J. Membrane Biol. 188, 183–197 (2002)
DOI: 10.1007/s00232-001-0184-0

The Journal of
Membrane
Biology

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Adenosine 5′-Monophosphate Is a Selective Inhibitor of the Brown Adipocyte Nonselective Cation Channel

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Received: 15 January 2002/Revised: 16 April 2002

Abstract. Calcium-activated nonselective cation channels (NSC_{Ca}) in brown adipocytes are inhibited by several nucleotides acting on the cytosolic side of the membrane. We used excised inside-out patches from rat brown adipocytes to identify important nucleotide structures for NSC-channel inhibition. We found that 100 μM 5′-AMP inhibited NSC-channel activity more than did ATP or ADP. Adenosine was a weak inhibitor, whereas adenine and ribose-5-phosphate had no effect. The channel activity was effectively blocked by 10 μM AMP, but it was unaffected by 10 μM cAMP, CMP, GMP, IMP, TMP or UMP. Dose-response studies yielded IC_{50}-values of 4 μM for AMP and 32 μM for cAMP. dAMP was as effective as AMP, but all 5′-phosphate group modifications on AMP dramatically lowered the inhibitory effect. 10 μM of the AMP precursor adenylosuccinate weakly inhibited the channel activity. An increase in AMP concentration from 1 to 10 μM shifted the EC_{50} for Ca^{2+} activation almost 1 order of magnitude; a Schild plot analysis yielded a K_B value of 0.3 μM for AMP. We conclude that AMP is the most efficacious endogenous nucleotide inhibitor of the brown adipocyte nonselective cation channel ($NSC_{Ca/AMP}$) yet identified and that there is functional competition between Ca^{2+} and AMP. The brown adipocyte $NSC_{Ca/AMP}$ thus appears to be functionally different from the $NSC_{Ca,PKA}$ in the exocrine pancreas and the $NSC_{Ca,cAMP}$ in the endocrine pancreas, but similar to the $NSC_{Ca/AMP}$ in the endocrine pancreas.

Key words: Brown adipocytes — NSC_{Ca} — Nonselective cation channels — Nucleotide regulation — Patch clamp — Single-channel recording

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Introduction

Whole-Cell Currents and Membrane Potential Changes in Brown Fat Cells

Brown adipose tissue is the organ responsible for nonshivering thermogenesis. The thermogenesis is induced by norepinephrine (NE) released from surrounding sympathetic nerves. NE binds to β_3-adrenergic receptors, which elevate cyclic AMP levels, activating protein kinase A, which activates hormone-sensitive lipase to release free fatty acids from stored triglycerides. This in turn leads to activation of the mitochondrial uncoupling protein 1 (UCP1), which depolarizes the mitochondria and thereby raises the respiration rate (reviewed in Nedergaard et al., 2001).

However, during stimulation with norepinephrine, in parallel to the activation of thermogenesis, the brown adipocyte also shows a triphasic change in membrane potential (Girardier, Seydoux & Clausen, 1968; Horwitz, Horowitz & Smith, 1969; Koivisto, Siemen & Nedergaard, 2000). The ion channels involved in the NE-induced membrane potential change have been partly analyzed by single-channel analysis (Lucero & Pappone 1990; Koivisto et al., 2000). The triphasic response starts with an initial, transient depolarization of ~25 mV, lasting for 10–30 sec (Lucero & Pappone 1990). This depolarization is mainly due to α_1-adrenergic stimulation and is probably caused by Cl^- efflux through Cl^- channels (Dasso, Connolly & Nedergaard, 1990; Lucero & Pappone 1990; Koivisto et al., 2000). The following repolarization may also include an ~5–10 mV hyperpolarization for 2–5 min (Lucero & Pappone 1990; Koivisto et al., 2000). This hyperpolarization is also activated by α_1-receptors and it is probably mediated by calcium-activated K^+ channels causing a K^+ efflux (Nånberg, Nedergaard & Cannon, 1984; Lucero & Pappone 1990).

The third phase is a fairly weak but sustained depolarization of about 5 mV, carried by an inward Na^+ current (Lucero & Pappone 1990; Koivisto et al., 2000), which continues as long as the adrenergic stimulation continues. This long-lasting depolarization was originally discussed as being activated by β-adrenergic stimulation (Girardier & Schneider-Picard, 1983; Horwitz & Hamilton, 1984; Lucero & Pappone 1990), but under conditions of adequate oxygen supply the depolarization is probably mainly due to α_1-adrenergic stimulation; the underlying Na^+-influx is probably mediated by Ca^{2+}-activated nonselective cation (NSC) channels (Koivisto et al., 2000). Temporally, the norepinephrine-induced increase in metabolic rate thermogenesis correlates well with this long-lasting depolarization, but these two events do not acutely depend on each other.

Nonselective Cation Channels in Brown Fat Cells

Calcium-activated nonselective cation (NSC) channels in brown fat cells were first described by Siemen and Reuhl (1987). In inside-out patches, these 30-pS channels have a high relative permeability to NH_4^+ (1.55) > Na^+ (1.0) > K^+ (0.8), but they are not measurably permeable to Ca^{2+} (< 0.002) (Weber & Siemen, 1989). These NSC-channels show a very low Ca^{2+} sensitivity in excised inside-out patches: more than 100 μM free Ca^{2+} is needed for a substantial activation (Koivisto et al., 1998; Halonen and Nedergaard, unpublished data). Brown adipocyte free Ca^{2+}-levels are normally around 0.1–0.2 μM in the resting state; if stimulated by norepinephrine or by ATP, the free Ca^{2+} level seems to plateau around 0.5–1 μM (Wilcke & Nedergaard, 1989; Lee, Nuccitelli & Pappone, 1993; Thonberg et al., 1994). The requirement of more than 100 μM cytosolic Ca^{2+} for a significant activation of the brown adipocyte NSC-channel in excised inside-out patches has therefore led to speculations about additional endogenous co-activators (Koivisto et al., 1998). A high and seemingly unspecific inhibition by nucleotides (Koivisto et al., 1998) should theoretically prevent NSC-channel activation under physiological conditions. However, the NSC channel is indeed activated by NE both in cell-attached and in whole-cell patch-clamp experiments, in ways not currently understood (Koivisto et al., 2000).

Due to its low Ca^{2+} sensitivity, the brown adipocyte NSC channel has mainly been characterized in solutions containing at least 1 mM Ca^{2+}. Under these conditions, the NSC channel is inhibited not only by nucleotides but also by micromolar concentrations of the fenamate type of nonsteroidal anti-inflammatory drugs (NSAIDs), by nanomolar concentrations of thiol reagents such as Cd^{2+} and Hg^{2+}, and by nitric oxide (Koivisto et al., 1993; Koivisto & Nedergaard, 1995; Koivisto et al., 1998; Halonen and Nedergaard, unpublished data). None of the protein subunits of the functional brown adipocyte NSC channel have as yet been cloned.

Nucleotide Modulation of Brown Adipocyte NSC Channels

In excised inside-out patches, 100 μM concentrations of the purine nucleotides ATP, ADP, AMP, GDP and GMP, as well as cAMP and cGMP, have been reported to block the brown adipocyte NSC-channel activity more than 90% (Koivisto et al., 1998). This seemingly unspecific nucleotide sensitivity seems to be a widespread feature of calcium-activated nonselective cation (NSC) channels (Sturgess, Hales & Ashford, 1987; Popp & Gögelein, 1992; Thorn & Petersen, 1992; Ono et al., 1994; Korbmacher et al., 1995; Koivisto et al., 1998), but no detailed investigation of the necessary structural components required for the nucleotide-induced inhibition of the brown adipocyte NSC-channel has been performed. Such a study is now reported here. In the absence of a molecular cloning of the NSC_{Ca} channels, such an investigation is also of significance for distinguishing the brown adipocyte NSC-channel nucleotide pharmacology from (or identifying it with) other, apparently similar, channels described in other tissues.

We have focused our studies on the regulation of the NSC channel by cytosolic nucleotides and have not examined the possibility of an indicated, indirect or direct, Ca^{2+}-independent NSC-channel regulation by extracellular nucleotides, which has been described by Lee & Pappone (1997).

We were able to identify adenosine 5′-monophosphate (AMP) as the most efficacious endogenous nucleotide inhibitor of the NSC channel known to date, and we show that there is functional competition between the Ca^{2+} activation and the AMP inhibition of the channel. The brown adipocyte NSC channel thus probably belongs to the same ion-channel family as one of the NSC channels ($NSC_{Ca/AMP}$) previously described in the endocrine pancreas (Sturgess, Hales & Ashford, 1986; Sturgess et al., 1987a; Sturgess, Hales & Ashford, 1987b; Reale, Hales & Ashford, 1994; 1995).

