observed.

out of the five patches tested, while a higher concentration (0.1 mM) reduced it in all four patches tested.

Parallel studies were carried out with R_p-cAMPS to see if it had any direct modulatory effects on the activity of the Ca-NS$^+$ channel prior to an assessment of its antagonistic actions. Figure 6*C* shows an example of exposure of a patch to 10 μM R_p-cAMPS which reduced the activity of the Ca-NS$^+$ channel to 72.6% of its control level. When the nucleotide was washed from the patch, in nucleotide-free solution, the channel activity increased and the patch showed refreshment. This concentration of R_p-cAMPS produced an average reduction of Ca-NS$^+$ channel activity to 53.7 ± 9.1% (n = 4) of control levels. At lower concentrations (0.1 and 1 μM) it produced no discernible effects on Ca-NS$^+$ channel activity (n = 4). The R_p-cAMPS thio-isomer was never observed to have an activating effect on channel activity, either at low or high concentrations (n = 8). Thus, its ability to antagonize the channel activating ability of both S_p-cAMPS and cyclic AMP was tested.

Figure 7 shows a continuous recording of Ca-NS$^+$ channel activity from a single inside-out patch which was exposed to increasing concentrations of R_p-cAMPS in the continuous presence of 1 μM S_p-cAMPS. Initially the patch was exposed to 0.1 μM S_p-cAMPS which increased channel activity. When this concentration of the nucleotide was washed from the patch, in nucleotide-free solution, channel activity initially declined but then showed a large refreshment. When the activity of the channel had declined to approximately control levels, the patch was exposed to a prolonged pulse of 1 μM S_p-cAMPS which immediately increased channel activity. Neither introducing 0.1 μM R_p-cAMPS into the solution together with the S_p-cAMPS, nor removing the R_p-cAMPS and leaving the patch exposed to the S_p-cAMPS, produced any further change in channel activity. However, when 1 μM R_p-cAMPS was introduced in the continued presence of S_p-cAMPS, then channel activity was reduced. It increased when the R_p-cAMPS was removed to leave the patch exposed to the continued presence of 1 μM S_p-cAMPS. A much more dramatic reduction in channel activity was observed when 10 μM R_p-cAMPS was introduced into the solution bathing the patch in the presence of 1 μM S_p-cAMPS. This effect was again reversible and the level of channel activity increased when the R_p-cAMPS was washed from the patch leaving the patch exposed to 1 μM S_p-cAMPS. This experiment (n = 3) indicates that the activation produced by S_p-cAMPS continues for as long as the nucleotide is applied to the inside surface of the patch. In parallel experiments (n = 4) (*not shown*) the addition of a combination of 10 μM R_p-cAMPS plus 1 μM S_p-cAMPS to the solution bathing the patch also blocked the activating effect of 1 μM S_p-cAMPS completely (*see* the Table for average activating effect).

Similar experiments revealed that the R_p-cAMPS

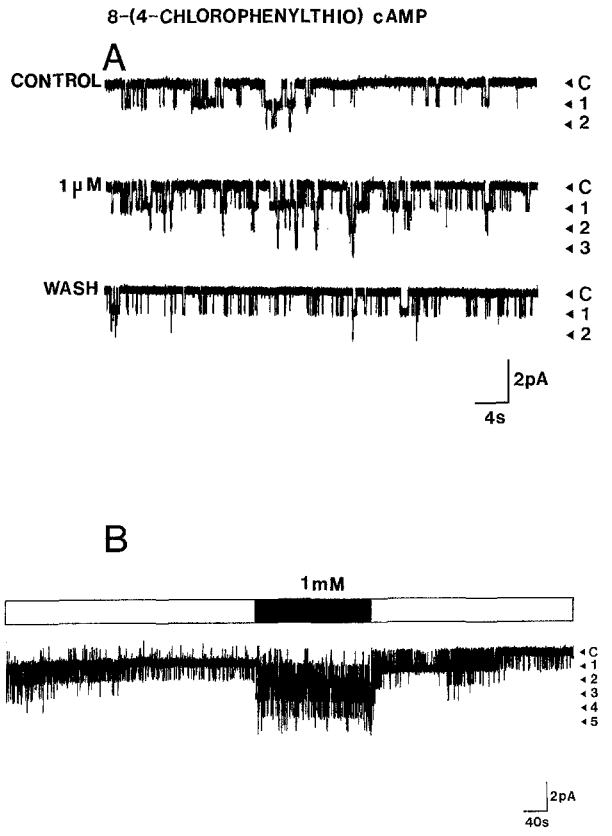
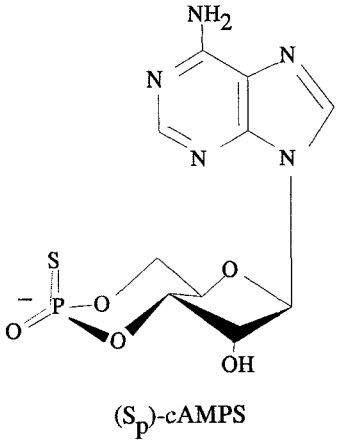
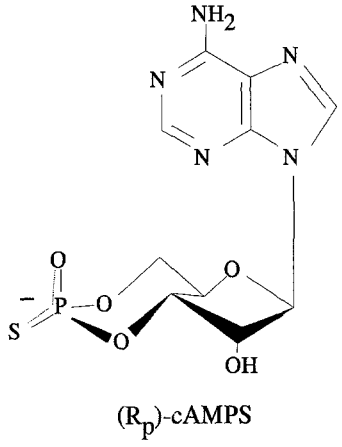