Materials and Methods

Cell Isolation

All experiments were based on an earlier described approach with freshly isolated mature brown fat cells from young male Sprague-Dawley rats (Siemen and Reuhl, 1987; Koivisto et al., 1993). The rats had a body weight of 87 ± 5 g, they were rapidly killed by inhalation of CO_2, and the interscapular brown adipose tissue was removed and minced to small pieces (< 1 mm^3) with a pair of scissors. The minced tissue was incubated in 5 ml of a HEPES buffer containing (in mM): NaCl 134, KCl 6, HEPES 100, glucose 5,

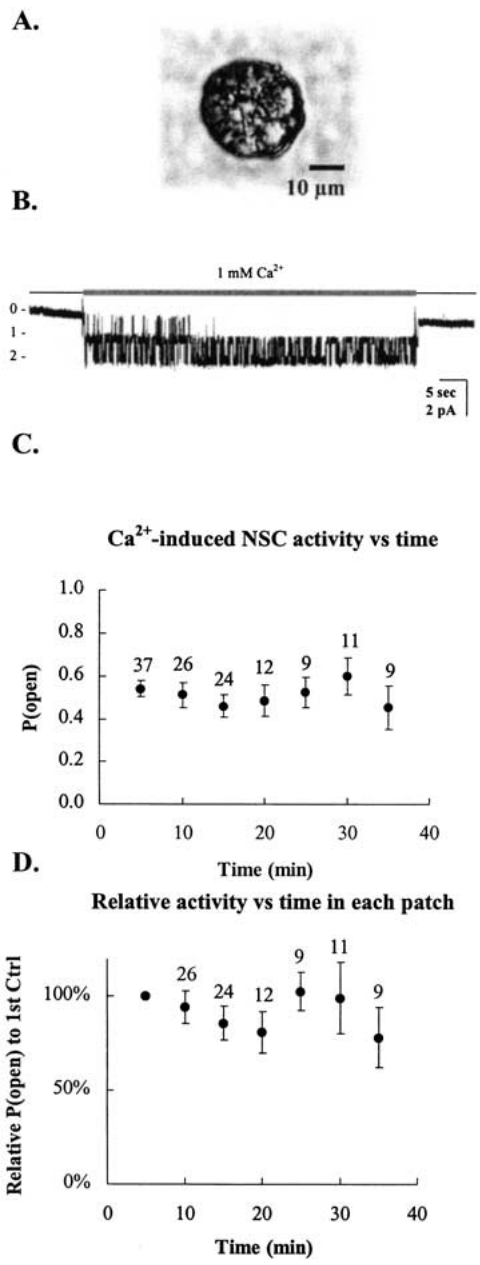


Fig. 1. (*A*) Microphotograph of a mature brown fat cell. (*B*) Sample trace showing NSC activity induced by 1 mM Ca^{2+} in an inside-out patch. The thin line indicates perfusion with the negative control (1 mM BAPTA); no NSC activity was observed. When the perfusion was changed to the positive control (1 mM Ca^{2+}), two NSC channels were immediately activated and the channels remained active for the duration of the perfusion (often with a tendency to a slight increase in activity over time). When the perfusion was changed back to the negative control, both channels inactivated instantly. (*C*) NSC-channel activity induced by 1 mM Ca^{2+} in inside-out patches with time. Each point represents an average *P*(*open*) value derived from analyzed 1-min fragments with a perfusion containing only 1 mM Ca^{2+} in KCl-Ringer, following and followed by a negative-control perfusion with 1 mM BAPTA. The number of fragments analyzed for each average is indicated above the data points; the total number of patches analyzed in this assay was 37. The fragments were taken from 1-min perfusions binned in 5-min intervals starting from zero time and having different test substances perfused in between the positive controls. Every analyzed recording has at least two 1-min fragments represented in the plot. However, all recordings are not represented in all 5-min intervals. (*D*) Average relative NSC activity induced by 1-min perfusions with 1 mM Ca^{2+} at various times during the same 37 inside-out patch recordings already described in *C*. 100% activity was here defined individually for each patch as the *P*(*open*) value obtained from the first 1-min perfusion.

$CaCl_2$ 1, $MgCl_2$ 1.2, and 2–3 mg of type II collagenase; pH was adjusted to 7.4 with NaOH. The buffer with minced tissue pieces was then gently shaken at 37°C for 30 min. The cell suspension was filtered through a silk filter (Precision Cells) into a 15-ml plastic test tube. The tube was then centrifuged 10 min at 20 × *g*, and the top layer (with brown fat cells) on the surface of the supernatant was collected by gentle pipette suction for a subsequent dilution in 5 ml culture medium. The centrifugation step was done twice to wash out remaining collagenase from the cell suspension. Finally, the homogenate was distributed equally into 6-well plates containing 3 ml culture medium per well. The culture medium was Dulbecco's modified Eagle's Medium with the following additions: 10% newborn calf serum, 4 nM insulin, 126 μM Na-ascorbate, 4 mM glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were kept in the culture medium with a piece of a plastic film (Biofoil-25^{TM} from Heraeus, Hanau, Germany) floating on the surface with the hydrophilic side down, to capture the mature brown fat cells when they floated up. The plates were kept in a standard incubator at 37°C and 8% CO_2. The cells were used 0–3 days after isolation. Cells were selected by visual criteria, i.e., multilocular fat droplets were present in all patched cells (*see* Fig. 1*A*).

Buffers

Only freshly prepared buffers were used and they were composed as follows: the bath solution, which was also the patch pipette solution, was Modified Ringer containing (in mM): NaCl 134, KCl 6, HEPES 10, glucose 5, $MgCl_2$ 1.2, $CaCl_2$ 1.2. The pH was adjusted to 7.40 with 1 M NaOH. The perfusion solutions were prepared from KCl-Ringer consisting of (in mM): NaCl 10, KCl 130, $MgCl_2$ 1.2, HEPES 10, reduced glutathione (GSH) 2 and oxidized glutathione (GSSG) 0.04. In the negative control, 1 mM 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) was added. In all other perfusion solutions, 1 mM $CaCl_2$ was routinely added as indicated. After the test agents had been dissolved in 50 ml of KCl-Ringer in small Erlenmeyer flasks, the pH was adjusted to 7.20 with KOH individually in each of the solutions. All indicated nucleotide additions were nominal additions, i.e., free nucleotide levels were not specifically calculated. The software Bound And Determined 4 (BAD4) (Brooks & Storey, 1992) was used to calculate the required nominal $CaCl_2$ additions to the KCl-Ringer (after a nominal nucleotide addition) in order to obtain the desired free Ca^{2+} levels. The calculated free Ca^{2+} concentration in KCl-Ringer with 1 mM nominal Ca^{2+} was 0.91 mM. If 1 mM AMP were added without compensatory Ca^{2+}-adjustments, the new calculated free Ca^{2+} level would be 0.88 mM. All nucleotides were added as Na^+ or K^+ salts. Mg^{2+} levels were nominal, but included in the calculations. In the Ca^{2+} concentration calculations, all nucleotide monophosphates were treated as AMP, all nucleotide diphosphates as ADP, and all nucleotide triphosphates as ATP. Unphosphorylated nucleotides were ignored in the calculations.

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma. $CaCl_2$, NaCl and KCl were of analysis quality and were

purchased from Merck. Rp-ADP-α-S and AMPS were from Biolog Life Science Institute, Bremen. Structural details of certain investigated compounds can be seen in the summary (*see* Fig. 9).

Electrophysiology

All experiments were made at room temperature (18–22°C), with the excised inside-out patch-clamp configuration (Hamill et al., 1981). Patch pipettes were freshly manufactured with a pipette puller (Mechanex, Geneva, Switzerland) from borosilicate glass capillaries of the type GC150–10 (Clark Electromedical Instruments, Reading, U.K.) after fire-polishing of each end and an external cleaning with 70% ethanol/water. The pipette resistances varied between 15 and 30 MΩ. The final tip shapes were long and thin, resulting in fairly large pipette capacitances but also in physically more stable patches. The NSC channel has very slow kinetic behavior, with open-time constants in the order of seconds (Koivisto, 1997); thus the rapid-event filtering resulting from high pipette capacitances was not considered a limitation. We used an 8-channel pinch-valve perfusion system (ALA Scientific Instruments, NY), which thus, in addition to one positive and one negative control perfusion, allowed the perfusion of 6 test substances.

A piece of the Biofoil-25™ with attached adipocytes was transferred to a small Petri dish, and the bath solution was added. When the patch pipette tip adhered to the adipocyte, a Giga-ohm seal was achieved within seconds. A +20 to +40 mV holding potential was applied before excision. Upon patch excision into the Ca^{2+}-containing bath, NSC-channel activity usually appeared immediately (Siemen & Reuhl, 1987; Koivisto & Nedergaard, 1995). In the inside-out patch mode, the membrane potential was clamped at −40 mV, a value close to that of a brown fat cell (Girardier et al., 1968). With the perfusion adjusted, a minimized back-leakage of Ca^{2+} from the bath was secured by repeated perfusions with the calcium-free, BAPTA-containing negative control between each test solution. This negative control usually inactivated all active NSC channels within 0–5 sec.

Experimental Protocol

When the perfusion had been properly adjusted, a 20–30-sec perfusion with the negative control started each recording, in order to allow for identification of the baseline. This perfusion was routinely followed by a 1-min perfusion with the positive control (1 mM Ca^{2+}) to monitor the maximal number of activatable NSC channels in the patch. In some cases, particularly with 10 mM Ca^{2+}, there was a small baseline shift when Ca^{2+} was introduced or removed. The reason for this phenomenon is unknown, but the baseline shifts were larger in patches from cells that did not give very tight seals, so they may in part result from increased leak currents. After the initial NSC-channel activation by the positive control, the negative control was perfused for 30 sec between each 1-min perfusion with test substances, and the positive control was regularly perfused every 5–10 min to monitor the general activity level in the patch. Under the conditions described, single-channel recordings of 20–40 min could regularly be made. Observed responses to Ca^{2+} and, where present, to nucleotides, were normally immediate and continued throughout the test minute, therefore this perfusion time was considered both reliable and time-efficient for these experiments. No significant rundown was detected for the activation by 1 mM Ca^{2+} during this period (*see* Fig. 1*C* and 1*D*).

Data Acquisition and Analysis

All recordings were made at gain 50 on the EPC-7 amplifier (List Electronic, Darmstadt). The signal was first filtered at 10 kHz by the amplifier, then it was recorded to a VHS video tape recorder for storage and filtered in parallel by an 8-pole Bessel-type filter at a cut-off frequency of 500 Hz for subsequent analysis. After the second filtration step, the signal was digitized by a pulse code modulator at a 14-bit amplitude resolution and 44-kHz time sampling. The digitized recordings were transferred to a PC computer by the software FETCHEX 6.0.4 (Axon Instruments, Foster City, CA) with a sampling rate of 2 kHz, i.e., 4 times the filter corner frequency, to avoid aliasing (Dempster, 1993). When the signal had been acquired by the computer, a plot of the whole data file, which was normally 5–10 MB, was first made for a visual overview of the recording. The perfusions were marked out and the 1-min fragments (corresponding to the full length of each perfusion) were identified for statistical analysis. Event-list files for each 1-min perfusion were created in FETCHAN 6.0.4 (Axon Instruments, CA) and open probabilities for all channels in each patch were calculated with PSTAT 6.0.4 (Axon Instruments). Relative open probabilities for individual fragments were calculated by defining the average value for all $P(open)$ values obtained with the positive control in each patch as 100% channel activity. The $P(open)$ obtained with each 1-min perfusion with a test substance was then compared individually to the above patch-average and expressed as relative activity. An overall average of the relative activities induced by given test substances in all patches was then calculated and plotted with standard errors and the number of represented fragments in bar diagrams. For statistical analysis, the mean value of these data was calculated for each patch and used in Student's paired t-tests with resulting differences expressed as $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results

NSC-Channel Activity in Inside-Out Patches

Figure 1*A* shows a typical mature brown adipocyte with multilocular fat droplets and smooth membranes, the main characteristics for our choice of cells to examine. Figure 1*B* shows the typical Ca^{2+}-induced NSC-channel activity in an inside-out patch, in accordance with earlier published results (Koivisto et al., 1998). Initially, a short perfusion with the negative control (1 mM BAPTA), marked out by the thin line, kept the NSC channels inactive. When the negative-control perfusion was replaced by the positive control (1 mM Ca^{2+}), as indicated by the heavy grey line, vigorous NSC-channel activity was observed here from two channels. When the positive control was substituted with the negative control, the observed NSC-channel activity rapidly disappeared, and no channel activity was observed as long as this perfusion continued. The diagrams in Fig. 1*C* and Fig. 1*D* show data from a total of 37 patches in which the positive control (1 mM Ca^{2+}) was added repeatedly (with other perfusions in between). As seen in Fig. 1*C*, the average $P(open)$ was about 0.5, which is in agreement with earlier published results for NSC activity induced by 1 mM Ca^{2+} (Koivisto et al., 1998), and it was not affected by time under these conditions. The absence of a rundown facilitated the evaluation of the following nucleotide trials, since it

did not matter at what time point during a 40-min recording a test substance was added.

Adenosine 5′-Triphosphate Derivatives

As earlier investigations have demonstrated a qualitative and apparently rather unspecific effect of different adenosine nucleotides (Koivisto et al., 1998), we first examined whether the degree of phosphorylation of adenosine was of significance. We used a concentration of 100 μM for each nucleotide, i.e., a nucleotide concentration that gave high inhibition in earlier studies (Koivisto et al., 1998). The sample trace in Fig. 2*A* shows an example of the nucleotide-induced inhibition of NSC channels. Initially, a short BAPTA perfusion kept the NSC channels inactive. When this negative control perfusion was replaced by 1 mM Ca^{2+} and 100 μM 5′-AMP, as indicated by the heavy black line, almost no NSC channel activity was observed. However, when 1 mM Ca^{2+} alone (the positive control, indicated by a heavy grey line) was perfused after the second BAPTA perfusion, three channels became active. The subsequent perfusions with 1 mM Ca^{2+} plus 100 μM 5′-ADP or 5′-ATP also yielded a smaller NSC-channel activity than perfusion with Ca^{2+} alone. Average relative *P*(*open*)-values for all analyzed 1-min fragments are shown in Fig. 2*B*. The diagram shows that 5′-AMP was the most potent NSC-channel blocker among the tested 5′-adenosine phosphates, followed by 5′-ADP and 5′-ATP. Regression analysis of *P*(*open*) versus number of phosphate groups indicated that this order of potency was statistically significant ($P = 0.05$).

As AMP was the most potent phosphorylated form of adenosine, we examined the potencies of different moieties of AMP. The lower sample trace in Fig. 2*A* is from another patch where the adenosine moiety in itself was tested. It shows an activation of six NSC channels by the positive control (1 mM Ca^{2+}) and also in the additional presence of 100 μM adenosine. The perfusion with 1 mM Ca^{2+} and 100 μM 5′-ADP demonstrated that ADP was an effective inhibitor of the NSC-channel activity also in this patch. In the compilation in Fig. 2*B*, it is seen that unphosphorylated adenosine at 100 μM had a small inhibitory effect, whereas neither adenine, ribose-5-phosphate or ribose were able to inhibit the channel. In conclusion, the order of potency of NSC-channel block by 5′-adenosine triphosphate derivatives was thus AMP > ADP > ATP ≫ adenosine; adenine, ribose-5-P and ribose had no effect.

AMP Analogs

With the information that 5′-AMP (AMP) was the most potent inhibitor among the adenosine phosphates, we continued with the same experimental protocol to investigate how different nitrogen bases could affect the blocking potency of the corresponding nucleotide monophosphate. Since the diagram in Fig. 2*B* showed an almost complete block of the NSC channel by 100 μM AMP, a nucleotide concentration of 10 μM (in 1 mM Ca^{2+}) was chosen to obtain en-

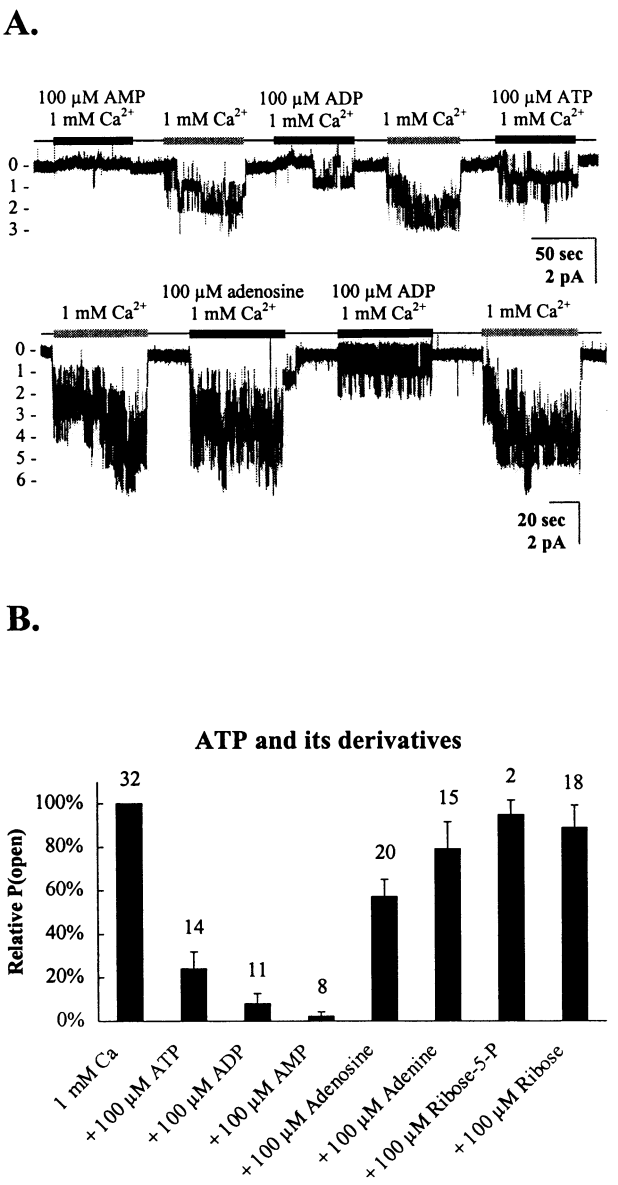


Fig. 2. Effects of adenosine 5′-triphosphate (ATP) and some of its derivatives on the NSC-channel activity. (*A*) Sample traces from two inside-out patches clamped at −40 mV. The thin line indicates perfusion with the negative control (1 mM BAPTA) and the heavy grey line indicates perfusion with the positive control (1 mM Ca^{2+}). (*B*) Average relative *P*(*open*) values from 9 inside-out patches with 1-min perfusions. The values above each bar indicate the number of analyzed 1-min fragments; not all substances were tested in all patches. 100% channel activity corresponded to $P(open) = 0.58 \pm 0.05$ in these experiments. Statistical significance *vs.* Ca^{2+} alone was obtained with AMP, ADP and ATP ($P < 0.001$) and with adenosine ($P < 0.01$).

hanced discrimination between different blocking potencies. Different nucleotide monophosphates with either purine bases (adenosine (AMP), guanosine (GMP), inosine (IMP), and xanthosine (XMP)) or pyrimidine bases (cytidine (CMP), thymidine (TMP), and uridine (UMP)) in 1 mM free Ca^{2+} were tested. As shown in the sample trace in Fig. 3*A* and further by the average relative *P*(*open*)-value diagram in Fig. 3*B*, 10 μM AMP abolished nearly all channel activity induced by 1 mM Ca^{2+}, while the other tested nucleotide monophosphates did not markedly affect the channel activity. 10 μM XMP gave an increased NSC-channel activity in some of the patches; however, this effect did not reach statistical significance. These results indicated a high specificity for AMP as an NSC-channel inhibitor among the nucleotide monophosphates. Structural details of these and other examined compounds are included in the summary figure (*see* Fig. 9).