Fig. 4. Single channel records from two different patches exposed to different concentrations of 8-[-4-Chlorophenylthio] cyclic AMP, (*A*) 1 μM and (*B*) 1 m*M*. Channel currents were recorded from excised inside-out patches held at a membrane potential of –45 mV in the presence of 0.2 mM Ca^{2+}. (*A*) $N_f \cdot P_o$ values are: Control, 0.162; 1 μM 8-[-4-Chlorophenylthio] cyclic AMP, 0.384; Wash, 0.225. (*B*) a continuous recording of single channel activity of the Ca-NS^+ channel from an inside-out patch held at a membrane potential of –45 mV. 8[-4-chlorophenylthio] cyclic AMP (1 mM) (black bar) was applied to the cytoplasmic face of the patch for the period indicated and the nucleotide was then washed from the patch in nucleotide-free solution. $N_f \cdot P_o$ values are: Control, 1.265; 1 mM 8-[-4-chlorophenylthio] cyclic AMP, 2.440; Wash, 0.968. In (*A*) the traces start 216 sec after patch formation. The gap between the top and middle traces was 232 sec, of which the patch spent 184 sec in the presence of 1 μM 8-[-4-chlorophenylthio] cyclic AMP. The gap between the middle and bottom traces was 444 sec, of which the patch spent 360 sec in the wash. In (*B*) the trace starts immediately after patch formation.



(S_p)-cAMPS



(R_p)-cAMPS

Fig. 5. Structures of (S_p) and (R_p)-cAMPS

can also block the activating effects of cyclic AMP on Ca-NS^+ channel activity. Figure 8 shows an experiment in which exposure of a patch to 1 μM cyclic AMP increased channel activity to 394% of the control level and increased the number of functional channels observed from one to three. When the nucleotide was washed from the patch, the channel activity returned to control levels (*not shown*). However, if the same concentration of cyclic AMP was now applied in the presence of 0.1 mM R_p-cAMPS the degree of activation of the channel was substantially reduced to only 127% of the control. When the R_p-cAMPS was washed off the patch, leaving the 1 μM cyclic AMP to bathe the patch, then the channel activity was again increased (to 1,076% of control levels).

Discussion

The Ca-NS^+ channel in the CRI-G1 cells has previously been suggested to have two separate sites for modulation by cyclic AMP, which produce opposite effects on the activity of the channel (Reale, Hales & Ashford, 1994*a*). However, there does appear to be some concentration dependence in the changes in channel activity induced by cyclic AMP, since high concentrations predominantly inhibit, while low concentrations predominantly activate, channel activity. Thus, it may be argued that there is only one cyclic nucleotide binding site on the channel and that at high concentrations of agonist the channel changes into a different response state. The evidence presented in the present paper, on the differential selec-