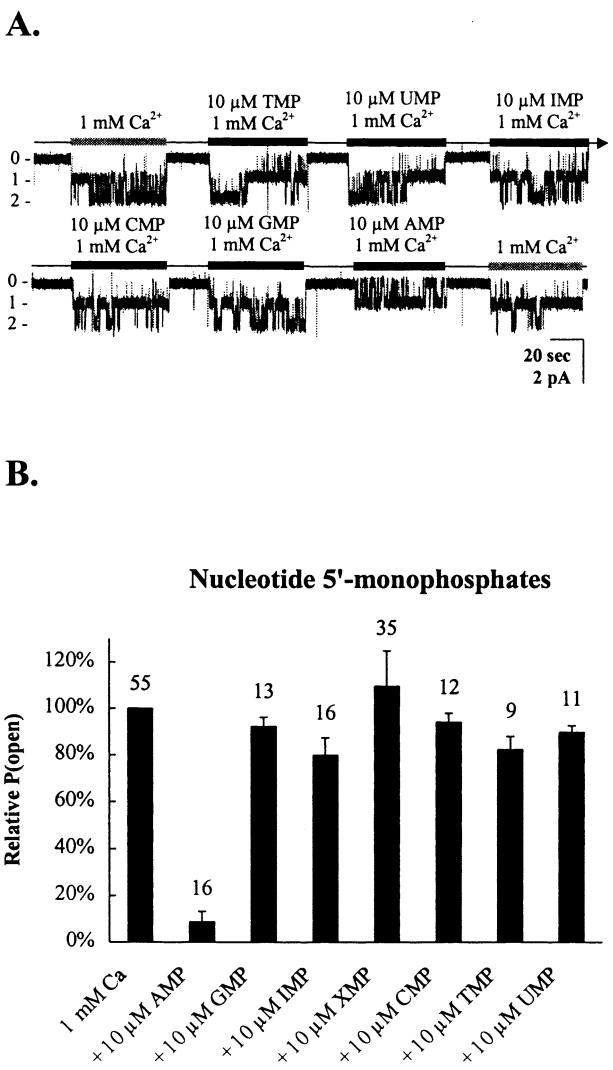


Fig. 3. Modulation of the NSC-channel activity by 10 μM of some nucleotide 5′-monophosphates. (*A*) A continuous signal from one patch, here presented in two parts for visual clarity. The thin line indicates perfusion with the negative control (1 mM BAPTA). (*B*) Average relative *P*(*open*) values from 20 inside-out patches with 1-min perfusions identify AMP as a selective NSC-channel blocker. 100% channel activity corresponds to $P(open) = 0.47 \pm 0.03$ in these experiments. Statistical significance *vs.* Ca^{2+} was obtained with AMP ($P < 0.001$) and with UMP ($P < 0.05$).

Ribose-modified AMP Analogs

Since AMP was a selective inhibitor among the nucleotide monophosphates, we examined to what extent modifications of the ribose moiety on the AMP molecule could affect the blocking potency. Selected nucleotide monophosphates in this assay were, in addition to 5′-AMP, 2-deoxy-AMP, 3′-AMP, the physiologically important cyclic adenosine monophosphate (3′,5′-cAMP), and the analogue 2′,3′-cAMP. The sample trace in Fig. 4*A* shows that in the presence of 10 μM 2-deoxy-5′-AMP and 1 mM Ca^{2+}, no NSC-channel activity was observed, whereas Ca^{2+} alone induced activity from two channels. Fig. 4*A*, lower trace, shows that neither 2′,3′-cAMP nor 3′-AMP could inhibit the NSC-channel activity dramatically. The average relative *P*(*open*) values presented in Fig. 4*B* confirm that, at 10 μM concentrations and in 1 mM free Ca^{2+}, 5′-AMP and 2-deoxy-5′-AMP were the only nucleotides that could block the NSC-channel activity completely. At this concentration, the two cyclic AMP analogues could weakly inhibit the NSC-channel activity, whereas 3′-AMP was without effect. These results indicate, together with the results in Fig. 2*C*, a critical role of the 5′-phosphate group in the AMP-mediated inhibition of the brown adipocyte NSC channel, rather than an importance of the intact ribose component.

Dose-Response Assay for cAMP and AMP

Given the important signalling role of 3′,5′-cAMP (hereafter referred to as cAMP) in brown adipocytes, e.g., for control of thermogenesis, (Zhao, Cannon & Nedergaard, 1997) and the weak inhibition by cAMP shown in Fig. 4*C*, it was interesting to clarify the dose-response relationships for the NSC-channel inhibition by AMP and cAMP. A sample trace with a complete block of the NSC-channel activity by 100 μM AMP and a smaller inhibition by 100 μM cAMP is shown in Fig. 5*A*. In Fig. 5*B*, dose-response curves are shown with curve fits following the Michaelis-Menten equation, with the Hill coefficient set to 1. The calculated half-maximal inhibition (IC_{50} value) for AMP was only 4 μM, whereas the IC_{50} value for cAMP was 32 μM. The results obtained in these experiments further indicate a high specificity for AMP as a nucleotide inhibitor of the NSC channel. cAMP is thus probably not a primary physiological inhibitor of the NSC channels.

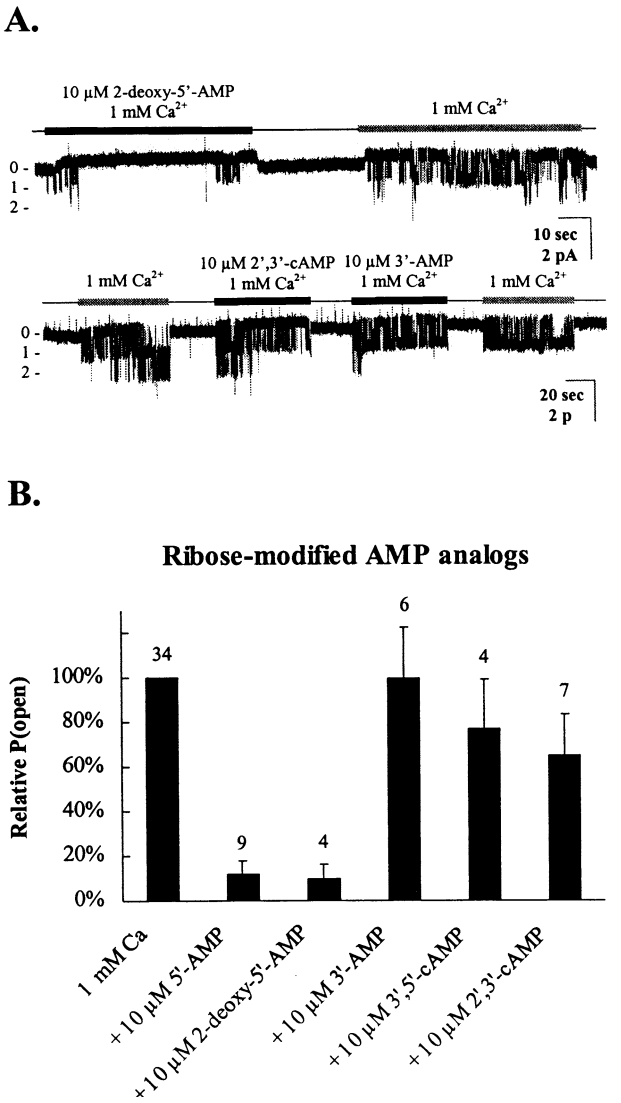


Fig. 4. Effects of ribose-modified AMP-analogs on the NSC-channel activity. (*A*) Sample traces from two inside-out patches showing the effects of 2-deoxy-5′-AMP (*upper*) and 2′,3′-cAMP or 3′-AMP (*lower*). The thin line indicates perfusion with the negative control (1 mM BAPTA). (*B*) Average relative *P*(*open*) values from 10 inside-out patches with 1-min perfusions. 100% channel activity corresponded to $P(open) = 0.40 \pm 0.04$ in these experiments. Statistical significance *vs.* Ca^{2+} alone was obtained with 5′-AMP ($P < 0.001$) and with 2′-deoxy-5′-AMP ($P < 0.01$).