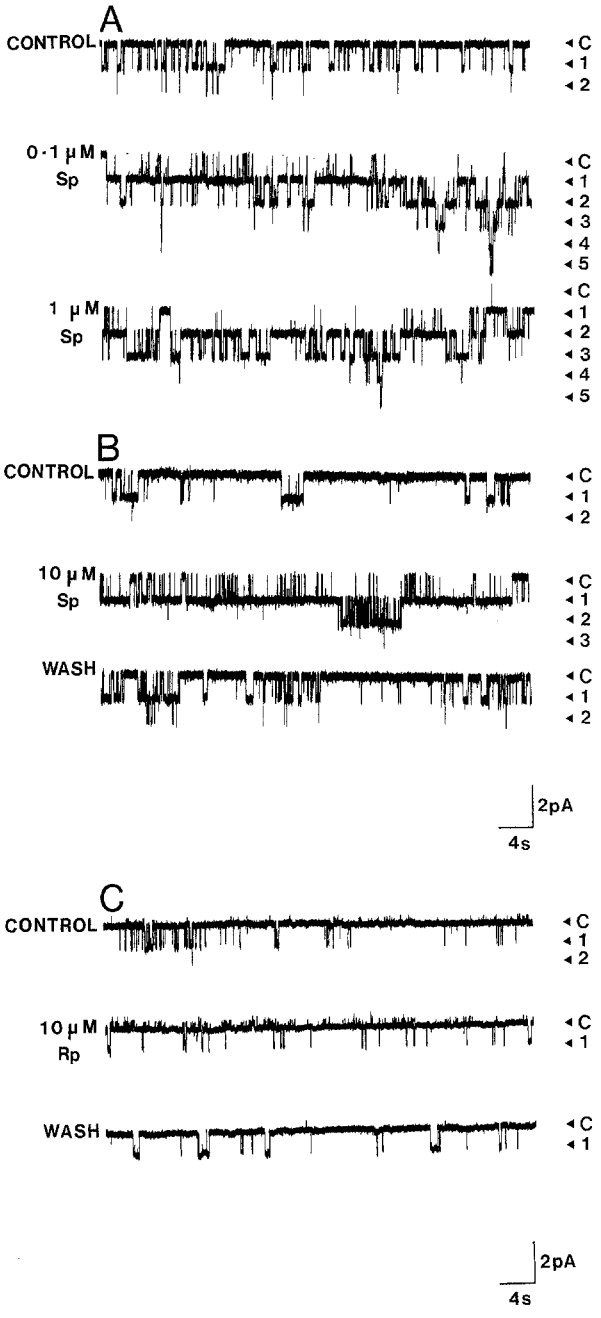


Fig. 6. Single channel records from three different patches exposed to S_p-cAMPS, (*A*) 0.1 and 1 μM, (*B*) 10 μM and (*C*) 10 μM R_p-cAMPS. Channel currents were recorded from excised inside-out patches held at a membrane potential of −45 mV in the presence of 0.2 mM Ca^{2+}. $N_f \cdot P_o$ values are: *A*, Control, 0.237; 0.1 μM S_p-cAMPS, 1.280; 1 μM S_p-cAMPS, 1.543. *B*, Control, 0.129; 10 μM S_p-cAMPS, 0.913; Wash, 0.172. *C*, Control, 0.034; 10 μM R_p-cAMPS, 0.024; Wash, 0.099. In (*A*) the traces start 48 sec after patch formation. The gap between the top and middle traces was 280 sec, of which the patch spent 180 sec in the presence of 0.1 μM S_p-cAMPS. The gap between the middle and bottom traces was 700 sec, of which the patch spent 132 sec in the presence of 1 μM S_p-cAMPS. In (*B*) the traces start 1332 sec after patch formation. The gap between the top and middle traces was 248 sec, of which the patch spent 52 sec in the presence of 10 μM S_p-cAMPS. The gap between the middle and bottom traces was 448 sec, of which the patch spent 288 sec in the wash. In (*C*) the traces start 6 sec after patch formation. The gap between the top and middle traces was 292 sec, of which the patch spent 40 sec in the presence of 10 μM R_p-cAMPS. The gap between the middle and bottom traces was 140 sec, of which the patch spent 88 sec in the wash.

tivity of the two responses of the channel to a range of cyclic nucleotide analogues, argues in favor of a two-site model for cyclic nucleotide modulation of the Ca-NS^+ channel and we will use this two-site model as a framework for the discussion of our results.

In a bid to provide a better description of the binding properties of these two sites, which might be useful in the development of selective agonists and antagonists, a preliminary survey has been made of the effectiveness of a range of cyclic AMP analogues substituted at various positions on the adenine ring, ribose sugar and cyclic phosphate group. Similar studies have provided a considerable amount of information about the cyclic nucleotide binding sites on a number of different proteins (Shabb & Corbin, 1992).