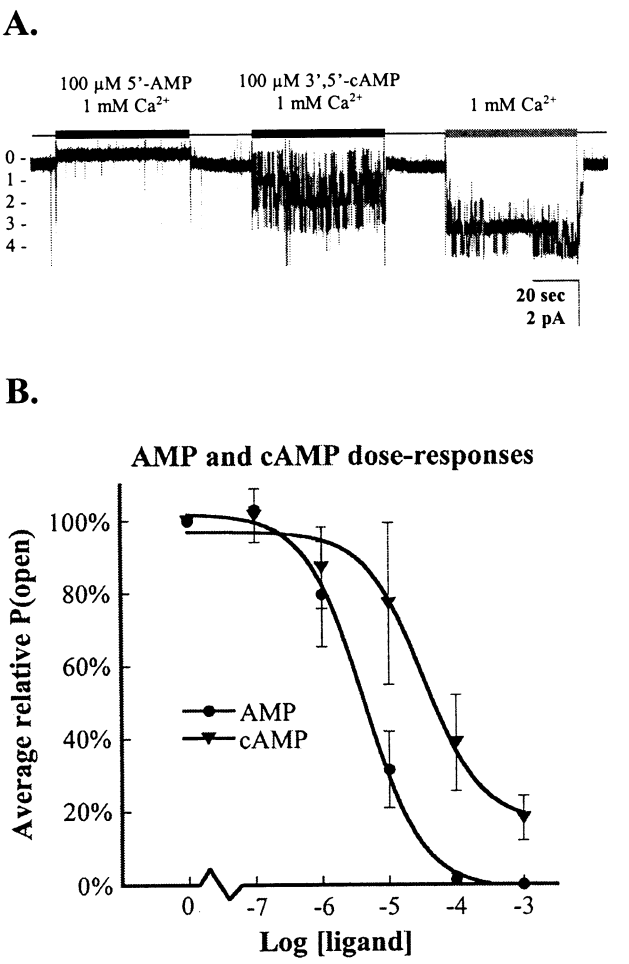


Fig. 5. Dose-response relationships for the NSC-channel inhibition by adenosine 5′-monophosphate (5′-AMP) and adenosine 3′,5′-cyclic monophosphate (3′,5′-cAMP). (*A*) Sample trace from an inside-out patch. The thin line represents 1 mM BAPTA. (*B*) Average relative *P*(*open*) values from 16 inside-out patches (3–14 analyzed fragments per point). The dose-response curves are shown as curve fits following the Michaelis-Menten equation with the Hill coefficient set to 1. The calculated IC_{50}-values were 4 μM for AMP and 32 μM for cAMP. 100% channel activity corresponded to $P(open) = 0.61 \pm 0.03$ in these experiments.

Effects of Low Micromolar Concentrations of AMP Analogs

Since it has been observed that a coactivation by low micromolar concentrations of AMP analogs and Ca^{2+} could occur in some phenotypically very similar NSC channels in pancreatic CRI-G1-cells (Reale et al., 1994; 1995), the possibility that the brown adipocyte NSC channel could be stimulated by very low nucleotide concentrations was examined in detail in a dedicated experiment. We tested 1 μM concentrations of ATP, ADP, AMP, cAMP and 3′,5′-cGMP, as well 1 mM 8-(4-chlorophenylthio)-3′,5′-cAMP (CPS-cAMP) in the 1 mM free Ca^{2+} solution. As shown by the sample trace in Fig. 6*A*, no additional activation to that obtained with 1 mM Ca^{2+} alone could be observed with any of the tested nucleotides. Individual fragments occasionally showed a slightly higher activity than Ca^{2+} alone, as did the one with ADP in the sample trace, but such an effect was not consistent throughout the recordings (Fig. 6*B*). The average relative open probabilities in Fig. 6*B* indicate that AMP or cAMP inhibited the NSC-channel activity weakly at 1 μM concentrations, although none of the results in Fig. 6*B* reached statistical significance. 1 μM cGMP had an even weaker or no effect, and 1 mM 8-(4-chlorophenylthio)-3′,5′-

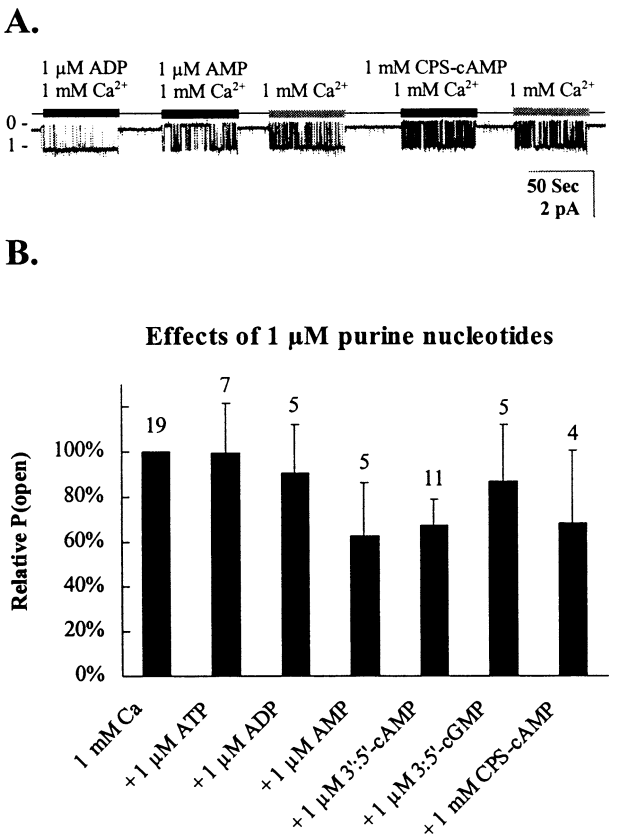


Fig. 6. Effects of 1 μM purine nucleotides on NSC-channel activity. (*A*) Sample traces from an inside-out patch showing effects of 1 μM 5′-ADP or 5′-AMP or 1 mM 8-(4-chlorophenylthio)-3′,5′-cAMP (CPS-cAMP) (*here:* 100-Hz filtration for visual clarity). (*B*) Average relative *P*(*open*) values from 6 inside-out patches with 1-min perfusions. 100% channel activity corresponded to *P*(*open*) = 0.50 ± 0.05 in these experiments. None of the samples in this test reached statistical significance *vs.* Ca^{2+} alone.

cAMP also inhibited rather than coactivated the NSC-channel.

Based on two types of responses to cAMP, Reale and colleagues divided the results of their inside-out patch studies into two groups of responses, in which a minority (6/26) of the patches responded positively to 0.1–5 μM AMP or ADP (Reale et al., 1994) and a majority (14/20) of the patches showed negative responses to 0.1–1 μM cAMP. We examined individual data from all our patches to see whether or not there was a difference between channels in individual patches, but we found no coactivation by Ca^{2+} and AMP or cAMP in any patch. Although a few individual 1-min perfusions with Ca^{2+} plus a nucleotide did show slightly higher activity than individual positive control perfusions (resulting in standard errors that indicate some larger relative activities underlying the statistics), this effect was never consistent within a given patch. Therefore, the brown adipocyte NSC channel appears to differ in its pharmacological properties from some of the NSC channels described in the CRI-G1-cells.

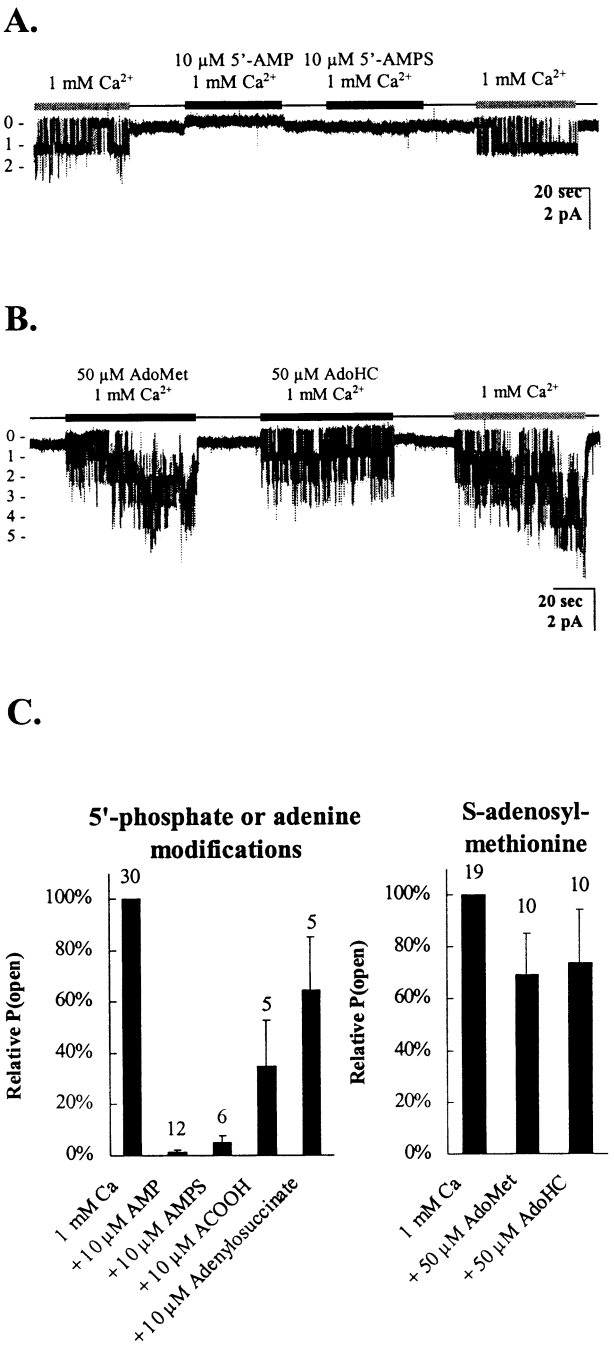
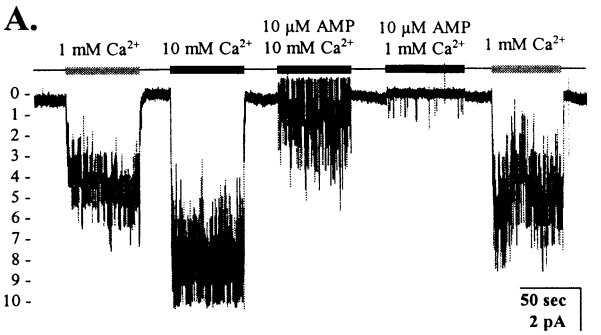
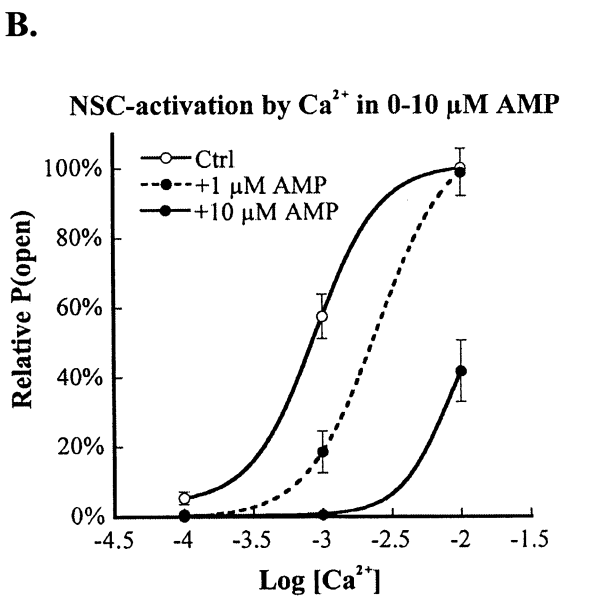


Fig. 7. Effects of 5′-phosphate group modifications. (*A*) Sample trace from an inside-out patch showing the effects of 10 μM 5′-AMPS. The thin line indicates perfusion with the negative control (1 mM BAPTA). (*B*) Sample trace from an inside-out patch showing the effects of S-adenosylmethionine (AdoMet) and 5′-deoxy-S-adenosyl-L-homocysteine (AdoHC). (*C*) (*Left*): Average relative *P*(*open*) values from 9 inside-out patches with 1-min perfusions (100% channel activity corresponded to *P*(*open*) = 0.32 ± 0.04 in these experiments. (*Right*): Average relative *P*(*open*) values from 6 inside-out patches. 100% channel activity corresponded to *P*(*open*) = 0.32 ± 0.05 in these experiments. In the experiments with AdoMet and AdoHC, 1 mM L-cysteine replaced glutathione as an antioxidant. Statistical significance *vs.* Ca^{2+} alone was obtained with AMP ($P < 0.001$) and with AMPS ($P < 0.05$).





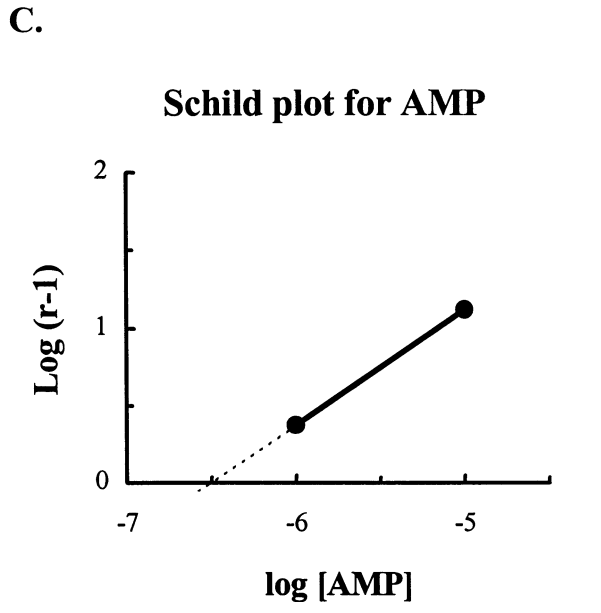


Fig. 8. Calcium dependence of the AMP-induced NSC-channel inhibition. (*A*) Sample trace from an inside-out patch. The thin line indicates perfusion with the negative control (1 mM BAPTA). (*B*) Average relative *P*(*open*) values from 2–19 analyzed fragments per point, from 7 inside-out patches. 100% channel activity corresponded to *P*(*open*) = 0.75 ± 0.04. The dose-response curves are shown as curve fits following the Michaelis-Menten equation with the Hill coefficient set to 2 (which gave a better correlation than 1). Calculated EC_{50} values for the dose-response curves were 0.90 mM (control), 2.1 mM (1 μM AMP) and 12 mM (10 μM AMP), respectively. (*C*) Schild plot for AMP-Ca^{2+} competition. *r* is the required *r*-fold increase in the dose of Ca^{2+} necessary to obtain the same activity as in the absence of AMP. A linear curve fit gave a horizontal intercept of −6.50, a slope value of 0.74 and a calculated K_B value for AMP of 0.3 μM.

α-Phosphate Group Modifications and Adenine Modifications

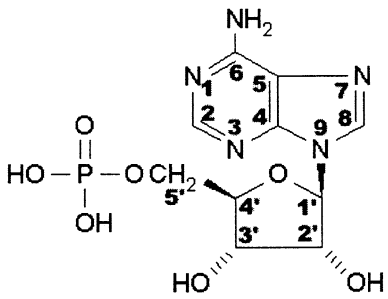
Given the necessity of the 5′-phosphate group for the inhibitory effects of adenosine (Fig. 2), we assessed more closely the importance of an intact α-phosphate group by comparing the inhibitory effects of 10 μM AMP versus 10 μM of adenosine-5′-O-monophosphorothioate (5′-AMPS), which has minimal modification on its α-phosphate group. The sample trace in Fig. 7*A* and the data in Fig. 7*C* show that the inhibitory properties of AMPS were nearly identical to those of AMP. To examine whether the phosphate could be mimicked by other substituents, we tested the protein methylation agent S-adenosylmethionine (AdoMet) and its unmethylated metabolite, S-adenosylhomocysteine (AdoHC). These molecules both contain a sulfide group in the 5′-position of adenosine. It has earlier been shown that the brown adipocyte NSC channel is sensitive to sulfhydryl reagents, and it has been suggested that the channel has free sulfhydryls exposed on the cytosolic side of the membrane (Koivisto, Siemen & Nedergaard, 1993). Nucleotides with reactive sulfides in the α-phosphate position could therefore possibly have a regulatory effect on the channel activity. However, as the sample trace in Fig. 7*B* and the data in Fig. 7*C* (*right*) demonstrate, 50 μM AdoMet or 50 μM AdoHC had almost no effect.

When the phosphate group was instead substituted with a carboxyl group, as in adenosine 5′-carboxylic acid (ACOOH), a weak inhibitory effect was still observed. Addition of two carboxyl groups, in the form of succinate, to the amino group of the adenine moiety of AMP forms the AMP precursor adenylosuccinate, which was unable to affect the channel activity at 10 μM (Fig. 7*C left*). Together, these results indicate that the α-phosphate group is a critical pharmacophore on AMP and that a carboxyl group cannot effectively substitute for the α-phosphate group to preserve a full blocking potency at 10 μM.

Ca^{2+} and AMP Competition Study

We finally examined the interaction between the inhibition by AMP and the activation by Ca^{2+}. In these experiments, free Ca^{2+} levels were adjusted to compensate for the indicated AMP additions. The

A.



B.

	Ribose					Adenine									
	1'	2'	3'	4'	5'	1	2	3	4	5	6	7	8	9	Effect vs. AMP
AMP	B	OH	OH		PO_4						NH_2			R	0
AMPS	B	OH	OH		PO_3S						NH_2			R	0
ADP	B	OH	OH		2 PO_4						NH_2			R	weaker
ATP	B	OH	OH		3 PO_4						NH_2			R	weaker
Adenosine	B	OH	OH		H						NH_2			R	weaker
ACOOH	B	OH	OH		COOH						NH_2			R	weaker
Ado-Met	B	OH	OH		Met						NH_2			R	weaker
AdoHC	B	OH	OH		hCys						NH_2			R	weaker
2'-deoxy-AMP	B	H									NH_2			R	0
3'-AMP	B	OH	PO_4		H						NH_2			R	weaker
3',5'-cAMP	B	OH	cycl		cycl						NH_2			R	weaker
2',3'-cAMP	B	cycl	cycl								NH_2			R	weaker
CPS-cAMP	B	OH	cycl		cycl						NH_2		CPS	R	weaker
cGMP	B	OH	cycl		cycl	H	NH_2				O			R	weaker
GMP	B	OH			PO_4	H	NH_2				O			R	weaker
IMP	B	OH			OH	H					O			R	weaker
XMP	B	OH			PO_4	H	O	H			O			R	weaker
Adenylosuccinate	B	OH			PO_4						NSucc			R	weaker

Fig. 9. An analysis of functional groups in the AMP structure, with respect to modulation of the $NSC_{Ca/AMP}$ channel activity. (*A*) General structure of AMP. (*B*) Overview of functional groups. The figure is based on the investigation presented in this manuscript; the principal necessity of an adenosine skeleton is not specified here. B = binding site for the base, R = Binding site for ribose. CPS = chlorophenylthio, NSucc = NH-succinyl, Met = methionine, hCys = homocysteine.