In the present study, one of the most marked changes in potency of the analogues used came when substitutions were made for the hydrogen atoms of the amino group attached to position-6 of the adenine ring. Both N^6-monobutyryl cyclic AMP and N^6,2′-O-dibutyryl cyclic AMP contrasted with cyclic AMP itself, in that they exclusively produced a reduction in the activity of the Ca-NS^+ channel at all concentrations tested, with the former analogue being more potent than the latter. This shows a parallel with the binding of analogues with bulky hydrophobic N^6-substituents to both protein kinase type I and II, where such analogues show a specificity for binding to site A, but are partially excluded from site B (Doskeland et al., 1983). (The carboxy terminus of each of the regulatory subunits of protein kinase contains two similar, but kinetically distinct cyclic AMP binding sites, A and B). In the present study the results support the exclusion of the large N^6-substituents from the activation site on the Ca-NS^+ channel due to their bulk. Substituting the amino group for a chloride in the 6-position, producing 6-chloropurine cyclic AMP, gives an analogue that is capable of both increasing and decreasing channel activity in different patches with a similar potency to cyclic AMP itself. The same analogue is also capable of binding to the A and B sites of both type I and type II cyclic AMP-dependent protein kinase, showing a slight preference for the B site in the type II kinase, but no selectivity in the type I kinase (Dostmann et al., 1990).

2′-O-monobutyryl cyclic AMP has a butyryl group added to the 2′-position of the ribose ring via an ether linkage. This again showed a selectivity for the site on the Ca-NS^+ channel causing a reduction in channel activity. It suggests that the availability of the 2′-OH on

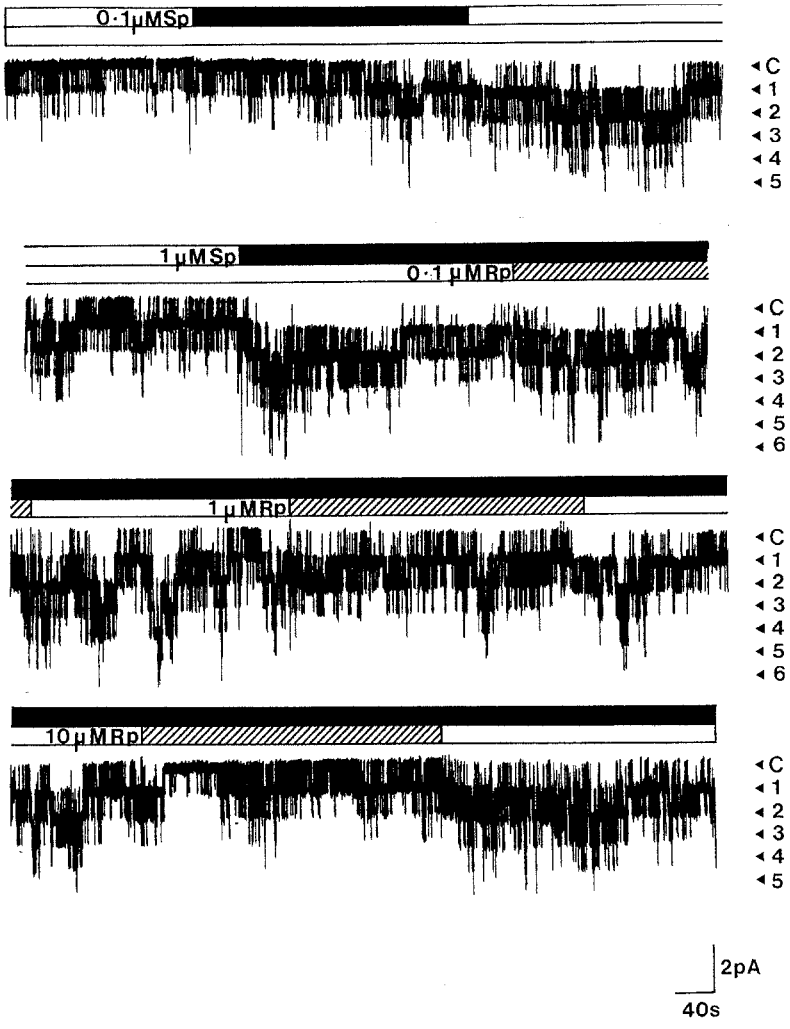


Fig. 7. The four traces form a continuous recording of single channel activity of the Ca-NS$^+$ channel from an inside-out patch held at a membrane potential of –45 mV illustrating the antagonistic effects of R_p-cAMPS on the activating effect of S_p-cAMPS. The patch was first exposed to 0.1 μM S_p-cAMPS (black bar) (S_p) and then washed in nucleotide-free solution. The patch was then exposed to a prolonged pulse of 1 μM S_p-cAMPS (black bar) during which time pulses of R_p-cAMPS (R_p) of increasing concentration (0.1, 1 and 10 μM) (hatched bars) were also applied to the patch. $N_f \cdot P_o$ values are: Control, 0.237; 0.1 μM S_p, 1.279; Wash, 0.841; 1 μM S_p, 1.593; 1 μM S_p + 0.1 μM R_p, 1.455; 1 μM S_p and wash 0.1 μM R_p, 1.542; 1 μM S_p + 1 μM R_p, 1.139; 1 μM S_p and wash 1 μM R_p, 1.659; 1 μM S_p + 10 μM R_p, 0.392; 1 μM S_p and wash 10 μM R_p, 1.645. The traces start 180 sec after patch formation.

the ribose ring for hydrogen bond formation may be an essential feature for binding of cyclic AMP to the activation site, but not to the inhibitory site for cyclic nucleotides on the Ca-NS$^+$ channel. The 2′-OH group is also essential for the binding of cyclic AMP to the regulatory domains of both type I and type II cyclic AMP-dependent protein kinase (Weber et al., 1987) and to the catabolite gene activator protein (Weber & Steitz, 1987), but is not important in the binding of cyclic AMP to the cell surface receptor of *Dictyostelium* (Van Haastert, 1983; Van Haastert & Kien, 1983) or to cyclic AMP-dependent phosphodiesterase (*see* Simon et al., 1973).

Substituents at the 8-position on the adenine ring were capable of interacting with both sites on the Ca-NS$^+$ channel. 8-Bromo cyclic AMP showed a similar activity pattern to cyclic AMP itself, while 8-(4-chlorophenylthio) cyclic AMP was remarkable in the high degree of activation it produced in many patches. The latter compound shows little selectivity between sites A and B in type II kinase (Dostmann et al., 1990). Since cyclic AMP analogues with large substituents in the 8-position are locked into the *syn*-configuration (Corbin et al., 1982; Doskeland et al., 1983; Dostmann et al., 1990) and they are capable of binding to both sites on the Ca-NS$^+$ channel, it means that cyclic AMP is likely to bind to both these sites in the *syn*-configuration. A similar conclusion has been drawn for the binding conformation of cyclic GMP to the cyclic nucleotide modulated channel of photoreceptors, based on studies with cyclic GMP analogues substituted in the 8-position (Zimmerman et al., 1985; Tanaka, Eccleston & Furman, 1989) and in most cases for the binding of cyclic AMP to both sites on cyclic AMP-dependent protein kinase (Doskeland et al., 1983; Dostmann et al., 1990). However, the reduced potency of C-8 substituted analogues at activating the cell surface receptors of *Dictyostelium* (Van Haastert & Kien, 1983) and of binding to the catabolite gene activator protein of *Escherichia coli,* suggests that cyclic AMP is bound in the *anti*-configuration in these two cases.

Most studies on the binding of cyclic nucleotides to their target sites agree on the importance of the exocyclic oxygen atoms on the cyclic phosphate for functional activation of the receptor protein (*see* Dostmann et al., 1990). If the exocyclic oxygen in the equatorial position