sample trace in Fig. 8*A* shows that 10 μM AMP inhibits the NSC-channel activity fully in 1 mM Ca^{2+} but incompletely in 10 mM free Ca^{2+}. Dose-response curves for the Ca^{2+} activation of the NSC channel at 0, 1 or 10 μM AMP are shown in Fig. 8*B*. The curve fits show that a shift of the Ca^{2+}-activation curves was caused by the presence of 1 or 10 μM AMP. This indicated a functional competition between Ca^{2+} and AMP. A calculation (with the BAD4 software) of free AMP-levels at 1 and 10 μM nominal AMP and 0.1, 1 and 10 mM Ca^{2+} showed that in all cases 40–50% of the AMP was in a free form (the major bound form of AMP under all conditions was K^+-AMP). The effect is thus not due to Ca^{2+}-complexing of AMP. To formally analyze the interaction between Ca^{2+} and AMP, the Schild plot in Fig. 8*C* was constructed, in which 1 minus the shift in EC_{50} for Ca^{2+} (*r*) was plotted as an effect of the AMP concentration. A linear curve fit yielded a slope value of 0.74 and a horizontal intercept of −6.50, corresponding to a K_B value of 0.3 μM for AMP. The K_B value indicates the AMP concentration needed to increase the apparent EC_{50} value for Ca^{2+} by a factor of 2.

Discussion

We have investigated here the regulation of the brown adipocyte calcium-activated nonselective cation channel by nucleotides in excised inside-out patches, at 1 mM free Ca^{2+} and at −40 mV membrane potential. Earlier investigations under similar

experimental conditions had shown that 100 μM purine nucleotides could seemingly unselectively block at least 90% of the Ca^{2+}-induced NSC-channel activity (Koivisto et al., 1998). Given the general importance of nucleotides in cellular metabolism, we have further examined the inhibitory properties of several purine and pyrimidine nucleotides. Our results confirmed the earlier published findings by Koivisto et al. (1998), but we also identified previously unknown characteristics as follows (*see also* Fig. 9).

AMP is a Selective Inhibitor of the Brown Adipocyte NSC Channel

The results in Fig. 2 demonstrate that AMP was the most potent NSC-channel inhibitor among different adenosine 5′-phosphates and adenosine moieties. In Fig. 3, we show that AMP was also a selective NSC-channel inhibitor among different nucleotide monophosphates. Fig. 5*B* shows that AMP was clearly selective over cAMP as an NSC-channel inhibitor, and the endogenous AMP-precursor adenylosuccinate was not nearly so potent as AMP itself (Fig. 7*C*); the unphosphorylated form of AMP, i.e., adenosine, was not effective as an NSC-channel inhibitor (Fig. 2*B*). The results in Fig. 2 also show that phosphorylation of AMP to ADP lowers the NSC-channel inhibitory potency of the nucleotide.

The above results raise questions about possible AMP contaminations in ATP or ADP, which could account for the inhibitory effect seen with these nucleotides. The results in Fig. 2*B* can be used to recalculate the amount of AMP that should give the same inhibitory effect as 100 μM of ATP or ADP. If one assumes a dose-response relationship with a Hill-coefficient of 1 (*confer* Fig. 5*B*), the result of such a calculation shows that only 7 μM AMP would be required to give the same inhibitory effect as 100 μM ATP. Thus, if 7% of the ATP were to decompose to AMP, the total ATP inhibition could occur due to this. Correspondingly, the required AMP-contamination to induce the same inhibition as 100 μM ADP was 28%. Even if contamination by AMP could thus theoretically account for the inhibition seen with ATP, this is nonetheless unlikely. The major nucleotide contaminants in the ATP (99% purity) used in this study are adenosine 5′-tetraphosphate and ADP, and the ATP should not decompose more than 0.1%/year (Labkemi Sigma AB technical support, personal communication). ATP hydrolyzes spontaneously in neutral aqueous solutions, but the half-life is many hours in the absence of catalysts (Mathews & van Holde, 1991). It has also been shown earlier that 1 mM of the nonhydrolyzable (Peterson et al., 1999) ATP analog β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) can block the NSC-channel activity to a similar extent as 100 μM ATP (Koivisto et al., 1998). Although neither of the results discussed above definitely resolves whether the ATP-induced NSC inhibition is partly mediated by AMP, our results, together with the results from Koivisto et al. (1998), indicate that ATP itself does exert its own (but weak) inhibitory action on the NSC channel.

Active Parts of AMP

In order to identify critical pharmacophores on the AMP molecule, we investigated the effects of base modifications, ribose modifications and phosphate modifications (summarized in Fig. 9). As seen in Fig. 3, AMP was the most potent NSC inhibitor among nucleotide monophosphates. Earlier results have shown similar inhibitory effects of 100 μM GTP, GDP and cGMP as well as of 100 μM AMP (Koivisto et al., 1998), but here we show that neither of the purine nucleotides GMP or IMP have an inhibitory effect at 10 μM. In Fig. 7*C*, we show that adenylosuccinate was not nearly so potent as AMP itself, although it did have some inhibitory effect at 10 μM. We understand these findings as indicating that an intact amino group in the 6-position on adenine may be one of the most important structures for the strong blocking potency of AMP. The possible requirement of an unaltered structure in positions 1 and 2 on adenine remains unclarified, since we did not test AMP analogs with selective modifications in these domains.

Ribose modifications on AMP (Fig. 4) showed that the high NSC-channel blocking effect of AMP was rapidly lost when the 5′-phosphate group was either moved to another position or made cyclic. There was no detectable difference in the NSC channel-blocking potency between AMP and 2′-deoxy-AMP, indicating that the 2′ part of the ribose domain is not critically involved in the AMP-NSC channel interaction. It was clear from the results in Fig. 2 that the 5′-phosphate groups were important for the nucleotide phosphate-NSC channel interaction.

The detailed study of phosphate modifications of the AMP-molecule (Fig. 7) showed that even when one of the oxygens on the 5′-phosphate group was substituted with a sulphur (in 5′-AMPS), no change in blocking potency could be observed. When the 5′-phosphate was substituted with a carboxyl group, a dramatic loss of NSC channel-blocking potency was observed. Also, when the 5′-phosphate group was substituted with a reactive sulphur on the ubiquitous protein methylation agent S-adenosyl-L-methionine, the blocking potency was dramatically decreased. The 5′-phosphate group is thus clearly a highly critical pharmacophore in the nucleotide-NSC channel interaction.

It is of relevance to relate this to the cellular biosynthesis and metabolism of AMP. AMP is synthesized from adenylosuccinate (Fig. 7), which was practically without effect at 10 μM. AMP itself blocks the NSC channel almost totally at the same concentration. Endogenous degradation of AMP can occur in two ways, either by deamination by the enzyme adenosine deaminase (ADA), which results in IMP, which was ineffective as an NSC-channel inhibitor at 10 μM (Fig. 3), or by hydrolysis to adenosine (Fig. 2) and free phosphate (*data not shown*), both of which were ineffective as NSC-channel inhibitors even at 100 μM. Taken together, our results therefore strongly indicate that 5′-AMP is the most efficacious endogenous modulator of the brown adipocyte NSC channel presently known.

Ca-AMP Interaction

We have shown that micromolar concentrations of AMP compete functionally with Ca^{2+} (Fig. 8). This competition was not evident when 2 mM ATP was tested (Koivisto et al., 1998). The K_B for AMP was 0.3 μM, which means that the interaction between Ca^{2+} and AMP can adequately be described by the equation: $P(open)_{Ca/AMP} = [Ca^{2+}]/([Ca^{2+}] + 0.9\ mM\ (1 + ([AMP] / 0.3\ \mu M))$. This formula indicates that a higher concentration of AMP is needed for 50% inhibition of channel activity (IC_{50}) if the Ca^{2+} concentration is increased, and the formula reasonably predicts the experimental outcomes in Fig. 5 and Fig. 8. The nature of the competition is not known. Experimental conditions were such that a chemical interaction between Ca^{2+} and AMP cannot explain the observations. It is in principle not likely that the very positively charged Ca^{2+} and the very negatively charged phosphate moiety of AMP bind to exactly the same site. However, these compounds could still force the channel into different exclusive configurations.

Two Highly Expressed Brown Adipocyte Proteins are Nucleotide-Sensitive

NSC-channel activity can be induced by norepinephrine in cell-attached patch-clamp recordings (Koivisto et al., 2000). Theoretically, this phenomenon is surprising, since the very low Ca^{2+} sensitivity of the NSC channel (Koivisto et al., 1998; Halonen and Nedergaard, unpublished results) and the high sensitivity for channel inhibition by endogenous nucleotides (as documented in this study) should in principle never allow the activation to occur. Given the assumption that cells keep the ratio of ATP to ADP and AMP as high as 10:1:0.1 (Lodish et al., 1995) and that ATP levels in both prokaryotic and eukaryotic cells are in the range 1–10 mM (Mathews & van Holde, 1991), ADP + AMP 1evels should easily reach 100 μM in the cytosol. With such high nucleotide concentrations, it should be impossible that the NSC channels are activated by norepinephrine/Ca^{2+}.