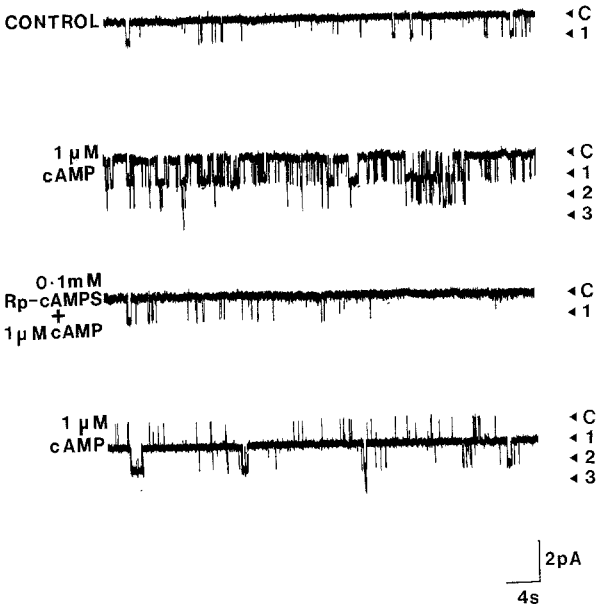


Fig. 8. Single channel records to illustrate the antagonistic effects of 0.1 mM R_p-cAMPS on the activating effect of 1 μM cyclic AMP. The control trace shows channel activity before exposure to the analogues. The patch was first exposed to 1 μM cyclic AMP alone, washed (*not shown*), and then to a combination of 0.1 mM R_p-cAMPS plus 1 μM cyclic AMP. In the last trace R_p-cAMPS has been withdrawn from the bathing solution leaving 1 μM cyclic AMP. Channel currents were recorded from an excised inside-out patch held at a membrane potential of −45 mV in the presence of 0.2 mM Ca^{2+}. $N_f \cdot P_o$ values are: Control, 0.094; 1 μM cyclic AMP, 0.371; 0.1 mM R_p-cAMPS + 1 μM cyclic AMP, 0.126; and 1 μM cyclic AMP, 1.013. The traces start 152 sec after patch formation. The gap between the first and second traces was 152 sec, of which the patch spent 92 sec in the presence of 1 μM cyclic AMP. The gap between the second and third traces was 828 sec, of which the patch spent 176 sec in the presence of 0.1 mM R_p-cAMPS + 1 μM cyclic AMP. The gap between the third and fourth traces was 960 sec, of which the patch spent 184 sec in the presence of 1 μM cyclic AMP.

is replaced by a sulfur atom, it produces an analogue that is known to act as an antagonist of the activation of cyclic AMP-dependent protein kinase, namely R_p-cAMPS. Axial substitution of the exocyclic oxygen produces S_p-cAMPS, an analogue with reduced effectiveness at activating cyclic AMP-dependent protein kinase (Dostmann et al., 1990). Both binding sites on the Ca-NS^+ channel are accessible to S_p-cAMPS, but R_p-cAMPS blocks the actions of both S_p-cAMPS and cyclic AMP itself on the site producing an increase in channel activity, while having very little intrinsic activity of its own on either Ca-NS^+ channel cyclic nucleotide site. This emphasizes the importance of the equatorial oxygen in the functional binding of cyclic AMP to both cyclic nucleotide sites on the Ca-NS^+ channel. Similar conclusions have been drawn from studies on the effectiveness of the S_p and R_p phosphorothioate derivatives of cyclic GMP on the cyclic GMP-activated nonselective cation channel of rod photoreceptors (Zimmerman et al., 1985). However, in contrast R_p-cAMPS is an agonist and S_p-cAMPS an antagonist of the catabolite gene activator protein of *Escherichia coli* (Scholuebbers et al., 1984).

The evidence presented above supports the idea that there are likely to be at least two cyclic nucleotide binding sites on the Ca-NS^+ channel or a closely associated protein from the insulinoma cell line. Since the adenine nucleotides AMP, ADP and ATP are also capable of reducing the activity of the Ca-NS^+ channel in this cell line, and have also been observed to increase channel activity in some patches, at low concentrations, this raises the question of whether they are actually working on the same sites as the cyclic nucleotides or on a different set of binding sites? Current evidence emphasizes the importance of the exocyclic oxygen atoms of the cyclic phosphate group of cyclic AMP in the binding to and activation of its target sites (Van Haastert, 1983; Van Haastert & Kien, 1983; Weber & Steitz, 1987; Weber et al., 1987; Dostmann et al., 1990). The similar observations in the present study of the importance of the equatorial exocyclic oxygen for the stimulation of both the activating and inhibitory effects of cyclic AMP on the Ca-NS^+ channel suggests that the two cyclic AMP regulatory sites will be distinct from the two sites described previously (Reale, Hales & Ashford, 1994*a*) mediating the modulatory actions of AMP, ADP and ATP on the Ca-NS^+ channel. The channel must also possess sites for regulation by intracellular Ca^{2+} levels and by pyridine nucleotides (Reale, Hales & Ashford, 1994*b*). It is not clear at present if the Ca-NS^+ channel functions as a homoligomer with all the nucleotide regulatory sites on a single subunit, or as a heteromeric channel complex, such as has been recently suggested for cyclic nucleotide activated channels in rods and olfactory receptors (*see* Yau, 1994), with its nucleotide regulatory sites differentially distributed on different subunits. The resolution of this question awaits cloning and expression studies on this channel.

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