Calcium-activated, nucleotide-sensitive nonselective cation channels are not the only nucleotide targets in brown adipocytes that have an unexplained, high sensitivity to purine nucleotides. Also UCP1 is highly sensitive for purine nucleotides (Nicholls et al., 1974; Huang & Klingenberg, 1995), but the specificity is different, with the di- and triphosphate nucleotides being the most effective inhibitors (Nicholls et al., 1974).

Comparison with Other Cell Systems

Calcium-activated, nucleotide-sensitive nonselective cation channels (NSC_{Ca}) with a 20–30-pS unitary conductance have been described in several cell types in addition to the brown adipocyte. In the following, we will compare some of them to the brown adipocyte NSC channel with respect to their reported Ca^{2+}-sensitivity and regulation. According to the results presented here (and earlier), brown fat cells contain a type of NSC which is activated only by Ca^{2+}, and which is inhibited by nucleotides. We will refer to this channel as $NSC_{Ca/AMP}$.

White Fat Cells

A white adipocyte 28-pS calcium-activated nonselective cation channel has been described and characterized in cell-attached and excised inside-out patch recordings (Koivisto et al., 1993; Ringer, Russ & Siemen, 2000). This channel is activated by millimolar concentrations of Ca^{2+} in excised inside-out patches (Koivisto et al., 1993; Ringer et al., 2000) and is totally blocked by 1 mM ATP and transiently inhibited by 100 μM ATP (Ringer et al., 2000). Thus, the ATP-sensitivity of the white adipocyte NSC channel appears to be similar to the sensitivity of the brown adipocyte NSC channel (Fig. 2*B*). Direct regulation of the white adipocyte NSC channel by either AMP or cAMP has not been reported. In cell-attached patches, the white adipocyte NSC channel is activated by norepinephrine. This activation is β-adrenergic, since it can be inhibited by propranolol (but not by the α-antagonist phentolamine) and mimicked by the selective β_3-adrenergic agonists BRL 37344 and BRL 35135A (Ringer et al., 2000). Given that β-adrenergic receptors are usually associated with cAMP rather than Ca^{2+} as a second messenger, whereas activation of the NSC channels in brown adipocytes is α_1-adrenergically mediated (Koivisto et al., 2000), it would be interesting to clarify how closely related the NSC channels in brown and white fat cells are.

Exocrine Pancreas

Ca^{2+}-activated NSC channels from acinar cells of the exocrine pancreas require >100 μM Ca^{2+} for maximal activation in inside-out patches (Maruyama & Petersen, 1982; 1984), but they can also be activated by low micromolar concentrations of Ca^{2+} if the patch is excised into a bath solution with 2 mM ATP (Thorn & Petersen, 1992). This is not the case for the brown adipocyte NSC channels (Koivisto et al., 1998). The acinar cell NSC channels are blocked by 0.2–4 mM concentrations of ATP and ADP when applied on excised inside-out patches (Thorn & Petersen, 1992; Suzuki & Koyama, 1996), but the catalytic subunit of PKA (+ATP) can reactivate these NSC channels in 1 μM free Ca^{2+}, after a total block by ATP (Suzuki & Koyama, 1996). We would refer to these channels as $NSC_{Ca,PKA}$. Brown adipocyte $NSC_{Ca/AMP}$ channels clearly differ from these pancreatic acinar $NSC_{Ca,PKA}$ channels by their insensitivity to PKA and by their inability to be activated by 1 μM Ca^{2+} (Koivisto et al., 1998).

Endocrine Pancreas

In pancreatic β-cells, the glucose-sensitizing hormone glucagon-like peptide 1 (GLP-1) activates whole-cell NSC currents carried by 30-pS NSC channels via a Ca^{2+} and cAMP-dependent pathway (Holz, Leech & Habener, 1995). These NSC currents have been discussed as mediators of the GLP-1-induced insulin release (Leech & Habener, 1998), although inside-out patch data on their regulation by Ca^{2+} or nucleotides have not been presented. However, such data exist from the insulin-secreting rat pancreatic islet cell line CRI-G1, which apparently contains two types of Ca^{2+}-activated, nucleotide-regulated nonselective cation channels. Both are inhibited by nucleotides, but one type also seems to be activated by very low concentrations of cAMP. These channels coexist with ATP-sensitive K^+-channels in the CRI-Gl cells and in human insulinoma cells (Sturgess et al., 1987). The NSC channels have a very low Ca^{2+} sensitivity in excised patches; 100 μM Ca^{2+} or more is needed for significant activation (Sturgess et al., 1987). AMP is the most potent inhibitory nucleotide of both channel types, with a full block at 100 μM and a significant inhibition at 10 μM. The relative blocking potency in excised patches is AMP > ADP > ATP > adenosine (Sturgess et al., 1986; Reale et al., 1994), which, together with the low calcium sensitivity, is in accordance with our data on the brown adipocyte NSC channel (Fig. 2*B*). AMP is a selective blocker among the nucleotide monophosphates AMP, UMP, CMP and GMP (Reale et al., 1994), which is also in good accordance with our more detailed data on the brown adipocyte NSC channel (Fig. 3). One of the endocrine pancreatic channels is thus clearly an $NSC_{Ca/AMP}$.

However, the second type of the CRI-G1 NSC channels, in contrast to $NSC_{Ca/AMP}$, can also be coactivated by Ca^{2+} and 0.1–5 μM of AMP or ADP (Reale et al., 1994). This was not the case for any of the brown adipocyte NSC channels (Fig. 6*B*; if there would have been any stimulatory effect to observe, we should have been able to measure it, since 1 mM Ca^{2+} alone only gave an average $P(open)$ around 0.5; *see* Fig. 1*C*). We therefore refer to the second NSC-channel type in the endocrine pancreas as $NSC_{Ca,cAMP}$, since, in contrast to the $NSC_{Ca/AMP}$ (Fig. *5B* and Fig. *6B*), the $NSC_{Ca,cAMP}$ channels can be coactivated by Ca^{2+} and 0.1–1-μM concentrations of cyclic nucleotides (Reale et al., 1994). However, nucleotide concentrations higher than 10–100 μM inhibit both $NSC_{Ca/AMP}$ and $NSC_{Ca,cAMP}$ activity in most patches (Reale et al., 1994), and 1 mM cAMP inhibits the activity of both channel types effectively in all patches (Reale et al., 1994; 1995). We did not observe any increased activity for the brown adipocyte NSC channel with 1 μM nucleotides, or even with 1 mM of 8-(4-chlorophenylthio)-3′,5′-cAMP, which appears to be a particularly effective nucleotide coactivator of the $NSC_{Ca,cAMP}$ in CRI-G1 cells (Reale et al., 1995). The authors also excluded protein kinase A (PKA) as a possible mediator of the cAMP-effect (Reale et al., 1994).

Both CRI-G1 NSC channels are inhibited by ATP (with a slightly higher potency than the coexisting ATP-sensitive K^+ channel; Sturgess et al., 1987). The reported IC_{50} value of 8 μM for the ATP inhibition of the CRI-G1 NSC-channel is identical to that reported earlier for the brown adipocyte NSC channel (Koivisto et al., 1998). From the data in Fig. 2B, an IC_{50} value for the ATP inhibition of the brown adipocyte NSC channel was estimated to be 32 μM. (This difference from the above results may depend on our use of 2 mM reduced glutathione in the KCl-Ringer and the absence of additional chelators (such as BAPTA)). However, the possibility should, of course, not be ruled out that a yet untested endogenous nucleotide can activate both the CRI-G1 NSC channels and the brown adipocyte NSC channel in only 1 μM free Ca^{2+}.

Kidney

Ca^{2+}-activated NSC channels of 25 pS conductance from the basolateral membrane of the thick ascending limb of Henle's loop require only 1 μM Ca^{2+} for significant activation in inside-out patches (Paulais & Teulon, 1989). These channels are inhibited by purine nucleotides with the inhibiting potency AMP > ADP ~ ATP≫adenosine. The low potency of cAMP as a channel modulator (Paulais & Teulon, 1989) and the high Ca^{2+} sensitivity suggest this channel to be similar to $NSC_{Ca/AMP}$, or possibly to $NSC_{Ca,PKA}$, but to our knowledge, it has not yet been shown to respond positively to PKA.

Eye

Ca^{2+}-activated NSC channels from acinar cells of the corneal endothelium also require at least 100 μM Ca^{2+} for significant activation in inside-out patches (Rae et al., 1990). These channels have a conductance of 21 pS and they are inhibited by purine nucleotides with the inhibiting potency AMP > ADP > ATP. The effects of cAMP or PKA on these channels have not, to our knowledge, been reported. However, based on the data above, the corneal endothelial NSC channel is a functional relative to the $NSC_{Ca/AMP}$ channel in brown adipocytes and in the endocrine pancreas.

Conclusions

There thus seem to exist at least three different types of NSC, as discussed above. $NSC_{Ca/AMP}$ is found both in brown adipocytes and in the endocrine pancreas. $NSC_{Ca,cAMP}$ is found in the endocrine pancreas, while $NSC_{Ca,PKA}$ is found in the exocrine pancreas. The white adipocyte NSC channel may be either an $NSC_{Ca,cAMP}$ or an $NSC_{Ca,PKA}$. More research is obviously needed to clarify the physiological activation mechanism between similar, but obviously not identical, NSC channels in tissues regulating different aspects of metabolism.

We thank Stig Sundelin and Rickard Ekström for their technical support during this project, and Barbara Cannon for valuable comments on the manuscript. This study was supported by grants from The Swedish Science Research Council.

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